

# Targeting the epigenome in in-stent restenosis: from mechanisms to therapy

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**Coronary artery disease (CAD) is one of the most common causes of death worldwide. The introduction of percutaneous revascularization has revolutionized the therapy of patients with CAD. Despite the advent of drug-eluting stents, restenosis remains the main challenge in treating patients with CAD. In-stent restenosis (ISR) indicates the reduction in lumen diameter after percutaneous coronary intervention, in which the vessel's lumen re-narrowing is attributed to the aberrant proliferation and migration of vascular smooth muscle cells (VSMCs) and dysregulation of endothelial cells (ECs). Increasing evidence has demonstrated that epigenetics is involved in the occurrence and progression of ISR. In this review, we provide the latest and comprehensive analysis of three separate but related epigenetic mechanisms regulating ISR, namely, DNA methylation, histone modification, and non-coding RNAs. Initially, we discuss the mechanism of restenosis. Furthermore, we discuss the biological mechanism underlying the diverse epigenetic modifications modulating gene expression and functions of VSMCs, as well as ECs in ISR. Finally, we discuss potential therapeutic targets of the small molecule inhibitors of cardiovascular epigenetic factors. A more detailed understanding of epigenetic regulation is essential for elucidating this complex biological process, which will assist in developing and improving ISR therapy.**

Coronary artery disease (CAD) represents the most significant cause of morbidity and mortality and poses a major health and economic burden worldwide.<sup>1</sup> The 2017 Global Burden of Disease (GBD) study noted that cardiovascular diseases were responsible for most deaths due to non-communicable diseases globally, with approximately 178,000 deaths, and the estimated cost of CAD treatment is expected to increase by 100% from 2015 to 2030.<sup>2</sup> Currently, percutaneous coronary intervention (PCI) is used for treating patients with CAD, which remains the focus of intensive research and development.<sup>3</sup> In the past, PCI has reduced the mortality accompanying CAD, particularly those cases related to acute myocardial infarction (AMI).<sup>4</sup> However, restenosis due to stent implantation is one of the major drawbacks of this therapy, which may lead to vascular injury and neointima formation, subsequently causing stent implantation failure, necessitating repeat intervention.

Restenosis, defined as the development of luminal narrowing at previously stented regions, usually happens several months after PCI and is accompanied by arterial injury and neointimal formation.<sup>5</sup> Initially, the restenosis rate of balloon angioplasty alone was approximately 40%, which was mostly associated with elastic recoil and neointimal hyperplasia.<sup>6</sup> Therefore, coronary stents were introduced to overcome these limitations. Subsequently, the introduction of bare metal stents (BMSs) minimized dissections and elastic recoil. Nevertheless, the application of BMSs is limited by the high rate of in-stent restenosis (ISR), which is mainly caused by the generation of a neointima.<sup>6,7</sup> The emergence of drug-eluting stents (DESs) (especially second-generation) and drug-coated balloons (DCBs) has further reduced the incidence of restenosis by <10%.<sup>8</sup> However, current DESs cannot effectively repress the proliferation and migration of vascular smooth muscle cells (VSMCs) and cannot induce adequate re-endothelialization within the vasculature, which increases the risk of restenosis.<sup>4</sup> Therefore, a better understanding of the underlying mechanisms and regulatory targets are required and warrant detailed investigation.

Epigenetic regulation has emerged as a novel direction to study human disease. Epigenetic modification is regulated on multiple sophisticated levels via various mechanisms, including DNA methylation, post-transcriptional histone modification, and non-coding RNAs (ncRNAs). Unlike genetic mutations, epigenetic alterations are largely reversible and vulnerable to nutritional and environmental factors, and they can return to their normal state via modifications and/or drug targeting.<sup>9</sup> Hence, their functions, both as potential biomarkers and as novel therapeutic targets, have been extensively studied in recent years. In particular, several aspects of the vascular response

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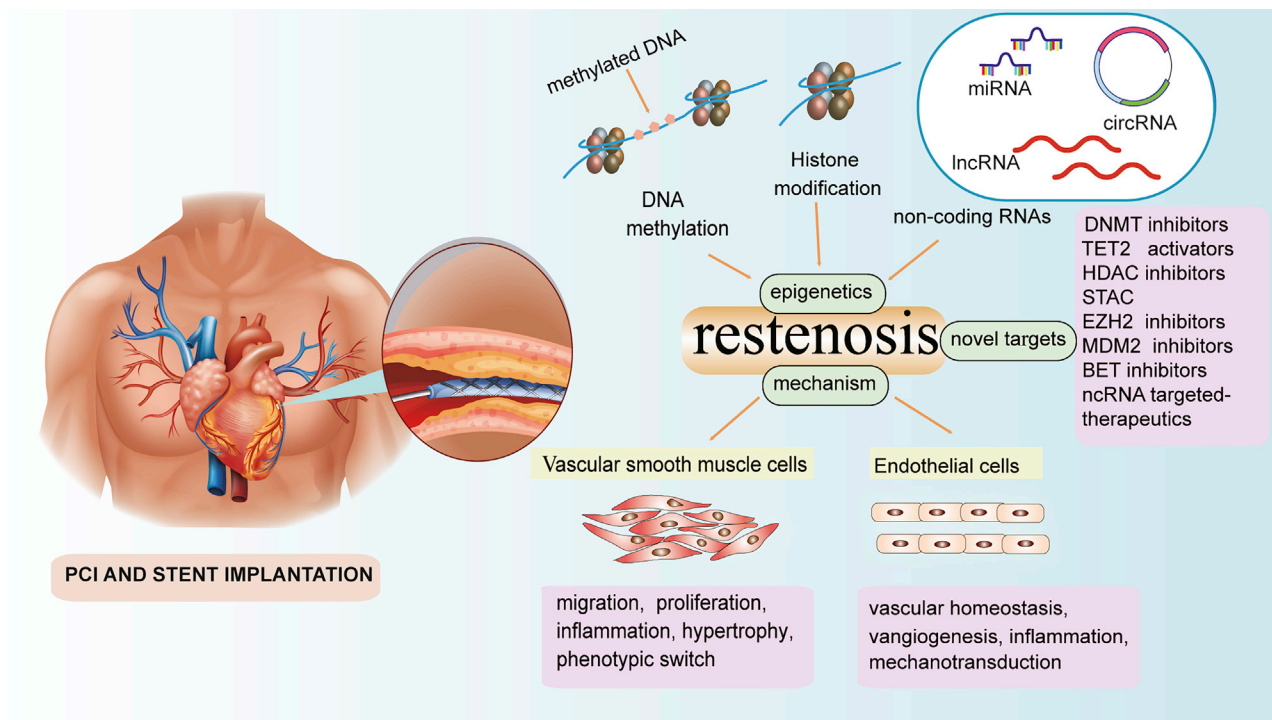
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**Figure 1. Overview of risk factors, pathogenesis, epigenetic regulation, and currently available targeted inhibitors associated with restenosis**

Targeted inhibitors are shown may address pathophysiological pathways associated with the formation and development of restenosis following stenting in coronary artery disease.

to injury are regulated via epigenetic mechanisms, which modulate not only cell proliferation, differentiation, apoptosis, and inflammation, but also thrombosis and vascular remodeling (Figure 1). Interestingly, changes in gene expression levels have been observed in cardiovascular disease, so these abnormal expression patterns are thought to be associated with restenosis.<sup>10</sup>

In this review, we provide a new perspective regarding the various epigenetic mechanisms regulating ISR and therapies on epigenetic factors targeting ISR. We expect that our observations will contribute to clarify the biological processes of epigenetic regulation of restenosis, as well as provide new ideas for the treatment of restenosis.

### Mechanisms of restenosis

Continuous efforts have been made to delineate the mechanism of ISR after stent implantation. Restenosis is a progressive phenomenon that begins after balloon dilation (barotrauma) is determined by PCI. The pathogenesis of restenosis can be subdivided mainly into three parts: (1) early elastic return (recoil) (ER), (2) vascular remodeling, and (3) neointimal hyperplasia. In addition, the presence of metallic struts leads to neointimal hyperplasia.<sup>8</sup>

### ER and vascular remodeling

The internal and external elastic laminae of the human epicardial coronary arteries contain elastin fibers, which may cause ER after balloon

hyperextension. ER usually occurs between a few seconds and a few minutes after the balloon is vented, resulting in a 40% reduction in the lumen area. Although angioplasty and stents themselves can cause overstretching injuries, stents can reduce acute recoil. Vascular remodeling is a process that occurs after acute arterial injury, constriction of the local arterial wall, and narrowing of the lumen in the injured vascular segment. However, the exact mechanism of arterial remodeling is not yet fully understood. There are several mechanisms that may explain arterial remodeling, including (1) changes in shear force, (2) oxidative stress, (3) endothelial dysfunction, (4) collagen metabolism, and (5) secretion of growth factors.<sup>11</sup> Studies have demonstrated that blood vessels self-remodel in response to long-term flow changes, thus changing the lumen area to maintain a pre-determined level of shear stress (OS).<sup>12</sup> Interestingly, the presence of stents almost eliminates the two mechanisms of ER and vascular remodeling, while the presence of metal struts promotes neointimal hyperplasia.

### Neointimal hyperplasia

The key pathophysiological phenomenon of restenosis after stent implantation is neointimal hyperplasia, which is predominantly due to VSMC proliferation and subsequent abluminal migration.<sup>13</sup> Under physiological conditions, VSMCs are a highly specialized, almost fixed cell population whose primary function is to maintain vascular tension and ensure the ability for vasoconstriction. VSMCs are

contractile in adult blood vessels and are characterized by a low proliferation rate (about 5%) and a group of specific contractile proteins, including smooth muscle myosin heavy chain (SMMHC),  $\alpha$ -smooth actin ( $\alpha$ -SMA), and calponin, among others. However, VSMCs have high plasticity and can easily change the phenotype from highly contractile to synthetic under specific stimuli, such as transforming growth factor (TGF)- $\beta$ , endothelin-1 (ET-1), hyperhomocysteinemia, fibroblast growth factor (FGF), monocyte chemokine protein 1 (MCP-1), prostaglandin D2, and cyclic stretch,<sup>14</sup> thus promoting abnormal proliferation and migration while reducing the expression level of contractile proteins, resulting in lumen reduction.<sup>15</sup>

ISR is primarily a nonspecific inflammatory response to vascular wall damage due to the continued “insult” of foreign elements (metal struts serving as a stent). The stent implantation causes a lesion in the endothelium, which is accompanied by consecutive endothelial denudation, re-endothelialization, and subsequent generation of the neo-endothelium.<sup>16</sup> Meanwhile, the endothelial cells (ECs) are denuded, resulting in a series of inflammatory responses, such as platelet activation and circulating leukocyte recruitment, and the release of cytokines and growth factors, including FGF-2, platelet-derived growth factors (PDGFs), TGF- $\beta$ , vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1).<sup>17</sup> These cytokines regulate the phenotypic transformation of VSMCs and change from contractile to synthetic, resulting in proliferation and migration, following which they enter the cell cycle and migrate to the intimal and extracellular matrix (ECM) to then form neointima.<sup>7,18</sup>

At present, paclitaxel, sirolimus, and zotarolimus are widely used as targeted drugs for restenosis.<sup>19</sup> Paclitaxel has a cytostatic effect. The mode of action is the  $\alpha$  and  $\beta$  unit polymerization of tubulin, thus stabilizing the microtubules needed for G2 conversion to the M phase. Paclitaxel inhibits the proliferation and migration of VSMCs by modifying the structure of the cytoskeleton.<sup>20</sup> Sirolimus, also known as rapamycin, is a macrocyclic antibiotic with strong immunosuppressive properties. Zotarolimus is an analog of rapamycin. Sirolimus and its analog could inhibit the proliferation and migration of VSMCs by blocking the cell cycle from the G<sub>1</sub> to S phase<sup>21,22</sup> through suppressing rapamycin mammalian targets. These drugs reduce the proliferation of VSMCs but harm the endothelialization of scaffold strut support. Also, delivery of these drugs does not eradicate ISR completely, and more beneficial therapeutic targets should be explored.

