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Epigenetic regulation of endothelial-cell-mediated vascular repair

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Maintenance of vascular integrity is essential for the prevention of vascular disease and for recovery following cardiovascular, cerebrovascular and peripheral vascular events including limb ischemia, heart attack and stroke. Endothelial stem/progenitor cells have recently gained considerable interest due to their potential use in stem cell therapies to mediate revascularization after ischemic injury. Therefore, there is an urgent need to understand fundamental mechanisms regulating vascular repair in specific cell types to develop new beneficial therapeutic interventions. In this review, we highlight recent studies demonstrating that epigenetic mechanisms (including post-translational modifications of DNA and histones as well as non-coding RNA-mediated processes) play essential roles in the regulation of endothelial stem/progenitor cell functions through modifying chromatin structure. Furthermore, we discuss the potential of using small molecules that modulate the activities of epigenetic enzymes to enhance the vascular repair function of endothelial cells and offer insight on potential strategies that may accelerate clinical applications.

Introduction

The derivation of embryonic stem cells (ESCs) and the identification of adult stem/progenitor cells from most adult tissues have opened the possibility that these cells could be used as a regenerative therapy for multiple diseases. However, with some exceptions (e.g. ocular disorders [1–3]) it has been difficult to translate our knowledge of stem cells into therapies. Furthermore, uncontrolled differentiation and/or proliferation of stem cells can potentially lead to malignant transformation [4]. Endothelial stem/progenitor cells present a particular interest for regenerative medicine. Indeed, the unique property of these cells to form new blood vessels *in vivo* highlights their remarkable potential for vascular regeneration in multiple diseases, ranging from acute limb ischemia, to heart attack, to stroke. Despite this potential, clinical trials using endothelial

Abbreviations
AML, acute myeloid leukemia; DNMT, DNA methyltransferase; EC, endothelial cell; ECFC, endothelial colony-forming cell; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; ESC, embryonic stem cell; EZH2, enhancer of zeste 2; HAT, histone acetyltransferases; HDAC, histone deacetylase; KLF2, Krüppel-like factor 2; LSD1, lysine-specific demethylase 1; MEF2C, myocyte-specific enhancer factor 2C; NFκB, nuclear factor κB; NRF2, NF-E2-related factor 2; OSS, oscillatory shear stress; PDGF, platelet-derived growth factor; PKD1, protein kinase D1; PRC2, polycomb repressive complex 2; PSS, pulsatile shear stress; TF, transcription factor; TGF-β, transforming growth factor β; TSA, trichostatin A; VE-cadherin, vascular-endothelial cadherin; VEGFR2, vascular endothelial growth factor 2; VPA, valproic acid; VSMC, vascular smooth muscle cells; vWF, von Willebrand factor.

progenitor cells (EPCs) have only been moderately successful [5,6]. The main limitation that currently prevents us improving the vascular repair function of EPCs is our lack of understanding of the molecular mechanism controlling cell fate. Indeed, if one can control cell fate determination, it will be possible to amplify EPCs *ex vivo* or *in vivo* and to force their differentiation exclusively towards the desired phenotype. In addition, it may be possible to generate 'enhanced' stem cells with additional properties (e.g. expressing a particular enzyme) to treat particular diseases. A plethora of studies, both in cell culture and in animal models, have shown that at the heart of cell fate determination lie transcription factors (TFs). These factors act, often in a cell-specific manner, to regulate cell fate determination. Specifically, TFs activate networks of specific genes that promote self-renewal or differentiation towards a particular cell fate while simultaneously inhibiting other competing cell fate(s). TFs act through the recognition of specific DNA sequences and through the recruitment of cofactors, including epigenetic enzymes that modify chromatin structure. Whilst it is possible to modulate cell fate through the modification of TFs (e.g. knockdown or overexpression), epigenetic enzymes are more easily amenable to clinical use since they provide the opportunity to be targeted by small molecule drugs. Therefore epigenetic drugs represent an unprecedented opportunity to control cell fate determination by acting directly on the transcriptional regulatory network.

Endothelial stem/progenitor cell therapy in vascular ischemic diseases

Vascular ischemic injuries, including limb ischemia, stroke and myocardial infarction, result in major organ failure and as such they represent an important therapeutic challenge. It has been well established that quickly restoring blood flow is essential to save organs [7,8]. Therefore, EPCs represent one of the most promising strategies for cell therapy after vascular ischemic injuries [9]. Since their initial discovery [10], several sub-types of EPCs have been established [5], among which endothelial colony-forming cells (ECFCs) [11] (also termed blood outgrowth endothelial cells [12] or late endothelial progenitors [5]) present a particular interest [13,14]. Indeed, ECFCs, which are derived from long-term (14–21 days) culture of human umbilical cord blood mononuclear cells, display the unique property of forming *de novo*, functionally active blood vessels *in vivo*. This is in contrast to 'early EPCs' that are derived from short-term (3 days) culture of cord blood mononuclear cells and display many characteristics of hematopoietic cells [13]. Importantly, ECFCs are able to promote vascular repair in a number of animal models of ischemia, including acute myocardial infarction [15], acute hind-limb ischemia [16–19], stroke [20], pulmonary arterial hypertension [21], ischemic retinopathies [22] and bronchopulmonary dysplasia [23]. Regulating ECFC activity in order to generate 'enhanced' cells ready to be injected into patients represents one of the most exciting future possible therapies for vascular ischemic disease treatment.

Transcriptional regulation of endothelial stem/progenitor cells

TFs have long been known to control the differentiation and activity of endothelial cells (ECs) [24,25]. However, little is known about the transcriptional network regulating ECFC differentiation and pro-angiogenic activity. Studies in mice and zebrafish have revealed that, rather than being determined by a single 'master' TF, the EC fate is established by the coordinated action of distinct families of TFs (i.e. Ets, GATA and forkhead) whose expression is not restricted to the endothelial lineage [24–27]. Specifically, these studies have revealed a hierarchy of TFs featuring several master regulators of the endothelial lineage that include ETV2, FOXC2, FLI1, GATA2, TAL1 and MEF2C (MEF2C, myocyte-specific enhancer factor 2C) [28]. Furthermore, it has been hypothesized that FOXC1/2 acts upstream of the Notch signaling pathway during EC sub-specification [29].