### Epigenetic mechanisms in ISR

Epigenetic regulation, which differs from genetic events in that the underlying DNA sequence is not changed, can have far-reaching effects on the transcription of genes and hence on cell lineage, function, and fate. In epigenetic regulation, DNA and histones are enzymatically modified by proteins known as “writers” (add modifying groups), “erasers” (remove modifying groups), and “readers” (recognize modifying groups).<sup>23</sup> Writers include DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), histone methyltrans-

ferases (HMTs), and E3 ligases, among others.<sup>23</sup> Erasers constitute ten-eleven translocation (TET) enzymes, histone demethylases (HDMs), histone deacetylases (HDACs), and deubiquitinases (DUBs).<sup>23</sup> Readers include ubiquitin (Ub)-like PHD and RING finger domain-containing 1 (UHRF1) and the bromodomain and extraterminal (BET) domain-containing family.<sup>24</sup> This multilateral regulation ensures robust execution of cellular functions, including VSMC proliferation and migration, and EC dysfunction.

### DNA methylation

DNA methylation is one of the main epigenetic modulations involving direct chemical modification of DNA. The extent of DNA methylation is regulated by the balance in the activities of DNA-modifying enzymes, which either add (DNMTs) or remove methyl marks (DNA demethylases) from the DNA. In the following sections, we discuss the regulation of VSMCs and ECs via DNA methylation both *in vivo* and *in vitro*.

DNA methylation is maintained by the DNMT family, which is classified into two types: DNMT1 mainly maintains the DNA methylation pattern, whereas *de novo* DNA methylation is typically mediated by DNMT3A and DNMT3B.<sup>25</sup> DNMTs are responsible for transferring methyl groups from S-adenosyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5-methylcytosine (5mC). DNA methylation or hypermethylation inhibits transcription, whereas hypomethylation is associated with transcriptional activation.<sup>25</sup>

Aberrant DNA methylation affects the function of different cell types, such as VSMCs and ECs. Previous studies have reported that increased methylation of the promoter regions of Src homology 2-containing protein tyrosine phosphatase 1 (*SHP-1*) in oxidative low-density lipoprotein (ox-LDL)-treated VSMCs inhibited *SHP-1* expression. The methylation level of the *SHP-1* promoter was positively related to the risk of restenosis, suggesting that methylated *SHP-1* is a novel target for preventing restenosis.<sup>26</sup>

Homocysteine (Hcy) is an independent risk factor for coronary and peripheral atherosclerosis and plays a key role in the regulation of vascular cell function. Hcy-induced VSMCs showed increased DNA methylation at the promoter of mitofusin-2 (*MFN2*), encoding a key transmembrane GTPase widely distributed in the mammalian mitochondrial outer membrane, which inhibited *MFN2* transcription, resulting in VSMC proliferation. These studies provided novel insights regarding DNMT1-regulated VSMC proliferation during restenosis.<sup>27</sup> In addition, DNA methylation coordinates with paracrine mechanisms to regulate cell function. Previous studies have shown that PDGFs modulate numerous pathophysiological events, including cell proliferation and migration, ECM accumulation, and production of pro- and anti-inflammatory regulators. Hcy-induced DNA hypomethylation is mediated mainly via inhibition of DNMT1 expression, which may increase the expression and paracrine secretion of PDGFs from ECs and further enhance the proliferation and migration of VSMCs. As discussed above, Hcy upregulates

PDGF expression via DNA demethylation in ECs, affecting the cross-talk between ECs and VSMCs and thereby activating VSMCs.<sup>28</sup> Previous studies have shown that VSMC contraction is dependent on ATP derived from mitochondrial respiration in animal arteries. DNMT1 translocates to the mitochondria and induces mitochondrial DNA (mtDNA) hypermethylation, resulting in repression of mtDNA transcription and mitochondrial dysfunction, and subsequently impairing VSMC contractility during restenosis. These results filled a knowledge gap between mtDNA methylation and VSMC function and may provide a molecular mechanism for further understanding of vascular homeostasis and dysfunction. In addition, they provide a potential therapeutic direction for the treatment of vascular disease.<sup>29</sup>

Furthermore, several studies have emphasized the importance of microRNAs (miRNAs) in the complex regulation of gene expression. Proteins harboring *Uhrf1* induce VSMC proliferation, bind directly to hemimethylated DNA, and play a key role in maintaining DNA methylation by recruiting DNMT1 to these DNA sites. A study has demonstrated that miR-145 overexpression attenuates *Uhrf1* expression. In a mouse model, intimal hyperplasia in the mouse carotid artery was attenuated by *Uhrf1* short hairpin RNAs (shRNAs) or *Uhrf1* VSMC-specific knockout. This clearly indicates that *Uhrf1* may be a therapeutic target in vascular pathologies.<sup>30</sup> In addition, the findings of this study provided insights regarding the miRNA-mediated regulation of DNA methylation, adding a new layer to the mechanisms regulating VSMC phenotype regulation.

Zhang et al.<sup>31</sup> observed that the expression of miR-143 is negatively correlated with both DNMT3a mRNA and protein levels in Hcy-induced VSMCs. The effects of DNMT3a on the methylation status of miR-143 further confirmed the correlation between the expression of miR-143 and DNMT3a. In addition, studies have reported mutual regulation between miR-125b and DNMT3b, which can mediate Hcy-induced VSMC proliferation. On the one hand, DNMT3b is a target of miR-125b. On the other hand, Hcy-induced reduction in miR-125b increased the DNMT3b level and eventually caused the proliferation of VSMCs.<sup>32</sup> Taken together, these studies indicated that complicated mechanisms of epigenetic crosstalk between DNA methylation and miRNA coordinately regulate VSMC proliferation.

A large body of evidence has shown that hemodynamic forces, including OS, regulate EC function, which is dysregulated by activation of mechanotransduction. Zhou et al.<sup>33</sup> observed that OS induced hypermethylation of ECs by upregulating the expression and promoting the nuclear translocation of DNMT-1. This was reversed by 5-aza-2'-deoxycytidine treatment, which inhibits DNMT-1 and thereby hypermethylation. Consistent with these observations, the DNMT1 level and methylation were upregulated in the OS regions of the partially ligated carotid artery of model rats.<sup>34</sup> This was the first study to demonstrate the disturbed blood flow-induced changes in ECs, which were regulated at the epigenomic level in a DNMT- and DNA methylation-dependent manner. Furthermore, this suggests that flow regulates gene expression in a DNMT-dependent manner and

reveals new targets for regulating EC function. In addition, Zhang et al.<sup>35</sup> also showed that OS-mediated DNMT1 overexpression and mammalian target of rapamycin (mTOR) were involved in this signaling pathway both *in vitro* and *in vivo*. DNMT1 regulates proliferative and inflammation molecules including proliferating cell nuclear antigen (PCNA), vascular cell adhesion molecule 1 (VCAM-1), and intercellular cell adhesion molecule 1 (ICAM-1). Furthermore, DNMT1 deficiency improved the dysregulation of ECs caused by disruption of EC proliferation and pro-inflammation. However, the detailed mechanism of OS inducing site-specific binding and distribution of DNMT1 in the genome remains to be elucidated.

Krüppel-like factor 4 (KLF4) is a transcription factor that is extensively expressed in various vascular cells, such as VSMCs, ECs, and other vascular cells. Jiang et al.<sup>36</sup> reported that OS promoted hypermethylation of the *KLF4* promoter by increasing the expression of DNMT3A and inhibiting KLF4 transcription in ECs. Furthermore, in accordance with *in vitro* data, the hypermethylation of the *KLF4* promoter and the *KLF4* and *NOS3* gene bodies decreased in the OS region of aortic ECs. The above studies showed that OS increased DNMT expression, resulting in alterations in DNA methylation, which further regulated EC function both *in vitro* and *in vivo*.

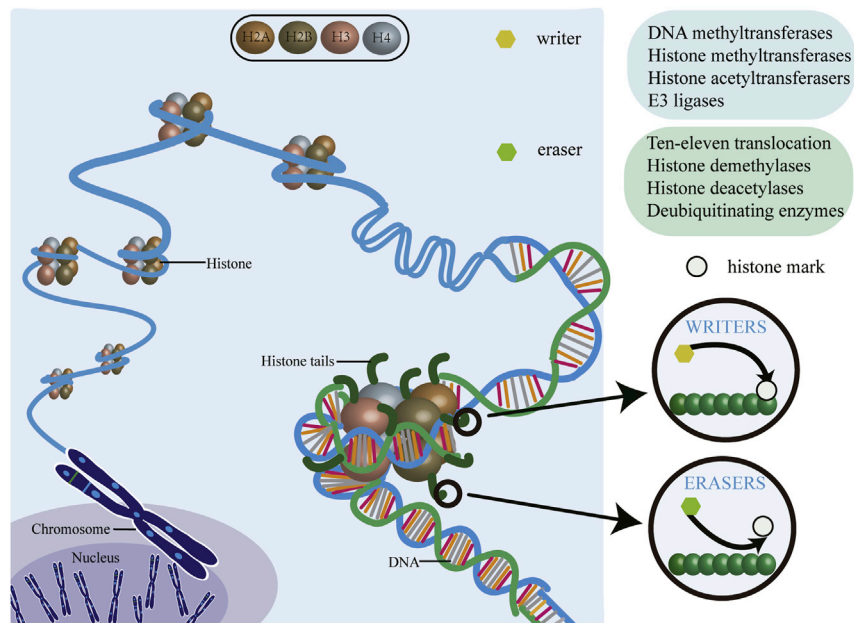
Lipopolysaccharide (LPS) was also involved in regulating EC function. Endothelial KLF2 is a key transcription factor involved in endothelium-dependent vascular homeostasis. LPS, which is a proinflammatory stimulus, increased hypermethylation of the *KLF2* promoter and subsequently downregulated *KLF2* and its downstream genes, including that encoding endothelial nitric oxide (NO) synthase (eNOS), in ECs. The loss of DNMT1 reversed *KLF2* downregulation in LPS-induced ECs, suggesting that *KLF2* methylation is associated with DNMT1 in mediating the inflammatory function of ECs.<sup>37</sup>

Overall, abnormal DNA methylation altered gene expression in VSMCs and ECs in ISR, leading to disease development. Furthermore, DNMT expression determines the state of DNA methylation, and miRNAs also play a key role in this process.

#### DNA demethylation

The Tet family, which includes Tet1, Tet2, and Tet3, is a set of enzymes capable of activating DNA demethylation. Tet enzymes promote DNA demethylation and gene transcription by catalyzing the conversion 5mC to 5-hydroxymethylcytosine (5hmC). Recently, the roles of TET in various diseases, particularly in cancer, were investigated extensively.<sup>38</sup> In addition, downregulation of *TET2* was observed in human atherosclerotic samples and in a mouse femoral artery wire injury model. Importantly, *TET2* overexpression dramatically improves intimal hyperplasia *in vivo* following vascular injury.<sup>39</sup>

*TET2*, a key enzyme in the DNA demethylation pathway, is highly expressed in VSMCs and is a key regulator of VSMCs.<sup>39</sup> *TET2* and 5hmC are upregulated during VSMC differentiation, whereas they are downregulated during VSMCs dedifferentiation.<sup>39</sup> Liu et al.<sup>39</sup>



**Figure 2. Illustrations of various epigenetic posttranslational modifications**

The nucleosome is comprised of approximately 147 bp of DNA comprising an octamer of two copies of each of the four core histone proteins H2A, H2B, H3, and H4. The histone tail is specifically modified by writing an enzyme that adds or erases the PTM shown.

downstream targets via the TET2-NF- $\kappa$ B signaling pathway.<sup>43</sup> Further studies should shed light on the other signaling pathways related to TET2 and EC function.

Considering that TET2 plays a critical role in EC dysregulation and phenotype transformation of VSMCs, these studies further suggest the possibility of TET2 as a new target for restenosis treatment.

#### Histone modification

Histone modification can be subdivided into the following categories: (1) methylation, (2) acetylation, (3) ubiquitination, (4) sumoylation, and (5) phosphorylation.<sup>44</sup>

Histone modifications comprise a complex set of multiple modifications that regulate chromatin by “writing” and “erasing” epigenetic marks.<sup>45</sup> In the eukaryote nucleus, 147 bp of DNA are wrapped around the octameric core of histones H2A, H2B, H3, and H4, called the nucleosome (Figure 2).<sup>46</sup> Each major globular (core) histone has a characteristic side chain or tail, rich in lysine and arginine residues.<sup>47</sup> The histone tail undergoes extensive covalent post-translational modifications (PTMs), which collaboratively regulate the chromatin state. A large body of evidence shows that histone PTMs can directly modify chromatin structure.<sup>48</sup> In addition, histone modifications also interact with DNMTs/TETs to drive or suppress transcription.

#### Histone methylation

Detailed studies are required to understand the effect of histone methylation/demethylation on VSMCs and ECs in the pathological process of ISR. Activation or inhibition of transcription by different histone lysine methylations depends on the site, degree of methylation, genomic location, and other co-existing PTM states.<sup>49</sup> It is generally thought that methylated histone H3 lysine 4, 36, and 79 (H3K4, H3K36, and H3K79) are closely related to transcriptional activation, while methylated histone H3 lysine 9 and 27 (H3K9 and H3K27) and histone H4 lysine 20 (H4K20) are involved in transcriptional repression.<sup>50</sup> In addition to transcriptional regulation, histone lysine methylation is associated with many other cellular processes, such as DNA damage repair, DNA replication, and mRNA splicing. The functions of HMTs in ISR are discussed in the following section.

To date, more than 50 lysine HMTs have been identified,<sup>51</sup> which are highly selective of the target histone lysine residue. Furthermore, histone lysine methylation states depend on activation or repression of

observed that TET2 is a master epigenetic regulator of VSMC plasticity, as it activates the key transcription factors such as myocardin (MYOCD), SRF, and MYH11 and inhibits KLF4. In addition, the loss of TET2 hampers the rapamycin-induced VSMC phenotype, whereas TET2 overexpression induces contractile differentiation. These findings showed that TET2 is a novel epigenetic regulator of the phenotypic transformation of VSMCs.

Emerging evidence indicates the importance of the relationship between DNA methylation and EC function. Peng et al.<sup>40</sup> demonstrated that elevated levels of TET2 repressed nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation in ox-LDL-induced ECs, downregulating pro-inflammatory chemotactic molecules, including ICAM-1 and VCAM-1, and protecting EC function, whereas TET2 knockdown induced the opposite effect. Interestingly, consistent with these results, previous studies have suggested that TET2 ameliorated low OS-mediated EC dysregulation,<sup>41</sup> increased autophagy, and decreased the expression of inflammatory factors in an ApoE<sup>-/-</sup> mouse model to hinder the development of atherosclerosis.<sup>42</sup> However, the exact molecular mechanisms of the TET2 effect and its role in signal transduction pathways have not been elucidated, which may help prevent or treat endothelial dysfunction in restenosis. A recent study investigated the relationships between TET2 and miRNA in modulating the abnormal function of ECs. miR-101-3p overexpression induced EC-mediated generation of reactive oxygen species (ROS), activated NF- $\kappa$ B, and subsequently dysregulated the ECs. Mechanistically, miR-101-3p contributed to EC dysfunction by repressing TET2 expression at the post-transcriptional level. Furthermore, elevated levels of TET2 attenuated cell death, ROS production, and inflammation caused by the miR-101-3p-induced ECs. This study presented a new approach to improving ISR lesions by targeting miR-101-3p and its

## Review

HMTs. In the following sections, we discuss the role of HMTs in vascular function and ISR development.

EZH2 is a member of the HMT family that plays important catalytic roles in the polycomb repressor complex 2 (PRC2). Studies have shown that PRC2 methylates H3K27, thereby causing transcriptional repression of the target genes.<sup>52</sup>

The long ncRNA (lncRNA) taurine-upregulated gene 1 (TUG1) was upregulated in synthetic VSMCs, accompanied by an increase in EZH2 expression. However, the level of lysine methylation was high in differentiated VSMCs. Further studies are required to understand the effects of methylation sites and amounts of lysine on VSMC biology.<sup>53</sup> Kolliputi and colleagues<sup>54</sup> and Liang et al.<sup>55</sup> observed that overexpression of EZH2 contributed to the proliferation and migration of SMCs and inhibited apoptosis, resulting in intima thickening. Mechanistically, an upregulated EZH2 level increased H3K27me3 levels, thereby inducing VSMC proliferation.<sup>56</sup> Conversely, Li et al.<sup>57</sup> concluded that elevated EZH2 facilitated VSMC growth, which depended on autophagy rather than proliferation. A reduced EZH2 level promoted autophagosome formation and led to aortic dissection. Furthermore, Hcy elevated the expression of EZH2 in the aorta in an ApoE<sup>-/-</sup> mouse.<sup>58</sup> Repression of EZH2 ameliorated thoracic aortic aneurysm in the mouse model of Marfan syndrome.<sup>59</sup>

In addition to regulating various VSMC functions, EZH2 is also associated with EC function. Previous studies by Smits et al.,<sup>60</sup> Floris et al.,<sup>61</sup> and Xu et al.<sup>62</sup> have shown that miR-101 expression led to pro-angiogenic effects by partially diminishing the EZH2 level, thereby promoting hypermethylation of H3K27 and altering the transcriptome. Furthermore, EZH2 acts not only as a target but also as a transcriptional inhibitor of miR-101 in ECs. Suppression of EZH2 reduces EC migration *in vitro*, indicating that EZH2 inhibitors can be used for treating abnormal vascularization. Xu et al.<sup>62</sup> and Maleszewska et al.<sup>63</sup> observed that fluid OS decreased the mRNA and protein levels of EZH2, leading to endothelial anti-inflammatory phenotype. Further research should focus on the precise role of EZH2 in endothelium-specific knockout mice using laminar flow induction. A recent study indicated that under ox-LDL treatment, lncRNA OIP5-AS1 promoted EC apoptosis by targeting GSK-3 $\beta$  via EZH2.<sup>64</sup> In addition, Tsou et al.<sup>65</sup> demonstrated that overexpression of EZH2 in ECs inhibited angiogenesis and restored normal angiogenesis in scleroderma.

These results implied that EZH2 plays a central role in vascular cells may be useful as a new therapeutic target for treating ISR.

### HDMs

To date, more than 20 HDMs have been identified and characterized.<sup>66</sup> Most importantly, aberrant expression of HDMs has been correlated with many diseases, including cardiovascular disease. The subsequent sections highlight the key HDMs that play crucial roles in the pathologic process of ISR.

JMJD3 (also known as KDM6B), a member of the JmjC-domain protein demethylase family, demethylates H3K27me2/3.<sup>67</sup> Activation of NF- $\kappa$ B is a feature of inflammation. JMJD3 increases the level of inflammatory cytokines (interleukin [IL]-1 $\beta$ , tumor necrosis factor [TNF]- $\alpha$ , IL-6) and adherence factors (ICAM-1, matrix metalloprotease [MMP]-9) and nuclear translocation of NF- $\kappa$ B/p65 in LPS-mediated ECs, subsequently leading to vascular cell inflammation.<sup>68,69</sup> Mechanistically, LPS promoted JMJD3 nuclear accumulation and assembly of JMJD3 and NF- $\kappa$ B at the promoter of target genes, indicating that both JMJD3 and NF- $\kappa$ B can activate the expression of target genes.<sup>69</sup> These findings revealed the epigenetic regulation of the LPS-induced NF- $\kappa$ B pathway in the endothelium and suggested Jmjd3 as a key regulator of the NF- $\kappa$ B pathway and potential therapeutic targets for NF- $\kappa$ B-related diseases. In addition, anti-inflammatory agents have been used to regulate JMJD3. Na et al.<sup>70</sup> reported that JMJD3 expression was inhibited in dexamethasone-induced VSMCs by negative glucocorticoid response element binding in the upstream region of the gene, subsequently resulting in suppression of *MMP-2*, *MMP-3*, and *MMP-9*. Furthermore, dexamethasone activates the expression of claudin 5 and occluding-encoding genes. This suggests that dexamethasone can maintain the integrity of ECs under inflammatory conditions. In addition, Lee et al.<sup>71</sup> demonstrated that JMJD3 is upregulated in ECs under oxygen-glucose deprivation/reperfusion injury. JMJD3-induced demethylation results in activation of *IL6*.

Recent studies have also reported the association of JMJD3 with NF- $\kappa$ B, resulting in the proliferation of bladder SMCs and collagen accumulation, which promotes bladder fibrosis.<sup>72</sup> Luo et al.<sup>73</sup> demonstrated that PDGF induced reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (*NOX4*) expression and elevated the JMJD3 level, which subsequently reduced H3K27me3 marks at the *NOX4* promoter and activated autophagy in VSMCs. An inhibitor of JMJD3 inhibited *NOX4* expression owing to H3K27me3 enrichment at the gene promoter. Furthermore, loss of JMJD3 and *NOX4* inhibited autophagic activation, thereby alleviating vascular remodeling after carotid injury. Notably, elevations in JMJD3 and *NOX4* levels are observed during the formation of human atherosclerotic plaques. Lack of JMJD3 blunts neointima formation after vascular injury by inhibiting the activation of *Nox4* autophagy signals, demonstrating that JMJD3 may be a prospective target for the prevention and treatment of vascular diseases. Overall, although increasing evidence shows JMJD3 to be associated with vascular cell function, the mechanism responsible for JMJD3 regulation remains to be completely elucidated.