While the above studies focused on the role of TFs during murine and zebrafish development, they did not permit direct versus indirect roles of TFs in human endothelial progenitors to be distinguished. A small number of studies have examined the role of TFs directly in human EPCs. For instance, it was shown that the TF HOXA9 is necessary for post-natal angiogenesis through the direct regulation of several genes that are critical for endothelial activation and maintenance including endothelial nitric oxide synthase (eNOS), vascular-endothelial cadherin (VE-cadherin) and vascular endothelial growth factor 2 (VEGFR2) in early human EPCs [30]. Another example is the TF Krüppel-like factor 2 (KLF2) that has been shown to be required for ECFC differentiation toward mature ECs [31]. In addition, we have recently shown that the TF TAL1 plays a critical role in ECFC-mediated vascular repair. Furthermore, we have identified the full spectrum of TAL1-target genes in ECFCs including HOXA9, SOX7, EFNB2 and VE-cadherin [19]. In Fig. 1 we provide a summary of the currently known

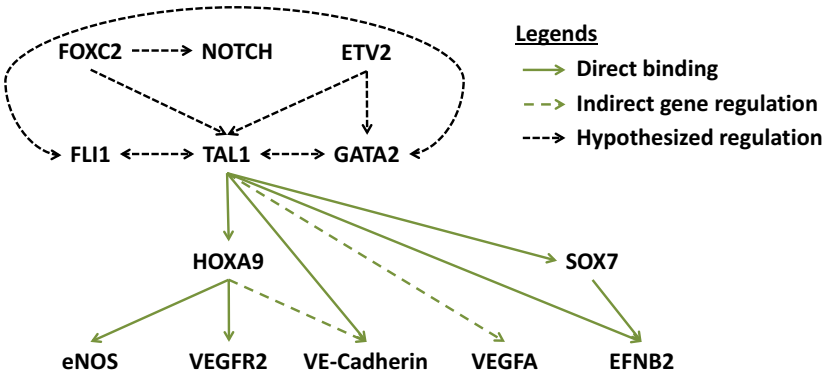

Fig. 1. Schematic representation of the transcription factor network hierarchy during endothelial cell differentiation and activation. Green full arrows represent direct binding, dotted green arrows represent indirect gene regulation and dotted black arrows represent hypothesized regulation.

TF network hierarchy in ECFCs. Additional studies are required to provide a better understanding of the transcriptional regulatory network in these clinically important cells.

Epigenetic factors regulating transcriptional activity in endothelial stem/progenitor cells

In eukaryotic cells, chromatin is composed of functional units called nucleosomes. Each nucleosome consists of a 146-bp DNA segment wrapped around an octamer of core histone proteins that includes two molecules of histones H2A, H2B, H3 and H4 associated with a single copy of histone H1. Epigenetics is defined as the study of stable alterations of gene expression without alterations of DNA itself. These alterations include the post-translational addition or removal of methyl groups to DNA as well as methyl, acetyl, sumoyl and phospho groups to histones. These changes participate in remodeling chromatin and modifying its accessibility to TFs and cofactors [32–36]. Epigenetic mechanisms play a critical role in regulating endothelial gene expression [37–40].

Non-coding RNAs

Non-coding RNAs including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) play important roles in the post-transcriptional regulation of gene expression.

miRNAs are small (20–24 nucleotides), highly conserved, non-protein-coding RNAs initially discovered for their role as negative post-transcriptional gene regulatory factors in *Caenorhabditis elegans*. It has been postulated that approximately 50% of genes are regulated by miRNAs. A single miRNA is able to target the 3′-UTR of several mRNAs to promote their degradation. The implication of miRNAs in EPC biology and the use of miRNAs to enhance EPC-mediated repair of ischemic injuries have been reviewed recently [41–43]. Briefly, microarray studies identified miRNA 21 (miR-21), miR-27a, miR-27b, miR-126 and miR-130a as being expressed in EPCs and downregulated in circulating EPCs with reduced angiogenic properties that were isolated from diabetic patients [44]. Furthermore, it was shown that inhibiting miR-126 in EPCs induces apoptosis, decreases proliferation and migration but does not affect differentiation. Conversely, restoring miR-126 expression in EPCs isolated from diabetic patients inhibits apoptosis and promotes EPC proliferation and migration. Specifically, miR-126 acts through inducing the downregulation of Spred-1 to activate Ras/ERK/VEGF and PI3K/Akt/eNOS signaling pathways improving the function of EPCs [44]. Recently, miR-126 has been shown to inhibit the transition from EPCs to mesenchymal cells via the PIK3R2-PI3K/Akt signaling pathway [45]. Collectively these data argue for a positive role of miR-126 in regulating EPC angiogenic properties. Another miRNA that is expressed at low levels in EPCs from diabetic patients is miR-130a. This miRNA is involved in repressing EPC differentiation while maintaining their proliferation, migration and colony formation properties through targeting the Runt-related transcription factor 3 (RUNX3), which results in the activation of ERK/VEGF and PI3K/Akt signaling pathways [46]. Furthermore, a similar signaling pathway involving miR-130a and Runx3 has been shown to inhibit EPC autophagy and to promote cell survival through Beclin1 downregulation and Bcl-2 upregulation [47]. Interestingly, miR10A* and miR-21 have been shown to mediate the aging process in EPCs. Indeed, it was shown that inhibition of these miRNAs results in EPC 'rejuvenation' associated with increased angiogenic properties of these cells *in vitro* and *in vivo* [48]. Finally, a recent study highlighted the upregulation of miR-221 and miR-222 in mature ECs and in ECFCs isolated from coronary artery disease patients.

Of importance in that regard, it was shown that overexpressing miR-221 and miR-222 in ECFCs reduces the expression of genes involved in the response to hypoxia, metabolism, transforming growth factor β (TGF-β) signaling, cell migration and capillary-like network formation [49].

Non-coding RNAs of more than 200 nucleotides length have been termed lncRNAs. Unlike miRNAs, lncRNAs are expressed at low levels, they are poorly conserved during evolution and they are mostly localized in the nucleus. lncRNAs are subdivided into five categories: sense, antisense (AS), bidirectional, intronic and intergenic based on their proximity to the closest coding gene [50]. The role of lncRNAs in endothelial biology has not been extensively studied. Interestingly, Robb *et al.* have discovered the existence of an AS lncRNA, termed sONE, that is derived from a transcription unit nitric oxide synthase 3-overlapping antisense (NOS3AS) and participates in regulating eNOS expression [51]. Three years later Fish *et al.* reported that sONE is responsible for eNOS downregulation in ECs under hypoxia [52]. More recently, a new AS lncRNA regulating the tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (tie-1) expression has been discovered [53]. Tie-1AS overexpression has been found in human samples presenting vascular anomalies and has been shown to impair EC junctions as well as tube formation [53]. Despite these interesting findings, additional studies are required to better understand the involvement of lncRNAs in vascular biology.