JMJD1 (KDM3a) is another HDM. Previous studies have reported that elevation of KDM3a expression promoted high glucose and angiotensin II (AngII)-induced proliferation and migration of VSMCs, which were reduced considerably under conditions of KDM3a deficiency. Furthermore, consistent with this *in vitro* result, elevated levels of KDM3a were promoted, whereas loss of KDM3a decreased neointima formation after vascular injury in a balloon injury model. Furthermore, JMJD1 expression was upregulated in

vessels of a diabetic rat. We observed that KDM3a may act as a novel target for regulating VSMC function and vascular neointimal hyperplasia.<sup>74</sup>

### Histone acetylation

Histone acetylation is a conserved reversible modification modulated by HATs and HDACs, which is associated with various cellular functions. Previous studies in animal models have shown that reversible acetylation plays a crucial role in cardiovascular disorders, such as vascular diseases, heart failure, hypertension, arrhythmia, and angiogenesis.<sup>75</sup> HATs are classified into three major families: (1) GCN5-related *N*-acetyltransferases (GNATs), (2) p300/CREB-binding protein (p300/CBP), and (3) and the MOZ, Ybf2, Sas2, and Tip60 (MYST) family.<sup>76</sup> Histone acetylation increases gene expression by “opening” the chromatin structure. In contrast, HDACs “close” the chromatin structure and inhibit gene expression. HDACs not only deacetylate histone, but they also bind to non-histone proteins to inhibit transcription. HDACs have been classified into four groups based on their sequence similarity: type I (HDAC1, 2, 3, and 8), type II (IIa, HDAC4, 5, 7, and 9; IIb, HDAC6 and 10), type III (NAD<sup>+</sup>-dependent sirtuin [SIRT]1–7), and type IV (HDAC11).<sup>77</sup> In the next section we focus on the role of HATs and HDACs in restenosis.

p300 is a major acetyltransferase and a transcriptional co-activator, which is closely related to many diseases, including cardiovascular disorder. A recent study reported that MFN2 exerts an anti-proliferative effect in VSMCs both *in vivo* and *in vitro*.<sup>27</sup> All-*trans* retinoic acid promotes *KLF4* acetylation by promoting the interaction of p300 with *KLF4*. Furthermore, the co-expression of p300 and *KLF4* activates the MFN2 promoter to induce MFN2 expression in VSMCs.<sup>78</sup> In addition, He et al.<sup>79</sup> showed that *KLF4* modulated the association between TGF- $\beta$ 1-induced gene transcription and H3 acetylation in VSMCs. These results describe a novel mechanism wherein all-*trans* retinoic acid upregulated *KLF4* expression and phosphorylation to eventually activate the MFN2 promoter and further expand the *KLF4* acting network in VSMCs. *KLFs* also regulate inflammation in VSMCs. Lu et al.<sup>80</sup> demonstrated that the loss of *KLF15* activated the inflammatory pathway in VSMCs and promoted atherogenesis. Mechanistically, *KLF15* deletion enhanced the acetylation status and activity of the NF- $\kappa$ B pathway due to interaction with p300. Furthermore, the marked decrease in the expression of *KLF15* in human atherosclerotic specimens supported this observation. de Jong et al.<sup>81</sup> demonstrated that p300/CBP-associated factor (PCAF) accelerated NF- $\kappa$ B-induced inflammation and played a key role in regulating subsequent VSMC proliferation after injury. Interestingly, three independent large prospective studies have shown an association between mutations in the –2481C allele of the PCAF gene and reduced vascular mortality, suggesting that PCAF is a possible diagnostic marker for restenosis.<sup>82</sup>

lncRNAs are also related to histone acetylation. lncRNA growth arrest-specific 5 (*GAS5*) inhibits proliferation, induces apoptosis, and promotes cell cycle arrest of VSMCs. Mechanistically, *GAS5* directly

binds to p300 and p53, stabilizing the interaction of p53 with p300 and regulating VSMC function.<sup>83</sup> Shah et al.<sup>84</sup> showed that atherosclerotic plaques predominantly exhibit oxidative DNA damage, mainly as 8-oxoguanine (8oxoG) lesions owing to decreased acetylation of OGG1. p300 was identified as the OGG1 acetyltransferase, which modulates the endogenous expression of OGG1 via acetylation. p300 was downregulated in the VSMCs of the human atherosclerosis plaque response to oxidative stress. Furthermore, p300 regulated both OGG1 stability and activity.

Shali et al.<sup>85</sup> showed that glucose upregulated p300 and caused nuclear DNA damage in ECs. p300 induced histone acetylation and activated various transcription factors by binding to the promoters of genes encoding angioactive factors and ECM proteins, subsequently leading to upregulation of vasoactive factors and ECM proteins. Data from these studies show that p300 upregulation is an important upstream epigenetic mechanism for regulating the expression of vasoactive factors and ECM protein genes in ECs. Altogether, p300 plays an important role in regulating inflammation, proliferation, and autophagy. Hence, understanding these mechanisms may contribute to the development of better strategies for treating restenosis.

### HDACs

HDAC5 is a class IIa HDAC. Previous studies have indicated that AngII-mediated HDAC5 phosphorylation and nuclear export lead to enhanced MEF2 transcriptional activity and VSMC hypertrophy.<sup>86</sup> This research demonstrates new roles for HDAC5 in AngII signaling in VSMCs. Tsou et al.<sup>87</sup> showed that HDAC5 knockdown enhanced the formation of the EC tube, thereby improving scleroderma. Furthermore, fluid OS also induced HDAC5 phosphorylation, and hence it downregulated HDAC5-induced *KLF2* and eNOS expression, with consequent anti-inflammatory effects.<sup>88,89</sup> Previous experiments have also indicated that HDAC5 plays a key regulatory role in EC proliferation and migration in a gene-deficient mouse model.<sup>90</sup> Taken together, the results presented in this section support the view that HDAC5 silencing can be a therapeutic target against neointimal hyperplasia and restenosis.

HDAC9 is a member of class IIa HDAC. Genome-wide association studies have shown that several genetic variants of HDAC9<sup>91</sup> are associated with large vessel ischemic stroke<sup>92</sup> and myocardial infarction.<sup>93</sup> Recently, Malhotra et al.<sup>94</sup> observed that loss of HDAC9 reduced VSMC proliferation and increased contractility. Furthermore, mutations in the VSMC cytoskeleton led to the formation of ternary complexes containing HDAC9, chromatin-remodeling enzyme BRG1, and lncRNA MALAT1. Knockdown of HDAC9 can restore contractility protein expression and improve aortic wall structure.<sup>95</sup> In ECs, HDAC9 expression was increased with ox-LDL exposure to apoptosis-inducing doses.<sup>96</sup> Han et al.<sup>96</sup> and Shi et al.<sup>97</sup> have observed that HDAC9 depletion significantly antagonizes ox-LDL-induced apoptosis and inflammatory response. Taken together, this evidence suggests that targeted inhibition of HDAC9 is a promising strategy to prevent restenosis development.

Knockdown of HDAC1 and HDAC3 (type I HDAC) decreased SMC proliferation and neointima formation in murine models of vascular injury.<sup>98</sup> Arginase 2 (Arg2) is a key target in the progression of atherosclerosis because it controls endothelial NO, proliferation, fibrosis, and inflammation. HDAC2 (type I HDAC) overexpression inhibited Arg2 expression in ECs, while knockdown of HDAC2 increased Arg2 expression. Furthermore, HDAC2 overexpression improves ox-LDL-induced vascular dysfunction.<sup>99</sup> These studies provide a novel therapy for endothelial dysfunction and restenosis by activation of HDAC2. HDAC3 is also critical for maintaining endothelial integrity through the phosphatidylinositol 3-kinase (PI3K)/Akt mechanism.<sup>100</sup> Zhang et al.<sup>101</sup> and Usui et al.<sup>102</sup> found that HDAC4 (type II HDAC) promoted the proliferation and migration of VSMCs mediated by PDGF-BB.<sup>102</sup> *In vivo*, the HDAC inhibitor MC1568 attenuated neointimal hyperplasia in a mouse model of carotid artery ligation.<sup>103</sup> These findings suggest that HDAC4 regulates VSMC proliferation and migration, and HDAC4 inhibitors are expected to be novel targets for the prevention of restenosis. HDAC6 is a member of the type IIb HDAC family. Knockdown of HDAC6 attenuates the proliferation and migration of VSMCs and reduces neointima formation after vascular injury.<sup>103,104</sup> In addition, Zhang et al.<sup>104</sup> observed that loss of HDAC6 prevents dedifferentiation of VSMCs *in vitro* and reduces the formation of new intima and restenosis *in vivo*. The mechanism is that inhibition of HDAC6 enhances SRF nuclear activity and maintains SMC contractile levels. These findings suggest that targeting HDAC6 represents an attractive therapeutic approach to treat proliferative vascular diseases, including restenosis. The role of HDAC in restenosis progression deserves further study in experimental animal models.

The SIRT6s belong to the family of class III NAD<sup>+</sup>-dependent HDACs, which catalyze deacetylation/deacylation on histones and protein substrates.<sup>105</sup> Seven closely related SIRT family members have been identified, that is, SIRT1–SIRT7. SIRT6 regulates numerous pathophysiological processes, for instance, energy metabolism, inflammation, genomic stability, and aging.<sup>106</sup> SIRT1 is the most widely studied member of SIRT6s, and accumulating studies have found that SIRT1 plays a protective role in CADs.<sup>105,107</sup> In the vascular system, SIRT1 negatively regulates vascular inflammation,<sup>108</sup> endothelial dysfunction,<sup>109</sup> and VSMC proliferation and migration,<sup>110</sup> thereby preventing vascular aging, intimal hyperplasia, and vascular proliferative disease. The expression of SIRT1 protein is decreased in the plaque area of atherosclerotic patients.<sup>111,112</sup> In addition, expression of the SIRT1 gene in mononuclear cells in patients with coronary atherosclerosis was reduced,<sup>113</sup> suggesting a systemic decline in SIRT1 levels in the disease. However, the question as to whether the impaired SIRT1 level in patients with coronary atherosclerosis is a result of the atherosclerotic process itself, or vice versa, remains to be investigated. SIRT1 expression was also decreased in ApoE<sup>-/-</sup> mice.<sup>114</sup> In addition, SIRT1 EC-<sup>115</sup> and VSMC-specific<sup>112</sup> overexpression can prevent the development of atherosclerosis in mice. These pieces of evidence suggest that SIRT1 has a protective effect on the cardiovascular system in general. Despite some controversy, overexpression of SIRT1 may be a new therapeutic target for cardiovascular diseases.

In human VSMCs, endogenous SIRT1 is gradually lost with aging, leading to cellular functional defects, including impaired stress response and reduced cell migration and proliferation ability.<sup>116</sup> The neointima formation was related to the decreased expression of SIRT1, while the overexpression of SIRT1 in VSMCs weakened the neointima formation after vascular injury.<sup>117</sup> Overexpression of SIRT1 in VSMCs inhibited AngII-<sup>118</sup> as well as injury-induced<sup>119</sup> VSMC hypertrophy and neointimal formation. Additionally, Li et al.<sup>117</sup> showed that SIRT1 inhibited the proliferation and migration of VSMCs through G<sub>1</sub>/S cell cycle stagnation, thus weakening the formation of neointimal formation. Furthermore, SIRT1 inhibits the uptake of ox-LDL in VSMCs by inhibiting expression levels of LOX-1 and NF- $\kappa$ B signaling pathways.<sup>112</sup> These findings offer a new view that the inhibitory effect of SIRT1 on VSMC proliferation and migration is the basis of neointima formation and suggest that SIRT1 may be a potential target for vascular disease intervention. Increasing SIRT1 activity should be further investigated as a promising treatment option for restenosis.