DNA methylation

Methylation of DNA is catalyzed by three different DNA methyltransferases (DNMTs) encoded by different genes on distinct chromosomes. DNA methylation is a relatively stable epigenetic modification that is almost exclusively restricted to cytosine residues present in CpG dinucleotides (5-mC). This modification has been associated with transcriptional silencing and is essential for embryonic development, genomic imprinting, X-chromosome inactivation and lineage specification [54]. DNA methylation can be removed through two different mechanisms: (a) passive demethylation whereby the 5-mC mark is not being maintained on the daughter strand during cell division; and (b) active demethylation whereby the ten eleven translocation (TET) enzymes catalyze the conversion of 5-mC to 5-hydroxymethyl cytosine (5-hmC), 5-formyl cytosine (5-fC) and 5-carboxyl cytosine (5-caC), which are then either lost through passive DNA replication dependent dilution or actively replaced by an unmodified C through base excision repair [55]. The contribution of DNA methylation/demethylation to endothelial gene regulation remains poorly understood. Most DNA methylation studies in ECs have been performed on the eNOS gene, a well known endothelial-restricted factor whose enzymatic activity is responsible for the production of vasoprotective and vasodilatative endothelial nitric oxide. Specifically, it was shown that the eNOS promoter is hypomethylated in ECs compared to non-endothelial cells (non-ECs) such as vascular smooth muscle cells (VSMCs) [37,40]. The functional relevance of eNOS promoter methylation was demonstrated by pharmacological inhibition of DNA methylation that resulted in the upregulation of eNOS in VSMCs [40]. Furthermore, inhibition of DNA methylation promotes EC differentiation of mouse embryonic stem cells (mESCs) [56]. Finally, a recent study identified DNA hypomethylation on most [VE-cadherin, CD31, von Willebrand factor (vWF), endoglobin, ICAM-2, P-selectin, Tie-2] but not all (VEGFR-1 and -2) endothelial genes in ECs compared to non-ECs. Interestingly, in non-ECs, inhibition of DNMT activity increased expression of the endothelial hypermethylated genes (CD31 and vWF) whereas their expression was not changed in ECs where these genes are already hypomethylated [57]. It has been shown that inducing DNA demethylation of GATA-2, GATA-3 and eNOS promoters in cultured human ESCs induces differentiation toward mature ECs [58]. Conversely, increasing the DNA methylation status of endothelial-specific genes allows the reprogramming of mature human ECs toward an ESC-like phenotype [59]. While these data argue for an important role of DNA methylation during endothelial differentiation, little is known about the DNA methylation status of genes in endothelial stem/progenitor cells. In fact, only one study has reported that the eNOS promoter is hypermethylated in early EPCs, which suggests a limited commitment of these cells toward the endothelial lineage [60]. The genome-wide DNA methylation status of endothelial stem/progenitor cells remains to be studied.

Histone modifications

Histone acetylation and deacetylation

Histone acetylation is regulated by a balance between the actions of antagonistic enzymes: histone acetyltransferases (HATs) that catalyze the addition of acetyl groups to lysine residues in histone tails, and histone deacetylases (HDACs) that remove acetyl residues from histones [61,62]. The addition of acetyl

groups to histone tails neutralizes their positive charges, which results in a weakened interaction with the negatively charged DNA, thereby facilitating access of TFs to their binding sites within chromatin [63,64]. Two categories of HATs have been described: (a) type A HATs that are localized in the nucleus and promote transcription through the acetylation of nucleosomal histones; and (b) type B HATs that are located in the cytoplasm and acetylate newly synthesized histones prior to their incorporation into nucleosomes [65]. The type A HAT family is made of several groups of enzymes, including the GNAT group (i.e. Gnc5, PCAF), the MYST group (i.e. Esa1, MOF), the p300/CREB-binding-protein (CBP) group, the TAF1 group and the ACTR/SRC1 group. CBP and p300 are usually considered as the more potent HATs since these enzymes do not strongly discriminate for histones present in the octameric nucleosomal core (H2A, H2B, H3 and H4) [63]. In the nucleus, the transcriptional activation function of HATs is counteracted by the action of HDACs that remove acetyl groups from histone lysine residues thereby increasing their negative charges, which leads to chromatin condensation and gene repression. Eighteen HDACs have been described in mammals and are divided into four distinct classes depending on their functional similarities and their homology with yeast HDACs. Class I HDACs comprise nuclear, ubiquitously expressed HDACs 1, 2, 3 and 8. Class II HDACs shuttle between the cytoplasm and the nucleus depending on specific cellular signals; they share a tissue-specific expression pattern and are divided into two subgroups: class IIa (HDACs 4, 5, 7 and 9) and class IIb (HDACs 6 and 10). Class III HDACs regroup the ubiquitously expressed NAD^+-dependent sirtuins (SIRT1 to 7) [66,67]. Interestingly, sirtuins display a differential pattern of cellular localization, with SIRT1, 6 and 7 being localized preferentially in the nucleus, SIRT3, 4 and 5 being found in the mitochondria and SIRT2 remaining predominantly cytoplasmic [68]. Finally, the class IV HDAC group only contains HDAC11 which shares similarities with class I and II HDACs but does not belong to any other HDAC classes [69]. The current paradigm is that HDAC inhibition increases the acetylation of core histones, which results in chromatin decompaction and increased gene expression [70]. In addition to their role in regulating histone acetylation and chromatin structure, HDACs and HATs are well known to deacetylate or acetylate non-histone proteins. For instance, HDAC3 deacetylates the p65 subunit of nuclear factor κB (NFκB) promoting its nuclear export [71], SIRT1 directly deacetylates Notch1 intracellular domain to induce its degradation in ECs [67], and other HDACs have been shown to directly deacetylate TFs such as p53, E2F1 and STAT1 [70]. The importance of histone acetylation has been shown for instance by upregulation of eNOS mRNA levels in VSMCs after treatment with an HDAC inhibitor named trichostatin A (TSA) [37]. Indeed, Fish *et al.* found that treatment with TSA de-represses the endothelial lineage-specific gene eNOS in non-endothelial VSMCs [37]. This result suggests that HDACs inhibit differentiation toward the endothelial lineage in non-ECs. Emerging evidence has highlighted the importance of histone acetylation in vascular ischemic injuries. For instance, it has been shown that HDAC inhibition with TSA or valproic acid (VPA) protects retina from ischemic injury in rats *in vivo* [72,73]. Moreover, Granger *et al.* reported that HDAC inhibitor treatment reduces myocardial ischemia-reperfusion injury in mice [74]. Altogether, these studies have shown that the process of acetylation/deacetylation of histones and non-histone proteins plays critical roles in the regulation of EC differentiation and function.

The role of HATs in ECs

Little is known about the role of HATs in vascular development and endothelial function. Interestingly, it has been shown that shear stress enhances HAT activity (i.e. histone H4 acetylation and histone H3 phospho-acetylation) in ESCs promoting their differentiation toward the endothelial lineage [75]. Furthermore, in ECs, shear stress increases p300-mediated acetylation of the p65 subunit of NFκB. And shear-stress-induced expression of eNOS is dependent on p300 acetyltransferase activity [76]. Consistent with these findings, p300 knockdown in ECs decreases NFκB expression as well as AP1 and CREB expression [77]. Altogether, these data suggest that p300 is essential for NFκB expression and nuclear translocation, and that HDAC3 counteracts p300 function through NFκB deacetylation. NFκB deacetylation by HDAC3 induces its nuclear export inhibiting shear-stress-induced eNOS expression. Mice expressing a myocyte-restricted transgene encoding p300 have been shown to develop spontaneous cardiac hypertrophy [78] with simultaneous compensatory blood vessel growth in response to cardiac hypertrophy blood flow overload [79]. This phenomenon is associated with angiogenic gene upregulation (VEGFA) and p300 binding to angiogenic regulator angiopoietin1 (Angpt1) and Egln3 enhancers [79]. On the other hand, p300 induces the expression of anti-angiogenic miRNAs such as miR-20a, providing an inhibitory

feedback on its own expression and VEGFA expression to finely tune the angiogenic and hypertrophic response to blood pressure overload [79]. These results suggest that p300 increases EC function in response to blood flow overload. In agreement with this study, p300/CBP complex is essential for mediating hypoxia-induced gene expression such as VEGFA in osteosarcoma cells suggesting its participation in tumor angiogenesis [80]. Finally, a recent study by our laboratory identified a critical role for p300 in regulating the *in vitro* and *in vivo* vascular repair function of ECFCs in a TAL1-dependent manner [19].

The role of class I HDACs in ECs

Blood flow induced shear stress is known to regulate EC function and differentiation. Specifically, pulsatile shear stress (PSS) that occurs in normal conditions of laminar blood flow is associated with endothelial growth arrest and differentiation while oscillatory shear stress (OSS), which is characteristic of atherosclerotic blood vessels, promotes inflammation and EC proliferation [81–84]. It has been shown that applying OSS to ECs leads to the upregulation and nuclear accumulation of HDAC1/2/3 which associate with NF-E2-related factor 2 (NRF2) resulting in NRF2