SIRT1 also exerts a protective role in ECs. SIRT1 is highly expressed in the growing vascular endothelium.<sup>120</sup> However, the expression level and activity of SIRT1 in inflammatory ECs were significantly decreased.<sup>121</sup> SIRT1 binds to the calmodulin of eNOS and thus enhances the production of endothelial NO. However, inhibition of SIRT1 results in decreased bioavailability of NO, suggesting that SIRT1 plays a key role in endothelium-dependent eNOS-mediated vascular homeostasis.<sup>122</sup> However, Guo et al.<sup>123</sup> found that no improvement in the bioavailability of NO was observed in SIRT1-overexpressed eNOS gene knockout mice. This suggests that eNOS plays an important role together with SIRT1, which has a beneficial effect on the bioavailability of NO. Besides NO, ET-1 (vasoconstrictor) and other factors may also play a key role in endothelial function. The reduction of SIRT1 was related to endothelial injury and increased expression of ET-1.<sup>124</sup> Although ET-1 is primarily a vasoconstrictor, it induces NO and prostaglandins to promote vasodilation. SIRT1 is very important to the regulation of NO and ET-1, respectively; thus, the question occurs whether SIRT1 can regulate the conversion between NO and ET-1. If so, how will SIRT1 regulate this process? Hcy induced EC injury, which is caused by downregulation of SIRT1 in the coronary artery and increased oxidative stress, in a mouse model of hyperhomocysteinemia.<sup>125</sup> Lu et al.<sup>126</sup> found that the high-fat diet-induced EC damage in rats was accompanied by an increased oxidative stress response and a decrease in eNOS and SIRT1 mRNA. Similarly, SIRT1 inhibition increases the expression of p53 and plasminogen activator inhibitor 1, leading to damage-induced neointimal formation and remodeling.<sup>127</sup> These studies demonstrated that the downregulation of SIRT1 may be a key factor mediating the endothelial inflammatory response and oxidative stress, leading to endothelial dysfunction. Therefore, activation of SIRT1 may provide a promising therapeutic strategy for cardiovascular diseases.

SIRT6 is a site-specific HDAC that regulates chromatin structure.<sup>128</sup> Similarly, in ApoE<sup>-/-</sup> mice<sup>129</sup> and human atherosclerotic plaques,<sup>130</sup>



the expression of SIRT6 was decreased. Furthermore, Yao et al.<sup>131</sup> observed that SIRT6 mediated by the TGF-1/Smad signaling pathway regulates VSMC differentiation. SIRT6 is an important regulator of EC function. The protective effect of SIRT6 on endothelial function is attributed to promoting endothelial-dependent vasodilation and bioavailability of vascular NO, reducing cell permeability, improving endothelial senescence and apoptosis, and promoting autophagy.<sup>132</sup> Accumulating research has demonstrated that SIRT6 deficiency enhances the expression of endothelial proinflammatory cytokines and the transcriptional activity of NF- $\kappa$ B.<sup>130,133,134</sup> Collectively, these studies suggest that SIRT6 plays a crucial role in regulating vascular cells, especially endothelial dysfunction, by regulating a variety of pathophysiological pathways.

AngII leads to cell migration, whereas depletion of SIRT2 inhibits cell migration. AngII induces microtubule redistribution and deacetylation through SIRT2 in ECs, implying that SIRT2 plays a new role in vascular remodeling induced by hypertension.<sup>135</sup> SIRT3 was stably expressed in mice VSMCs, while AngII increased SIRT3 in VSMCs.<sup>136</sup> SIRT3 elevations significantly inhibit VSMC proliferation and neointimal hyperplasia.<sup>137</sup> Yet, SIRT3 silencing will further increase cell proliferation.<sup>136</sup> However, the role of SIRT in restenosis needs to be studied more extensively in experimental animal models.

#### Histone ubiquitination

Ubiquitin is a small highly conserved globular protein of 76 aa, which can be covalently attached to proteins by ubiquitin ligases. Ubiquitination is an ATP-dependent three-stage enzymatic cascade. Ubiquitin is activated by the ubiquitin E1 enzyme, bound by the E2 enzyme, and ligated to the target protein by the E3 enzyme.<sup>46</sup> While evaluating the effect of histone ubiquitination and deubiquitination in ISR, Zhao et al.<sup>138</sup> reported that interferon (IFN)- $\gamma$  exacerbates neointimal hyperplasia by facilitating ubiquitin-dependent liver X receptor- $\alpha$  (LXR- $\alpha$ ) degradation. Consistent with the *in vitro* results, neointimal hyperplasia was observed after 4 weeks of carotid ligation in ApoE<sup>-/-</sup> mice after treatment with LXR agonist (T0901317). Furthermore, IFN- $\gamma$  promoted inflammation by inducing ubiquitination. Ubiquitination is closely regulated by E3 ligases, which participate in the regulation of various cellular functions. In subsequent sections, we discuss the specific roles of E3 ligases in ISR.

The E3 ubiquitin ligase mouse double minute 2 homolog (MDM2) is the main negative regulator of p53, as it induces p53 ubiquitination and degradation and impedes p53 transcriptional activity.<sup>139</sup> A large number of studies have revealed the correlation between MDM2 and the p53 signaling pathways in vascular ECs and VSMCs.

The function of MDM2 in VSMCs has received considerable attention. In particular, although MDM2 is a vital regulator of p53 activity, the transcription of MDM2 itself also depends on p53, forming a p53/MDM2 negative feedback loop. Hashimoto et al.<sup>140</sup> have indicated that disruption of MDM2-p53 interaction inhibited neointimal hyperplasia after vascular injury. Furthermore, Li et al.<sup>141</sup> reported that p55 $\gamma$ , a regulatory subunit of PI3Ks, clearly blunted the

MDM2-mediated ubiquitination of p53 in VSMCs. *In vitro*, overexpression of p55 $\gamma$  inhibited VSMC proliferation, increased the p53 protein level, and reduced the amount of ubiquitinated p53. This study indicated that p55 $\gamma$  stabilizes p53 by hindering the ability of MDM2 to interact with p53, thereby hampering p53 ubiquitination. *In vivo*, loss of p55 $\gamma$  increases neointimal thickening following injury. Furthermore, lncRNAs are also involved in ubiquitination. Long intergenic non-coding (linc)RNA-p21 inhibits VSMC proliferation and induces apoptosis *in vitro*. Mechanistic studies have indicated that lincRNA-p21 and p53 compete for binding to MDM2 in VSMCs. lincRNA-p21 directly binds to MDM2 and promotes p53-p300 interaction, enhancing p53 transcriptional activity. Reduced lincRNA-p21 expression was observed in the atherosclerotic plaque of ApoE<sup>-/-</sup> mice and in patients with CAD.<sup>142</sup> Thus, these studies demonstrated that disruption of the MDM2-p53 interaction impedes VSMC proliferation and neointimal hyperplasia.

MDM2 also plays a significant role in ECs. OS and VEGF-A stimulation upregulated MDM2 phosphorylation on serine 166.<sup>143,144</sup> Furthermore, overexpression of a Ser166-MDM2 phosphorylation mimetic increased the binding of MDM2 to FoxO1, decreased the FoxO1 level (known to promote an angiostasis gene), and promoted EC migration.<sup>143</sup> These results highlighted the importance of MDM2 in maintaining survival and responsiveness to pro-angiogenic stimuli in ECs.

Overall, E3 ubiquitin ligases are regulated in VSMCs and ECs and may act as “druggable” targets for the treatment of ISR.

#### Histone deubiquitination

DUBs are peptidases that reverse ubiquitination by removing ubiquitin molecules from target proteins.<sup>145</sup> The human genome encodes approximately 100 DUBs, which can be divided into two main groups according to the mechanism of enzymatic cleavage: (1) cysteine proteases, and (2) zinc metalloproteases. Furthermore, cysteine proteases can be classified into four groups: ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), and Machado-Joseph disease protein domain proteases (MJDs).<sup>145</sup> In the next section, we describe DUBs, particularly USPs, in the context of vascular cell function and ISR development.

USPs are the most abundant DUBs, with approximately 60 proteases in humans.<sup>146</sup> USP20 inhibits NF- $\kappa$ B activation via Toll-like receptor (TLR)4,<sup>147</sup> IL-1R, and the TNF<sup>148</sup> inflammatory pathway in VSMCs. Intriguingly, USP20 impedes TLR4-induced NF- $\kappa$ B activation by deubiquitinating TNF receptor-associated factor 6,<sup>147</sup> whereas it inhibits IL-1R and TNF-triggered NF- $\kappa$ B activation by deubiquitinating receptor-interacting protein kinase 1.<sup>148</sup> Consistent with the results of the *in vitro* experiments, USP20 decreased neointimal hyperplasia caused by carotid endothelial denudation in a transgenic mice model.<sup>147</sup> In addition, USP20 attenuated atherosclerosis in an LDLr<sup>-/-</sup> mouse model by inhibiting NF- $\kappa$ B activation *in vivo*.<sup>148</sup> Therefore, increased expression/activity of USP20 may offer protection against inflammatory pathways in patients with ISR.

### BET

The BET protein family consists of two tandem domains (reader for lysine acetylation) and one extra-terminal domain, including BRD2, BRD3, BRD4, and BRDT. The BET family proteins use bromodomain-targeted modifications of histones and regulate transcription through interactions with transcription mechanisms.<sup>24</sup> Mechanical force, injury, and cytokine response to VSMC stimulation can lead to (re)stenosis and other proliferative vascular lesions, all involving BET, especially BRD4.<sup>24</sup> Wang et al.<sup>149</sup> found that BRD4 is dramatically elevated in the neointima of injured rat carotid arteries as well as human vein and artery grafts. However, (+)-JQ1 (BET-specific inhibitor) can reduce the proliferation and migration of SMCs *in vitro*, thus effectively mitigating the neointima formation.<sup>149,150</sup> Further studies suggest that (+)-JQ1 induces G<sub>0</sub>/G<sub>1</sub> cell cycle arrest and prevents VSMC proliferation.<sup>150</sup> In ECs, VEGF stimulation enhances the acetylation of ETS-1 (v-ETS avian erythroblastosis virus E26 oncogene homolog 1), causing its binding to BRD4, resulting in angiogenesis.<sup>151</sup> Thus, decreasing BRD4 expression/activity may provide protection against restenosis patients.

### ncRNAs

The relationship between ncRNAs and epigenetics has opened up new domains of research, as they have been shown to modulate gene expression. In addition to DNA methylation and histone modifications, accumulating evidence has highlighted the role of ncRNAs in modulating ISR.<sup>152</sup> ncRNAs are mainly categorized into small RNAs (<200 bp) (small ncRNAs) or long RNAs (>200 bp) (lncRNAs) based on transcript size. Small ncRNAs consist of miRNAs, small nucleic RNAs (snRNAs), small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), QDE-2-interacting RNAs (qiRNAs), and piwi-interacting RNAs (piRNAs).<sup>153</sup> lncRNAs consist of lincRNA, circular RNA (circRNAs), natural antisense transcripts (NATs), and enhancer RNAs (eRNAs).<sup>154</sup> Interestingly, increasing evidence proved that ncRNAs are critically involved in atherosclerosis, particularly ISR.<sup>155–159</sup> The human ncRNAs circulate in the bloodstream and can act as ISR biomarkers for the diagnosis and prediction of disease outcomes. Several studies have shown the critical role of ncRNAs in ISR regulation. The following section discusses the relationship between ISR and ncRNAs, primarily miRNAs, lncRNAs, and circRNAs.

miRNAs are small endogenous RNAs 19–25 nt in length, which modulate gene expression by targeting the 3' untranslated region of specific mRNAs, resulting in post-transcriptional gene silencing.<sup>153,160</sup> miRNAs, for example, miR-125b1 and miR-124, are involved in epigenetic regulation in combination with the incorporation of DNA methylation, inhibitory histone marks, or deficiency of transcription factors.<sup>161,162</sup> Furthermore, various miRNAs mediate the expression of epigenetic elements, such as DNMTs (regulated by miR-152, miR-30, and miR-148a/b), HDACs (regulated by miR-140, miR-1, and miR-449a), and a polycomb group of genes (regulated by miR-101 and miR-26a).<sup>163–171</sup> Previous studies have indicated that miRNAs are expressed in the vascular system. As shown in Table 1, numerous studies have indicated that miRNAs impinge on a wide variety of cellular activity networks, related to

the ISR biological pathway, including VSMC and EC function. miRNAs regulate VSMC proliferation, migration, phenotypic switch, calcification, inflammation, and apoptosis. In ECs, miRNAs modulate inflammation, leukocyte adhesion, and eNOS-dependent NO production. Several recent studies have shown that miRNAs can be detected in multiple body fluids, such as cerebrospinal fluid, saliva, and urine; furthermore, miRNAs are stable in blood circulation,<sup>190</sup> indicating the potential of miRNAs as biomarkers (Table 2) of restenosis diagnosis, prognosis, and treatment. Thus, the regulation of miRNA levels represents an exciting opportunity for the development of new treatments for ISR.

Until now, studies have shown that lncRNAs play a vital role in gene regulation during the development of cardiovascular diseases. lncRNAs can indirectly regulate DNA methylation at CpG sites, thereby modulating gene expression. For instance, lncRNA HOXB13-AS1 recruits DNMT3b to methylate and increase transcription from the HOXB13 promoter.<sup>195</sup> Similarly, lncRNA SPRY4-IT1 recruits the *de novo* DNA demethylase DNMT1 to regulate cholangiocarcinoma development.<sup>196</sup> lncRNAs can be used as a scaffold to promote interactions among proteins, RNA, and DNA, leading to gene activation or inhibition.<sup>197</sup> Similarly, the expression of many VSMC- and EC-related genes involved in the ISR process is modulated via lncRNAs (for example, MALAT1, MANTIS, and NEAT1).<sup>95,198,199</sup> lncRNAs are not only involved in epigenetic regulation such as DNA methylation and histone modification, but also in the regulation of gene expression in vascular systems. Similar to miRNAs, lncRNAs such as MYOSLID and NEAT1 regulate the VSMC phenotype switch,<sup>199,200</sup> XR007793 regulates proliferation and migration,<sup>201</sup> and GAS5 regulates apoptosis.<sup>83</sup> lncRNAs modulate endothelial function and dysfunction, including proliferation (by CRNDE),<sup>202</sup> migration (by HOTTIP),<sup>203</sup> apoptosis (by OIP5-AS1),<sup>64</sup> and inflammation (by TGFB2-OT1).<sup>204</sup>

In addition to these classic linear RNAs, recent studies have indicated that some lncRNAs can generate circRNAs. Interestingly, circRNAs are more stable than lncRNAs and are relatively tissue-specific.<sup>205–207</sup> Owing to their stability and tissue specificity, circRNAs might become a research hotspot for CAD—in particular, ISR—in the future.<sup>208</sup> Currently, the role of circRNAs in ISR has attracted considerable attention. Microarray chip analysis has revealed a specific circRNA expression profile in rat carotid artery after balloon injury. Studies have shown the presence of circDiaph3 in injured carotid artery tissues, which regulates VSMC differentiation, proliferation, and migration by targeting *Igf1r*.<sup>209</sup> Furthermore, numerous studies have reported that alterations in DNMTs and the methylation status of some genes that host circRNAs can lead to circRNA silencing, suggesting that circRNAs are involved in epigenetic regulation.<sup>210</sup> Furthermore, circRNAs can act as sponges for miRNAs and protect mRNAs from degradation, serve as scaffolds, and interact directly with proteins.<sup>205</sup> Similarly, circRNAs also regulate vascular cells in ISR. circRNAs mediate EC proliferation (hsa\_circ\_0003575)<sup>211</sup> and migration (circ\_0003204),<sup>212</sup> as well as VSMC proliferation (circ\_Lrp6),<sup>213</sup> migration (circTET3),<sup>214</sup> and phenotypic transformation (circ-SATB2).<sup>215</sup> In the future, ISR may be treated by targeting circRNAs.

**Table 1. Role of miRNAs in vascular pathophysiology**

miRNA	Mechanism	Cell type	Function	Animal models	Ref.
miR-145	KLF5	VSMC	phenotypic switch	rat carotid artery balloon injury model	172
miR-100	mTOR	EC, VSMC	EC proliferation, VSMC migration	femoral artery occlusion in mice model	173
miR-143		EC, VSMC	↑EC migration, angiogenesis, ↑VSMC proliferation, ↓VSMC apoptosis	calf models of PAH	174
miR-221/222	p27 (Kip1)/p57 (Kip2)	VSMC	↑proliferation	rat carotid artery balloon injury model	175
miR-24	Chi3l1	VSMC	↓proliferation	murine models of AAA	176
miR-92b-3p	TSC1	VSMC	↑proliferation		177
miR-22	EV11	VSMC	phenotypic switch	mouse femoral arterial injury models	178
miR-663	JunB/My19	VSMC	phenotypic switch, ↓proliferation, ↓migration, ↓neointimal formation	rat carotid artery ligation injury	179
miR-133	Sp-1	VSMC	phenotypic switch, ↓proliferation, ↓neointimal formation	rat carotid artery balloon injury model	180
miR-128-3p	KLF4	VSMC	phenotypic switch, ↓proliferation, ↓migration	mice model of stenosis	181
miR-223	PDGFRβ	VSMC	phenotypic switch, ↓neointimal formation	mice femoral artery wire injury model	182
miR-10	FLT1	EC	angiogenic	zebrafish	183
miR-30	DLL4	EC	angiogenic	zebrafish	184
miR-133a	GCH1	EC	endothelial dysfunction	mice or rat models of hyperglycemia, hyperhomocysteinemia, and dyslipidemia	185
miR-92a	SOC5	EC	endothelial dysfunction	LDLR <sup>-/-</sup> mice	186
miR-126-5p	DLK1	EC	↑proliferation		187
miR-424	CUL2	EC	angiogenic	rat myocardial infarction models	188
miR-181b	NF-κB	EC	inflammation	APOE <sup>-/-</sup> mice	189

Ref., reference; KLF5, Krüppel-like factor-5; VSMC, vascular smooth muscle cell; EC, endothelial cell; mTOR, mammalian target of rapamycin; Chi3l1, chitinase 3-like 1; AAA, abdominal aortic aneurysm; TSC1, consisting of tuberous sclerosis 1; EV11, ecotropic virus integration site 1 protein homolog; My19, myosin light chain 9; FLT1, fms-related tyrosine kinase 1; DLL4, Delta-like 4; GCH1, GTP cyclohydrolase 1; SOC5, suppressor of cytokine signaling 5; DLK1, Delta-like 1; CUL2, cullin 2; NF-κB, nuclear factor κB.

### Epigenetic drugs in treating ISR

Despite our understanding of the pathophysiological mechanisms underlying ISR, the incidence of ISR remains high, indicating that extensive efforts are required for developing a new treatment approach. The advent of epigenetics not only helps us to understand the mechanisms underlying restenosis but also provides new targets for ISR drug development. However, it is uncertain whether evidence exists that epigenetic molecular regulators are suitable for treating diseases. At present, several molecular regulators have been approved by a regulatory authority or are undergoing evaluation in clinical trials (Table 3). In most cases, these drugs are currently under evaluation or have been approved for the treatment of various malignancies.

As the epigenome is reversible and vulnerable to environmental factors, it may become a new avenue for ISR-related research. Designing clinically meaningful targets for treating ISR now appears feasible, as the interplay between epigenetic regulatory pathways is being revealed. Recent studies on epigenetic regulation have confirmed their potential as druggable targets in clinical medicine. To date, several epigenetic regulators have been considered effective in regulating vascular cells, raising the possibility of treating ISR by targeting epigenetic regulation in humans (Figure 3; Table 4). The subsequent sections discuss in detail the potential of using epigenetic drugs.

### DNMT inhibitors (DNMTis)

DNMTis are of two types, that is, nucleoside-based and non-nucleoside inhibitors.<sup>260</sup> DNMTi, which includes 5-Aza and 5-aza-2'-deoxycytidine (decitabine, DAC), has been approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome and acute myeloid leukemia (AML).<sup>261</sup> Dunn et al.<sup>220</sup> reported that 5-Aza decreased OS-mediated EC inflammation. Furthermore, 5-Aza inhibited atherosclerosis in an ApoE<sup>-/-</sup> mouse model. However, nucleoside inhibitors, including 5-Aza, are incorporated into DNA and are hence cytotoxic.<sup>262</sup> Non-nucleoside inhibitors, including hydralazine, procaine,<sup>263</sup> and mithramycin A,<sup>264</sup> as well as RG108,<sup>263</sup> also suppress the activity of DNMTs. Notably, some natural products have also been indicated to inhibit the activities of DNMTs, such as (-)-epigallocatechin-3-gallate (EGCG),<sup>265</sup> curcumin,<sup>252</sup> and genistein.<sup>266</sup> These findings provide further support regarding the possibility of treating vascular diseases with DNMTi.

### TET2 activators

Recent studies have indicated that vitamin C is not only an antioxidant but also a cofactor for dioxygenases of DNA methylation. Several studies have shown that vitamin C decreased EC barrier permeability to protect the integrity and function of ECs.<sup>267</sup> Furthermore, Barabutis et al.<sup>268</sup> reported that the combination of vitamin C and

**Table 2. Biomarkers of miRNA in the development of restenosis**

miRNA	Expression	ISR patients	Stent type	ISR	Sample	Reference
miR-21	↑		DES	coronary	plasma	<sup>191</sup>
miR-100	↓		DES	coronary	plasma	<sup>191</sup>
miR-143	↓		DES	coronary	plasma	<sup>191</sup>
miR-145	↓		DES	coronary	plasma	<sup>191</sup>
miR-19a	↓		DES	coronary	plasma	<sup>192</sup>
miR-126	↓		DES	coronary	plasma	<sup>192</sup>
miR-210	↓		DES	coronary	plasma	<sup>192</sup>
miR-378	↓		DES	coronary	plasma	<sup>192</sup>
miR-93-5p	↑		DES/BMS	coronary	plasma	<sup>193</sup>
miR-146a	↑		DES	coronary	plasma	<sup>194</sup>
miR-146b	↑		DES	coronary	plasma	<sup>194</sup>

ISR, in-stent restenosis; DES, drug-eluting stent; BMS, bare metal stent; ↑, upregulated; ↓, downregulated.

hydrocortisone prevented and repaired the LPS-mediated dysregulation of the pulmonary endothelial barrier. Altogether, the above results are due to vitamin C's ability to preserve NO and scavenge reactive oxygen. Furthermore, vitamin C enhances DNA methylation by promoting TET2 expression.<sup>269</sup> Nevertheless, the precise mechanism of action of vitamin C on vascular cells remains to be elucidated. In addition to vitamin C, evidence indicates the involvement of several factors related to the expression and activity of TET2, including Fe<sup>2+</sup>,<sup>270</sup> vicenin-2,<sup>271</sup> α-ketoglutarate,<sup>272</sup> VprBP,<sup>273</sup> and ndp300.<sup>274</sup> In particular, p300 acetylates TET2, which consequently partners with DNMT1.<sup>274</sup> Collectively, an increase in TET2 level protects vascular cells.