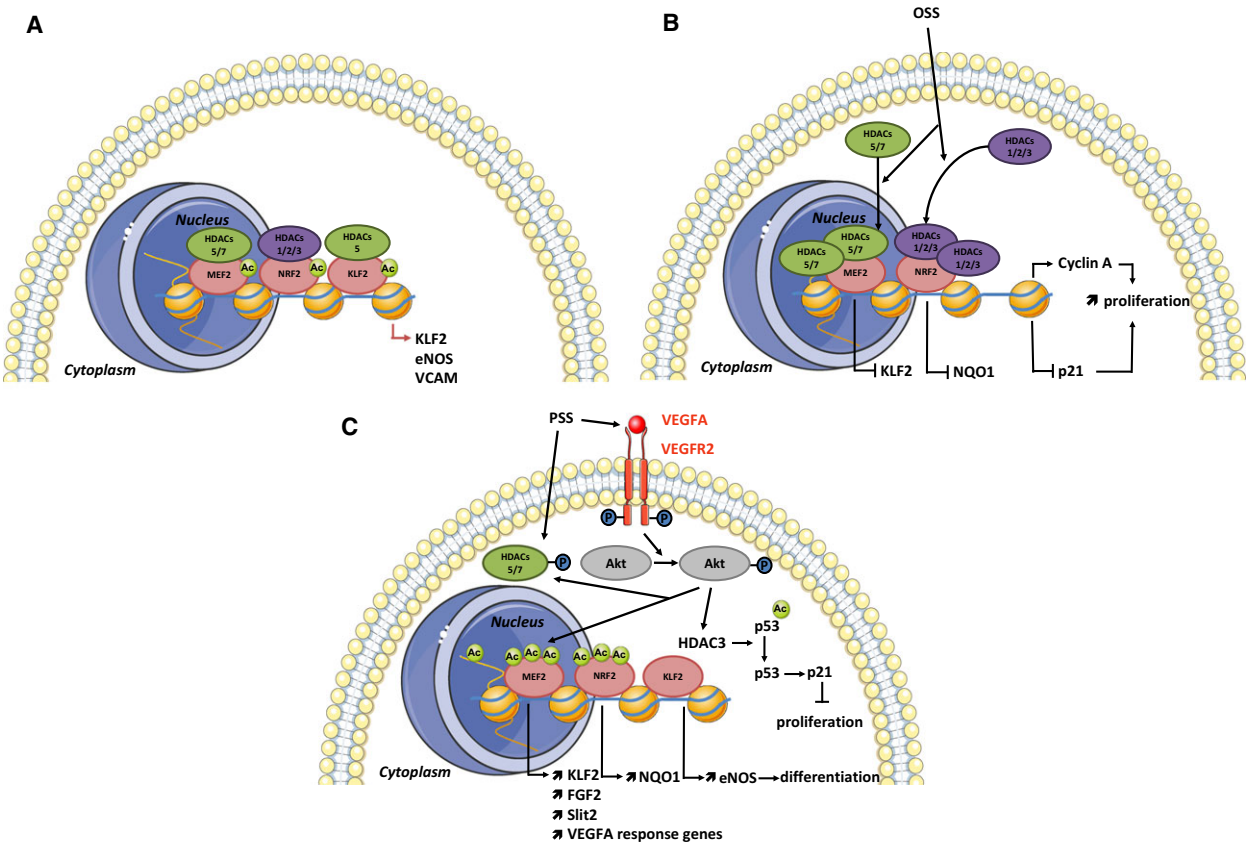

Fig. 2. Schematic representation of HDAC-mediated gene regulation by shear stress induction in ECs. (A) In the absence of shear stress, class I HDACs (1/2/3) and class II HDACs (5/7) are expressed at low levels, and are bound to NRF2 and MEF2, respectively, while HDAC5 is also bound to KLF2. As a result, these TFs display low levels of acetylation (Ac). (B) Applying oscillatory shear stress (OSS) increases the expression and nuclear translocation of class I HDACs (1/2/3) and class II HDACs (5/7). This, in turn, leads to the deacetylation of NRF2 and MEF2, respectively, and indirectly to the inhibition of NQO1 and KLF2 target genes. At the same time, HDAC nuclear translocation downregulates p21 expression while it upregulates cyclin A expression, resulting in increased EC proliferation. (C) Applying pulsatile shear stress (PSS) induces phosphorylation of class II HDACs (5/7) (but not class I HDACs (1/2/3)) resulting in their nuclear export. This, in turn, allows NRF2 and MEF2 hyperacetylation, which results in the upregulation of their respective target genes NQO1 and KLF2. KLF2 upregulation then leads to increased eNOS expression and endothelial differentiation. In addition, EC stimulation with VEGFA induces HDAC5 nuclear export, which allows MEF2 acetylation and transcriptional activity resulting in its target genes expression: FGF2 and Slit2 as well as VEGFA-response genes. Altogether, these effects lead to EC growth arrest and differentiation. PSS is also responsible for ESC growth arrest and differentiation toward endothelial lineage. Indeed, PSS activates VEGFR2 inducing Akt signaling pathway activation, resulting in HDAC3 stabilization and activation, which in turn deacetylates and activates p53 leading to p21 activation that is crucial for endothelial growth arrest and differentiation.

deacetylation (Fig. 2A,B). This phenomenon has been shown to be at least partially responsible for the upregulation of cyclin A, the downregulation of p21 and KLF2, which ultimately leads to EC proliferation [85] (Fig. 2B).

HDAC1 (but not HDAC2 and 3) has been shown to bind to the eNOS proximal promoter in non-EC cells (VSMCs [37], Hela [86]) suggesting its implication during TSA-mediated endothelial differentiation in non-ECs (VSMCs) [40]. Thus, HDAC1 seems to be an essential inhibitor of EC differentiation. Moreover, HDAC1 upregulation in ECs induces eNOS protein deacetylation leading to a critical decrease in basal and endothelin-1 stimulated NO production. On the other hand, HDAC1 knockdown in ECs results in an exacerbated increase of both basal and endothelin-1 stimulated NO production while not changing eNOS acetylation level, which suggests the existence of an acetylation-independent mechanism for regulating eNOS activity, possibly through histone deacetylation [87]. Thus, increasing eNOS expression or activity through HDAC1 inhibition is of interest as a potential method to promote EC function. Interestingly, eNOS expression is also regulated by blood flow. Indeed, it has been shown that applying PSS induces eNOS expression [88] and activity (NO production) in ECs [89]. Furthermore, PSS also induces HDAC1-dependent p53 deacetylation, which results in the activation of p21 and EC growth arrest [90]. Altogether these results suggest that HDAC1 is involved in arresting cell growth and inhibiting EC differentiation and function.

HDAC2 has been shown to act as a negative regulator of arginase2 (Arg2) expression in ECs. Arg2 is a competitive enzyme for eNOS substrate L-arginine and therefore induces eNOS uncoupling due to a lack of substrate, which subsequently decreases endothelial NO production. HDAC2, but no other member of class I HDACs, binds to the Arg2 promoter to inhibit its expression, which leads to an increase in NO production [91]. This demonstrates that HDAC2 is an indirect eNOS activator through the inhibition of Arg2 expression. Interestingly, inhibiting HDAC2 has been shown to protect retina from ischemic injury [92]. Collectively, these data suggest that HDAC2 plays an important role in the maintenance of EC function and aortic relaxation (through NO production) in atherosclerotic cardiovascular disease and vascular ischemic injury.

HDAC3 is one of the most studied class I HDACs in ECs. First, it was shown that HDAC3 plays a pivotal role during PSS and VEGF-induced differentiation of ESCs toward the endothelial lineage [93,94]. Specifically, HDAC3 knockdown in ESCs abolished the expression of PSS-induced eNOs, p53, p21 and VEGFR-2 as well as the expression of VEGFA-induced EC markers (eNOS, VEGFR-2 and vWF). PSS and VEGFA stabilize and activate HDAC3 through phosphorylation by the Akt signaling pathway, leading to HDAC3 association with and subsequent deacetylation of p53. This, in turn, results in p21 activation, growth arrest and differentiation towards the endothelial lineage. HDAC3 knockdown abolished VEGFA-induced ESC differentiation toward ECs [94]. Altogether these results suggest that HDAC3 plays an essential role in mediating PSS and VEGFA-induced stem cell differentiation toward ECs through a VEGFR-2/Akt/HDAC3/p53/p21 signaling pathway (Fig. 2C). Similar results were obtained in ECs, where it was shown that PSS increases HDAC3 expression [95]. Interestingly HDAC3 knockdown in ECs increases apoptosis in static and shear stress conditions leading to endothelial loss in aorta. Pharmacological inhibition showed that shear stress stabilizes HDAC3 protein by phosphorylation on serine and threonine residues through VEGFR-2 and Akt signaling pathways that are known to promote EC survival [95] (Fig. 2B). Application of OSS to ECs induces MEF2 association with HDAC3, leading to MEF2 deacetylation and subsequent KLF2 downregulation [85]. Altogether these data argue for a pivotal role of HDAC3 both in promoting EC differentiation during development and in maintaining the integrity of the endothelium in response to disrupted blood flow. Interestingly, it has recently been demonstrated that HDAC3 undergoes splicing during stem cell differentiation, which leads to the formation of four splicing variants called HD3α, β, γ and δ. Among these splicing variants, HD3α has been shown to be implicated in embryonic vasculogenesis [96]. HD3α directly interacts with the fully spliced HDAC3 and Akt to activate TGF-β2 secretion and cleavage, which is responsible for human aortic EC reprogramming into mesenchymal cells through endothelial-to-mesenchymal transition [96].

No evidence is currently available regarding a potential role for HDAC8 in ECs.

The role of class IIa HDACs in ECs

The importance of class IIa HDACs in the cardiovascular system has been highlighted by mice knockout studies. For instance, HDAC5 or HDAC9 deficiency in mice led to cardiac hypertrophy [97] while the lack of HDAC7 causes embryonic lethality due to endothelial cell–cell adhesion failure and subsequent blood vessel leakage [98]. ECs express relatively high levels of

HDACs 4 and 7 and comparatively low levels of HDACs 5 and 9. Interestingly, the pro-angiogenic factor VEGFA induces phosphorylation of class IIa HDACs resulting in their export from the nucleus, thereby decreasing their nuclear function [99]. Furthermore, a recent study showed that HDAC4 is involved in decreasing the production of VEGFA in chondrosarcoma cells, which in turn decreases angiogenesis through Runx2 downregulation [100]. Altogether these data argue for the existence of a regulatory loop between VEGFA and HDAC4, and are suggestive of an anti-angiogenic role for HDAC4. Similarly, HDAC5 has been shown to play an anti-angiogenic role in ECs. Indeed, knocking down HDAC5 in ECs induces cell migration and sprouting while HDAC7 and HDAC9 knockdowns show the opposite effect. HDAC5 overexpression results in an MEF2-dependent decrease of EC sprouting. This effect is mediated, at least in part, through the binding of HDAC5 to the promoters of FGF2 and slit2 genes, which decreases their expression [101]. Moreover, protein kinase D1 (PKD1) has been shown to mediate HDAC5 phosphorylation leading to its nuclear export, which allows for de-repression of VEGFA-dependent genes and subsequent angiogenesis [102]. Shear stress is another pathway regulating HDAC5/7 activity. On the one hand, PSS induces phosphorylation of both HDAC5 and HDAC7, which results in their nuclear export and respective dissociation from MEF2 and KLF2. This, in turn, enhances the activity of the TFs, leading to an increased expression of KLF2 and eNOS genes [85,99,103] (Fig. 2C). On the other hand, OSS induces the association of HDAC5/7 with MEF2 leading to its deacetylation and subsequent KLF2 target gene downregulation [85]. Moreover, EC stimulation with NO induces HDAC4 and HDAC5 translocation to the nucleus, preventing shear-stress-dependent histone acetylation [104]. This result suggests that NO can either act as part of a negative regulatory loop to modulate EC function (e.g. shear stress induces eNOS expression and NO production) or enhance endothelial function (e.g. deacetylation of anti-angiogenic genes). Thus, HDAC5 could be a potential useful target to inhibit for therapeutic vascular repair.

In contrast to the anti-angiogenic role of HDACs 4 and 5, knockdown studies have suggested that HDAC7 plays a pro-angiogenic role. Indeed, both Urbich *et al.* [101] and Mottet *et al.* [105] found that the knockdown of HDAC7 impairs EC migration and tube-like formation. Furthermore, HDAC7 knockdown leads to the overexpression of platelet-derived growth factor B (PDGF-B) and its receptor, the platelet-derived growth factor receptor β (PDGFR-β), which is at least partially responsible for the inhibition of EC migration. Interestingly, pharmacological induction of HDAC7 nuclear export mimics the HDAC7 knockdown phenotype suggesting that HDAC7 export from the nucleus is a key event in modulating endothelial migration through PDGF-B pathway activation [105]. Turtoi *et al.* recently showed that the mechanism of HDAC7-mediated EC migration and tube-like formation involves downregulation of the angiogenesis suppressor gene A-kinase anchor protein 12 (AKAP12) [106]. Overexpression of HDAC7 inhibits EC proliferation through direct binding to β-catenin preventing its nuclear translocation (Fig. 3A) [107]. Consistent with these findings, HDAC7-deficient mice display important vascular defects including cell–cell adhesion failure, blood vessel enlargement and leakage [98]. The profound vascular defects observed in HDAC7 knockout mice are in line with the important role played by HDAC7 splicing in promoting differentiation towards smooth muscle cells (SMCs) and blood vessel formation during embryonic development. Indeed, Margariti *et al.* demonstrated that PDGF-induced HDAC7 splicing is essential for ESC differentiation toward SMCs [108]. It should be noted that an anti-angiogenic role for HDAC7 has also been suggested. Indeed, it has been shown that VEGFA can induce HDAC7 phosphorylation and nuclear export, which enhances EC proliferation, migration [99], matrix metalloprotease expression and angiogenesis [109]. Furthermore, VEGFA-PKD1-induced HDAC7 nuclear export promotes EPC migration and tube-like formation [110]. Altogether, these findings strongly suggest that HDAC7 plays a key role in regulating EC proliferation and migration in response to VEGFA. In conclusion, the role of HDAC7 in inhibiting versus promoting endothelial function is more complex than originally proposed based on knockout mice studies, and appears to strongly depend on specific stimuli (PSS, OSS, VEGFA) received by ECs (Fig. 3B).

Despite its strong similarity with HDAC5, HDAC9 is clearly pro-angiogenic as shown by the fact that HDAC9 knockdown in ECs decreases both EC migration and tube-like formation *in vitro* [101,111] and *in vivo* [111]. Furthermore, HDAC9 overexpression enhances EC tube-like formation while HDAC9 mutants lacking the catalytic domain, the sumoylatable domain or the nuclear localization signal have no effect. This suggests that HDAC9 activation through sumoylation, nuclear translocation and deacetylation activity are all required to induce HDAC9 pro-angiogenic effect. Moreover, silencing or pharmacological inhibition of HDAC9 in ECs upregulates the