#### HDAC inhibitors (HDACis)

HDACis are divided into five main types according to their structure: hydroxamic acids, benzamides, depsipeptides, short-chain fatty acids, and cyclic peptides.<sup>275</sup> Findeisen et al.<sup>98</sup> showed that Scriptaid, an HDACi, caused mediation of cell cycle arrest, suppressed proliferation of VSMCs, and repressed neointima formation following vascular injury in a murine model of endovascular endothelial denudation. (R)-trichostatin A (TSA) is a natural compound, which belongs to the class of hydroxamate antibiotics.<sup>276</sup> Previous studies have indicated that TSA exacerbates neointima formation in the LDLr<sup>-/-</sup> mouse model,<sup>233</sup> and it furthermore facilitates myocardial hypertrophy in neonatal rats<sup>234</sup> and neointimal thickening after vascular injury in a murine model.<sup>235</sup> The HDACi activity of butyrate was first identified in HeLa and Friend erythroleukemia cell lines in 1977.<sup>277</sup> A recent study has demonstrated that butyrate inhibits proliferation via the HDAC and PI3K/Akt signaling network.<sup>278</sup>

In addition, miRNAs also inversely regulate HDACi. Zhang et al.<sup>101</sup> reported that miR-149-5p inhibits VSMC proliferation and migration by targeting HDAC4, suggesting that miR-149-5p also suppresses HDAC4. These studies clearly indicate that HDACi can be potentially used for prevention of vascular cell damage as a bioactive component of dietary fiber and in therapeutic intervention of restenosis.

#### SIRT-activating compounds (STACs)

According to the overall protection of SIRT against VSMCs and ECs, several natural and synthetic SIRT activators have been developed and have shown protective effects against cardiovascular disease. Currently, the best studied activator of SIRT1 is resveratrol, a plant polyphenol plant antioxidant.<sup>132</sup> In a wire-injured artery mouse model, oral administration of resveratrol significantly inhibited intimal hyperplasia.<sup>225,279</sup> However, oral resveratrol is highly absorbable in the human body, but bioavailability is low. This raises the question of whether other mechanisms are available to mediate indirect SIRT1 activation.<sup>280</sup> In a rat wire-injured artery model, intraperitoneal injection of the phytochemical resveratrol suppresses the development of intimal hyperplasia.<sup>236,238</sup> Yet, recent studies have shown that relatively high concentrations of resveratrol exhibit arginase inhibitory activity in VSMCs, which can enhance the vasoconstriction response.<sup>281</sup> However, recent studies have shown that relatively higher concentrations of resveratrol enhance vasoconstriction by inhibiting arginase activity in VSMCs. Thus, the optimal delivery method of resveratrol *in vivo* should be fully studied. In addition, multiple SIRT6 activators have been reported to have inhibited VSMC proliferation, including fluvastatin<sup>282</sup> and fucoidan<sup>283</sup> (a rat wire-injured artery model). However, the control of SIRT1 and SIRT6 activity in restenosis by specific activators remains a challenge, and although SIRT1 has made some progress, *in vitro* experiments on SIRT6 are still limited.

#### HMTis (EZH2 inhibitors)

A series of inhibitors of EZH2, including 3-deazaneplanocin A (DZNep), EPZ005687, EI1, GSK126, GSK343, or UNC1999,<sup>284</sup> have been developed and studied.<sup>285</sup> GSK126 inhibits monocyte adhesion to ECs and thus protects from endothelial inflammation.<sup>62</sup> Furthermore, treatment with GSK343 recovered SM22α expression and subsequently improved aortic performance in the Marfan mouse model.<sup>59</sup> Liang et al.<sup>55</sup> indicated that application of UNC1999 protected against the neointimal formation and VSMC proliferation in a carotid wire-guided injury model. Overall, these results indicate that EZH2 inhibitors can be used potentially as candidate drugs for vascular diseases.

#### E3 inhibitors (MDM2 inhibitors)

The effect of E3 ubiquitin ligase inhibitors, such as MDM2 inhibitors, has been extensively investigated. MDM2 and the MDM2-p53 pathway are targeted for preventing and/or treating cancer. Nutlins, one of the earliest MDM2 antagonists, were identified in the early 21st century. The nutlin-3 treatment alleviates neointimal hyperplasia and the intimal/media area ratio following vascular injury in mice.<sup>140</sup> A phase 1 trial experiment of another MDM2 antagonist, DS-3032b, in combination with quizartinib, has been conducted for patients with AML (ClinicalTrials.gov: NCT03552029). Similarly, the MDM2 antagonist, SAR405838, is under evaluation in a phase 1 clinical trial in combination with pimasertib for the treatment of malignant neoplasms (ClinicalTrials.gov: NCT01985191).<sup>286</sup> So far, several natural products, namely, genistein,<sup>287</sup> oroxylin,<sup>288</sup> apigenin,<sup>289</sup> ginsenosides<sup>290</sup> (25-OCH3-PPD, 25-OH-PPD, and 20(S)-ginsenoside

**Table 3. Drugs that target epigenetic regulation that have been approved or are in clinical trials**

Drug	Epigenetic mechanisms	Phase	Disease	Reference or <a href="https://clinicaltrials.gov">ClinicalTrials.gov</a> identifier
Azacitidine	DNMTi	FDA approved	MDS	<sup>216</sup>
Guadecitabine	DNMTi	III	AML	NCT02920008
Entinostat	HDACi	III	breast adenocarcinoma	NCT02115282
Vorinostat	HDACi	FDA approved	CTCL	<sup>217</sup>
Romidepsin	HDACi	FDA approved	CTCL	<sup>216</sup>
Panobinostat	HDACi	FDA approved	multiple myeloma	<sup>218</sup>
Givinostat	HDACi	II	chronic myeloproliferative neoplasms	NCT01761968
Mocetinostat	HDACi	II	non-small cell lung cancer	NCT02954991
Valproic acid	HDACi	I	CLL	NCT00810680
Belinostat	HDACi	FDA approved	PTCL	<sup>219</sup>
CXD101	HDACi	I	advanced cancer	NCT01977638
MPT0E028	HDACi	I	advanced solid malignancies	NCT02350868
CPI-1205	EZH2 inhibitor	I	B cell lymphoma	NCT02395601
Tazemetostat	EZH2 inhibitor	II	diffuse large B cell lymphoma	NCT02875548

DNMTi, DNA methyltransferases inhibitor; FDA, US Food and Drug Administration; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; HDACi, histone deacetylases inhibitor; CTCL, cutaneous T cell lymphoma; CLL, chronic lymphocytic leukemia; PTCL, peripheral T cell lymphoma; EZH2, enhancer of zeste 2.

Rg3<sup>291</sup>), matrine,<sup>291</sup> and gambogic acid,<sup>292</sup> have been shown to suppress MDM2 expression in cancer cells. Despite the anti-cancer activities of these natural products, the use of MDM2 inhibitors in ISR remains to be assessed. However, these findings offer an important opportunity to advance current understanding regarding the role of MDM2 inhibitors as ISR protection agents in clinical practice.

#### **BET inhibitor (BETi)**

(+)-JQ1, a BET bromodomain inhibitor, displays antiproliferative effects in several animal models, including intimal hyperplasia of the carotid artery in rats with balloon injury<sup>149</sup> and in the mouse carotid artery model of reendothelialization.<sup>150</sup> In addition, intraperitoneal injection of (+)-JQ1 ameliorated hypertension, hypertrophy, and inflammation in mice induced by AngII.<sup>257</sup> Rvx-208 (also known as apabetalone) is another BETi with cardiovascular benefits.<sup>293</sup> Oral administration of RVX-208 reduces LDL levels, thereby lowering atherosclerosis in ApoE<sup>-/-</sup> mice. Furthermore, RVX-208 suppresses TNF-induced increase of MCP-1 and VCAM-1 in human ECs.<sup>258</sup> In addition, the BRD4-specific inhibitor ZL0513 suppressed angiogenesis by regulating activating protein-1 expression.<sup>294</sup> Interfering with the function of BET through the use of such inhibitors, which are already clinically used to treat different diseases, may be an attractive approach with the great translational potential to support current interventional therapy by minimizing the negative vascular remodeling process.

#### **ncRNA targeted therapeutics**

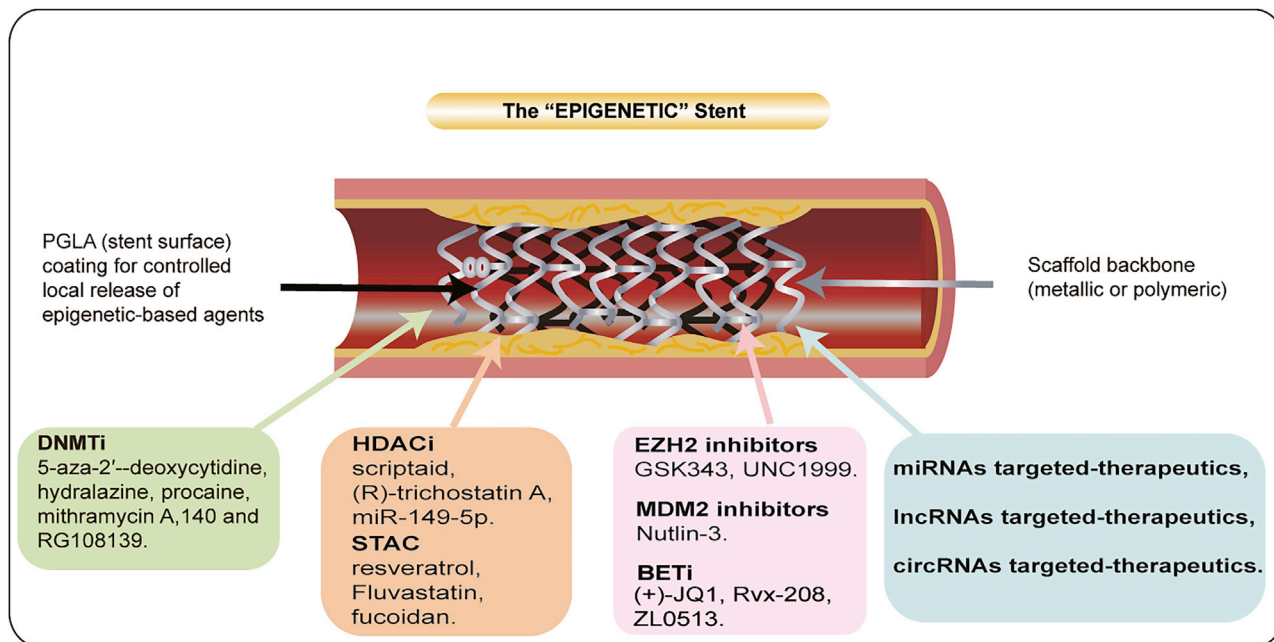
As reviewed above, ISR is a complicated disease that includes diverse mechanisms and multiple cell types. The emergence of ncRNAs opens a new avenue for ISR treatment. A considerable number of miRNAs have been successfully demonstrated to act as biomarkers or therapeutic targets. Similar evidence for lncRNA is also available. On the basis of therapeutic purpose, two approaches to changing

ncRNA levels in diseases have been developed.<sup>295</sup> Depending on their activity (deleterious or advantageous), ncRNA function can be enhanced or mimicked via delivery strategies, or their function can be suppressed using antisense oligonucleotides (ASOs) targeting RNA transcripts.<sup>295</sup>

Wang et al.<sup>296</sup> have indicated the merit of locally delivering the miR-21 sponge for inhibiting VSMC proliferation and neointima formation *in vivo*.<sup>296</sup> AntimiR therapies have some advantages, including the coordinated regulation of multiple gene targets via single miRNAs.<sup>297</sup> Nevertheless, a major hurdle in individual miRNA targeting is the off-target effect of antimiRs.<sup>297</sup> Use of stable, synthetic, and biodegradable miRNA carriers, and optimal delivery approaches, are being assessed to ensure the efficiency of miRNA targeting without toxicity.<sup>298</sup>

lncRNA is regulated by injecting oligonucleotides that inhibit their expression.<sup>299</sup> Furthermore, two main approaches are followed for inhibiting lncRNA expression in preclinical models,<sup>300</sup> namely, the RNA interference (RNAi)-based methods<sup>301</sup> and the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) system.<sup>302</sup> CRISPR represses the expression of miRNA and lncRNA, such as GAS5, MALAT1, UCA1, lncRNA-21A, and miRNA-21.<sup>303</sup> In the context of ISR, antisense oligonucleotides or CRISPR-Cas9 can be used to remove or activate/inhibit the expression of lncRNA to establish a restenosis therapeutic target.