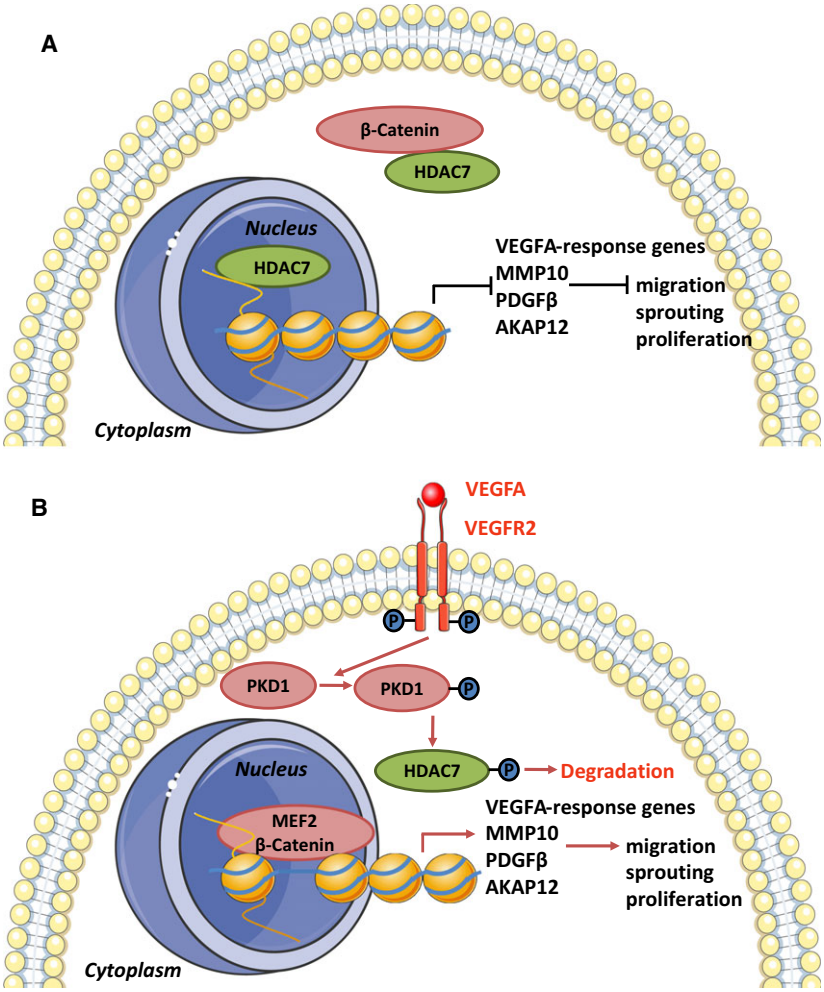

Fig. 3. The dual role of HDAC7 in ECs. (A) HDAC7 represses angiogenesis through both histone deacetylation and inhibition of transcription factor nuclear translocation. (B) VEGFA stimulation induces HDAC7 phosphorylation by PKD1 resulting in its nuclear export and subsequent degradation. HDAC7 nuclear export allows for histone acetylation, which favors transcription factor binding and endothelial gene expression.

anti-angiogenic miR-17-192. Consistent with this, overexpression of HDAC9, but not its mutated counterpart which lacks an active catalytic domain, reduced the expression of the miR-17-92 cluster activator region suggesting that HDAC9 directly inhibits the miR-17-92 cluster. Among the members of the miR-17-92 cluster, miR-20a has been shown to be responsible for mediating at least in part the anti-angiogenic effect in the absence of HDAC9 suggesting that it constitutes an HDAC9 preferential target to inhibit in ECs [111].

Collectively, the findings described above indicate that class IIa HDACs (including mostly HDACs 4, 5 and 7) play essential roles in mediating VEGFA-dependent angiogenesis through HDAC phosphorylation and nuclear export that prevents their inhibitory effect on VEGFA-dependent genes. In contrast, HDAC9 seems to be typically pro-angiogenic through targeting the miR-17-92 cluster, particularly miR-20a. As such, members of class IIa HDACs represent promising targets for EPC based therapy.

The role of class IIb HDACs in ECs

HDAC6 is predominantly cytoplasmic. In ECs, HDAC6 is upregulated to induce angiogenesis in response to hypoxia. Furthermore, the downregulation of HDAC6 decreases endothelial migration and sprouting *in vitro* as well as angiogenesis *in vivo* supporting its pro-angiogenic role. Specifically, it was shown that HDAC6 induces EC migration and sprouting through direct interaction and subsequent deacetylation of cortactin [112]. Currently, there is no evidence that HDAC6 regulates EC functions through nuclear translocation and/or deacetylation of histones. Finally, the potential role of HDAC10 in angiogenesis, EC function and/or differentiation has not been investigated.

The role of class III HDACs in ECs

All sirtuins are expressed at high levels in ECs. However, so far, a pro-angiogenic role has only been

demonstrated for SIRT1. Indeed, SIRT1 has been shown to regulate EC sprouting, tube-like formation and migration *in vitro* [113]. While overexpression of SIRT1 increases EC sprouting and migration, an enzymatically dead SIRT1 did not display any pro-angiogenic activity, suggesting that SIRT1 deacetylase activity is required for its pro-angiogenic function [113]. Further characterization of the role of SIRT1 in vascular development highlighted its essential role in both embryonic and post-natal neovascularization *in vivo*. Indeed, loss of SIRT1 activity leads to the downregulation of several important angiogenic factors, including Flt1 and CXCR4 [113]. Furthermore, SIRT1 has been shown to activate eNOS and to promote endothelium vasodilatation [114]. Also importantly, SIRT1 is involved in preventing apoptosis [115], inducing proliferation and preventing senescence [116] of ECs. Interestingly, the mechanism through which SIRT1 regulates EC sprouting and elongation involves direct binding and deacetylation of Notch1. Indeed, it has been demonstrated that SIRT1 directly binds to Notch1 intracellular domain and induces its deacetylation and subsequent degradation. Consistent with this finding, SIRT1-deficient ECs display an increased sensitivity to Notch signaling, which leads to a switch from a sprouting-cell-like phenotype to a stalk-cell-like phenotype, and results in an overall reduction of vascular branching and density [67]. Overall, SIRT1 is important for regulating mature EC functions [68]. Finally, a recent study highlighted the important role of SIRT1 in preventing senescence in ECFCs. Specifically this study showed that ECFCs that are derived from the umbilical cord blood of preterm infants display impaired endothelial functions and increased senescence that are due to the downregulation of SIRT1 [117]. Collectively these data highlight the important role played by SIRT1 in mediating endothelial functions (proliferation and sprouting) in ECFCs.

The role of class IV HDACs in ECs

Currently, there is no evidence that HDAC11 is involved in regulating the vascular system.

Altogether the data described above highlight that acetylation/deacetylation cycles of histone and non-histone proteins play critical roles in regulating EC functions and angiogenesis. Notably, flickering transport of HDACs between the nucleus and the cytoplasm is a major event that regulates TF activity both directly through TF deacetylation and indirectly through the modulation of chromatin structure (Fig. 4). Importantly, the role of HDACs is highly dependent on the cellular context since the same HDAC can play different, sometimes opposite, roles depending on the cellular environment and external stimuli (Table 1).

Histone methylation

Histone methylation is a complex epigenetic event that leads to different transcriptional outputs depending on