However, certain unanswered problems in ncRNA-based treatment persist, including safe and effective means of administration, the long-term effectiveness of their effects, and side effects after long-term treatment. Future studies addressing these problems are therefore recommended. We anticipate that in the coming years,



**Figure 3. Image representing the hypothetical structure of a stent capable of eluting a targeted inhibitor**

The scaffold surface is coated with poly(lactic acid-glycolic acid) copolymer (PLGA). At the bottom of the figure are listed targeted regulatory inhibitors that have the potential to be regulated for therapeutic use.

advancements in biological research will improve our understanding and provide more insights regarding ncRNA-based therapies.

### Discussion

Although the progress of the current generation of DESs has solved several problems associated with the use of DESs, restenosis is still an evolving clinical problem. Even with the latest advances in coronary angioplasty (e.g., the introduction of bioabsorbable scaffolds), the problems of restenosis have not been solved. Whereas the understanding of the mechanisms of restenosis is positive in itself, the increasing incidence of CAD and the grim prognosis of those who develop restenosis in CAD require a serious effort to identify new treatment opportunities.

Ideally, PCI should eliminate restenosis by preventing VSMC proliferation and enhance EC regeneration. However, there is no effective therapeutic approach at this time. The advent of epigenetics may help us to understand the mechanism underlying restenosis and provide new targets for ISR drug development.

Restenosis pathogenesis can be mainly attributed to complex interactions between aberrant proliferation and migration of VSMCs and dysfunction of ECs. Epigenetic mechanisms, consisting of DNA methylation, histone modifications, and ncRNAs, may integrate these interactions. Hence, epigenetics may provide new insights for understanding ISR. Several histone modifications, DNA methylation, and numerous ncRNAs have been shown to be involved in regulating the expression of genes responsible for proliferation, migration, and

inflammation in ISR. In this review, we have discussed the mechanism underlying the pathological process of ISR and the diverse epigenetic changes that regulate VSMC gene expression and function (migration, proliferation, inflammation, hypertrophy, and phenotypic switch), as well as EC function (vascular homeostasis, angiogenesis, inflammation, and mechanotransduction). Furthermore, epigenetics enzymes regulating vascular cells and the major mechanisms via which they respond to varying stimuli have been described. Based on their significance, the epigenetic enzymes are considered as potential targets in the treatment of ISR. For instance, DNMT1 maintains the synthetic state of VSMCs through hypermethylation, thus promoting the proliferation and migration of VSMCs,<sup>304</sup> whereas TET2 has the opposite effect by producing 5hmC demethylation.<sup>39</sup> Interestingly, the DNMT inhibitor 5-Aza can reverse DNA hypermethylation in the atherosclerotic aorta of ApoE<sup>-/-</sup> mice by not only inhibiting DNMT activity but also by restoring the TET2 expression. Similarly, several studies have shown that EZH2 promotes not only VSMC proliferation and migration,<sup>53-55,57</sup> but also Es migration and angiogenesis.<sup>57,61</sup> Consistent with these reports, administering the EZH2 inhibitor (such as UNC1999) orally or perivascularly in the model of carotid wire-guided injury, the neointima formation in rats was significantly reduced.<sup>55</sup> Thus, it has the potential to be used as a stent-coating drug for restenosis. Moreover, p300 plays an epigenetic role mainly through the interaction with a variety of pro-inflammatory genes in vascular cells and the regulation of NF-κB-dependent expression.<sup>77</sup> In HDACs, depletion of HDAC5<sup>90</sup> and HDAC9<sup>94</sup> inhibits VSMC proliferation and migration, and inhibition of HDAC9<sup>96,97</sup> reverses ox-LDL-induced EC inflammatory

**Table 4. Drugs that target the epigenetic process of restenosis and associated vascular diseases**

Compound	Type	Animal models	Reference
5-Aza-2'-deoxycytidine	DNMTi	(1) ApoE <sup>-/-</sup> mice	220
		(2) LDLr <sup>-/-</sup> mice	221
Quercetin	DNMTi	(1) LDLr <sup>-/-</sup> mice	222
		(2) ApoE <sup>-/-</sup> mice	223,224
		(3) mice with a carotid injury model	225
Vitamin C	TET2 activator	(1) rabbits+HCD	226
		(2) ApoE <sup>-/-</sup> mice	227,228
		(3) pig coronary balloon injury	229
Scriptaid	HDACi	murine model of endovascular endothelial denudation	98
SAHA	HDACi	ApoE <sup>-/-</sup> mice	230
Valproic acid	HDACi	(1) LDLr <sup>-/-</sup> mice	231
		(2) ApoE <sup>-/-</sup> mice	232
Phenylbutyrate	HDACi	LDLr <sup>-/-</sup> mice	231
(R)-trichostatin A	HDACi	(1) LDLr <sup>-/-</sup> mice	233
		(2) neonatal rat	234
		(3) murine carotid ligation model	235
		(1) wire-injured carotid artery model	236,237
Resveratrol	SIRT1 activator	(2) rat carotid artery balloon angioplasty model	238
		(3) ApoE <sup>-/-</sup> /LDLr <sup>-/-</sup> mice	239
		(4) Marfan mouse model	240
		(5) ApoE <sup>-/-</sup> mice	241
		(1) HFHS diet	242
SRT1720	SIRT1 activator	(2) ApoE <sup>-/-</sup> mice+AngII infusion ApoE <sup>-/-</sup> mice	243
SRT3025	SIRT1 activator	(1) ApoE <sup>-/-</sup> mice	244
		(2) rabbit iliac artery in-stent restenosis model	245-247
Fucoidan	SIRT6 activator	ApoE <sup>-/-</sup> mice	248
Cyanidin	SIRT6 activator	ApoE <sup>-/-</sup> mice	249
Icariin	SIRT6 activator	rabbits+HFD	250,251
		(1) ApoE <sup>-/-</sup> mice	251
		(2) ApoE*3-Leiden mice	252,253
Statins	EZH2 inhibitors	(3) LDLr <sup>-/-</sup> mice	254
			255
		(4) rat carotid artery balloon angioplasty model	256
GSK343	EZH2 inhibitors	Marfan syndrome mouse	59
UNC1999	EZH2 inhibitors	murine carotid ligation model	55
(+)JQ1	BET inhibitor	(1) rat carotid artery balloon angioplasty	256
		(2) AngII-infused mice	257
RVX-208 (apabetalone)	BET inhibitor	(1) ApoE <sup>-/-</sup> mice	258
		(2) CAD patients	259
Nutlin-3	MDM2 inhibitors	murine carotid ligation model	140

ApoE, apolipoprotein E; LDLr<sup>-/-</sup>, LDL receptor-deficient; TET2, Tet methylcytosine dioxygenase 2; HCD, high-cholesterol diet; SAHA, suberoylanilide hydroxamic acid; HFHS, high-fat, high-sucrose; AngII, angiotensin II; HFD, high-fat diet; CAD, coronary artery disease; MDM2, mouse double minute 2 homolog.

responses both *in vivo* and *in vitro*. These studies supported that HDAC inhibitors are promising potential targets for the treatment of restenosis. In ubiquitination, MDM2, by interacting with p53, not only inhibits neointimal hyperplasia after vascular injury in mice,<sup>140</sup> but it also supports the response of spinal cord cells to angiogenic stimulation.<sup>144</sup> Targeting MDM2-p53 interaction is expected to provide the potential target for the treatment of restenosis. In addition to DNA methylation and histone modification, non-RNAs also play significant roles, mainly including miRNAs, lncRNAs, circRNAs, and others. Changes in the expression of different epigenetic factors, particularly miRNAs,<sup>305</sup> can be detected from their concentrations in body fluids such as the bloodstream, and hence they can be used as biomarkers for ISR in the future. Interestingly, we found that ncRNAs could act as both epigenetic enzyme regulators and epigenetic enzyme targets. For example, the downregulation of miR-101 in ECs promotes angiogenesis by reducing EZH2 inhibition.<sup>60</sup> Studies have demonstrated that miR-145 can attenuate the proliferation and migration of VSMCs and neointimal hyperplasia by inhibiting the expression of UHRF1.<sup>30</sup> These data provided a cross-regulating network in vascular cells and may unveil a novel dimension of ISR biology.

Considering the importance of epigenetic modification in restenosis, the enormous therapeutic potential of molecular regulators with epigenetic alterations have been reported recently. Consequently, we have discussed several promising molecules herein, including DNMTi, TET2 activators, HDACi, STAC, HMTi, MDM2 inhibitors, BETi, and ncRNA-targeted therapeutics, which might contribute to the prevention or reversal of restenosis. As mentioned earlier, we summarized these targeted therapeutic agents in Table 3, including natural products and chemical compounds.

However, there are still some unanswered questions that deserve further deep studies, such as safety, effective delivery systems, off-target effects, the dose and duration of action, and side effects after long-term treatment. First, the application of ncRNAs is hindered by insufficient targeting specificity, insufficient site-specific delivery, and possible off-targets.<sup>306</sup> How can miRNA be delivered to the target in a stable and precise manner? Whether miRNA or miRNA inhibitors can be delivered to the required targeted vascular cells through the development of new vectors (e.g., nanoparticles, ultrasound-targeted microbubbles) is critical for the implementation of location-specific drug delivery and therapeutic effects.<sup>307</sup> Second, the optimal dose or treatment time also needs to be considered. Low doses of DNMT drugs have persistent antitumor effects on blood and epithelial tumor cells.<sup>308</sup> However, epigenetic regulation takes time, especially if the drug targets the writer protein: epigenetic markers are lost as a result of passive demethylation or deacetylation.<sup>309</sup> Does this mean the patient needs to be treated for life? Finally, these drugs may have adverse effects and, although tolerated in the context of cancer, are still unacceptable for the treatment of chronic diseases. For example, the most common adverse reactions of the HDAC inhibitor (vorinostat) are diarrhea, nausea, anorexia, fatigue, thrombocytopenia, and dysgeusia.<sup>310</sup> Furthermore, some adverse reactions to HDACi may result in the impairment of other important

biological processes. For example, specificity class I HDACs show a strong antitumor effect, but inhibition of class I HDACs leads to a DNA damage response.<sup>311</sup> Other major problems include cardiotoxicity<sup>312</sup> of some HDACs and cellular resistance to some HDACs. Hence, it is crucial to develop drugs that are tolerated and specific enough to treat complex chronic diseases.

Although the problems of restenosis have not been fully solved, the prevailing mood in the field of epigenetics for restenosis is one of optimism. Clearly, the path to effective restenosis treatment is long and tortuous. Nevertheless, what we may now have is a promising road to follow.

### Conclusions

In conclusion, a better understanding of the relationship between vascular cells and epigenetics will not only highlight their role in ISR but will also offer new targets for the prevention and treatment of ISR. Therefore, epigenetic drugs for cardiovascular diseases such as ISR may complement the standard DES treatment in clinical practice. Hence, understanding epigenetics and ISR is expected to yield new insights into the pathogenesis of ISR that, in turn, can be translated into novel biomarkers and therapeutic modalities.

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### AUTHOR CONTRIBUTIONS

X.Y., Y.Y., J.G., X.L., T.Y., and Y.L. wrote the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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