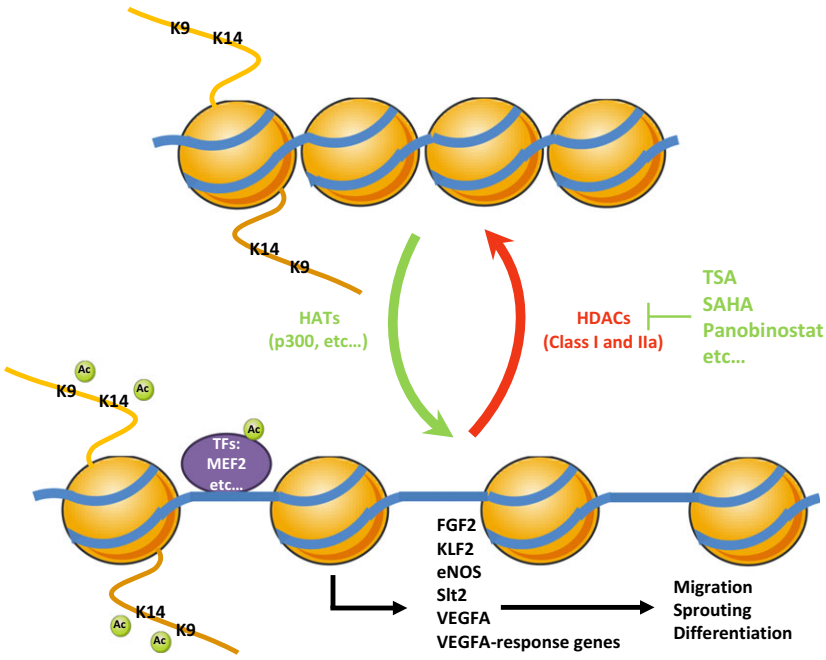

Fig. 4. In ECs, HDACs regulate gene expression both directly through the deacetylation of transcription factors and indirectly through histone deacetylation and chromatin condensation. HDACs directly deacetylate transcription factors (TFs), which reduces their activity. HDACs also deacetylate histones such as H3K14 and K9, which induces chromatin condensation and decreases TF DNA binding site accessibility. HATs counteract the activity of HDACs, which facilitates transcription factor activity and leads to the upregulation of genes that are important for EC differentiation and function, including eNOS, FGF2 and VEGFA. Treatment with HDACs inhibitors (e.g. TSA, SAHA, panobinostat) is expected to increase EC function and/or differentiation.

Table 1. The role of HDACs and HATs in angiogenesis is context dependent.

Type	Name	Localization	Pro-angiogenic role	Anti-angiogenic role
Class I	HDAC1	Nuclear	Mediates OSS proliferation	eNOS promoter binding, eNOS binding/ deacetylation, mediates PSS growth arrest
	HDAC2	Nuclear	Arg2 promoter binding/downregulation, ↗ NO production, mediates OSS proliferation	–
	HDAC3	Nuclear/cytoplasmic	Mediates PSS and VEGFA-induced endothelial differentiation, and survival	–
	HDAC8	Nuclear	–	–
Class IIa	HDAC4	Nuclear/cytoplasmic	↘ PSS-dependent histone acetylation after NO stimulation	VEGFA-induced nuclear exportation ↘ VEGFA expression and angiogenesis
	HDAC5	Nuclear/cytoplasmic	↘ PSS-dependent histone acetylation after NO stimulation	PSS, VEGFA-induced nuclear exportation FGF2 promoter binding/downregulation ↘ Migration, sprouting and angiogenesis
	HDAC7	Nuclear/cytoplasmic	Essential for vascular development *in vivo* ↗ Migration, tube-like formation ↘ Proliferation ↘ PDGF-B/PDGFR-β, AKAP12	PSS, VEGFA-induced nuclear exportation, ↘ VEGFA target gene expression and angiogenesis
	HDAC9	Nuclear/cytoplasmic	↗ Migration, tube-like formation ↗ miR-17-92	VEGFA-induced nuclear exportation
Class IIb	HDAC6	Cytoplasmic	↗ Hypoxia-induced angiogenesis ↗ Migration, tube-like formation	–
	HDAC10	Nuclear/cytoplasmic	–	–
Class III	SIRT1	Nuclear/cytoplasmic	↗ Migration, proliferation, tube-like formation, ↘ apoptosis, senescence, ↗ eNOS, Flt-1, CXCR4 Required for post-natal angiogenesis	–
Class IV	HDAC11	Nuclear/cytoplasmic	–	–
HAT	p300		PSS ↗ activity and endothelial differentiation, ↗ eNOS, and VEGFA, Angpt1 promoter binding/downregulation	–

the lysine (K) residue that becomes methylated (e.g. K4, K9, K27, K36) and its degree of methylation (mono-, di- or tri-methylation). For instance, histone H3 methylated on K4 or K36 is generally associated with active genes while K9 and K27 are mostly linked to gene repression. Similarly to histone acetylation, histone methylation is reversible through the action of highly specialized enzymes (i.e. histone demethylases) and the dynamics of histone methylation/demethylation is important during cell differentiation and is often deregulated in disease [118]. Distinct methylation marks are also differentially enriched at specific genomic locations. For instance, promoters of active genes are highly enriched in H3K4me3/2 (and histone acetylation) while enhancers of active genes are enriched in H3K4me1/2 (and H3K27 acetylation). On the other hand, promoters of inactive genes are enriched in H3K27me3, H3K9me3 (and DNA methylation) while enhancers of inactive genes are enriched in H3K9me3/2 (and DNA methylation) [119,120]. Interestingly, some gene promoters are simultaneously enriched for both active (i.e. H3K4me3) and repressive (i.e. H3K27me3) marks [121]. Similarly, enhancers can also be enriched simultaneously for active (H3K4me1) and repressive (H3K27me3) marks [122,123]. Genes whose promoters and/or enhancers are simultaneously enriched for both active and repressive marks have been called 'bivalent'. Interestingly, bivalent genes are expressed at extremely low levels, and it has been suggested that, while the H3K27me3 mark is important to maintain these genes in a repressed state, the presence of H3K4me3/1 may poise them for rapid activation upon induction of differentiation by external signals. Since bivalent genes are enriched for developmental regulators including TFs and signaling proteins, it has been proposed that the state of bivalency on promoters underlies pluripotency in ESCs or multipotency in adult stem cells [124]. Importantly, bivalent genes may constitute potentially useful targets to enhance the function of stem cells through epigenetic therapy since these genes may be particularly responsive to epigenetic drugs that boost the function of stem cells. The full extent of bivalent genes (enhancers and promoters) in ECFCs or EPCs is currently

unclear. Furthermore, the importance of bivalent genes for post-natal angiogenesis remains to be determined.

H3K4 methylation in the endothelial lineage

Methylation of H3K4 is mediated by the SET-domain-containing family of enzymes that includes SET1A/B and the TrxG proteins MLL1/2 (homologs of *drosophila* Trx) and MLL3/4 (homologs of *drosophila* Trr) [125–127]. While MLL1 is mostly known for its role during embryonic hematopoiesis, hematopoietic stem cell renewal and ESC differentiation through the epigenetic control of the Hox family of TFs (i.e. HoxA7, HoxA9 and HoxA10) [128–130], MLL1 is also essential for EC sprouting [131]. Indeed, it was shown that pharmacological inhibition or knockdown of MLL1 in ECs led to decreased migration and sprout formation. Furthermore, it has been proposed that this effect is mediated through MLL1 regulation of HoxA9 and HoxD3/EphB4 gene expression [131].

Demethylation of H3K4me1 and H3K4me2 is mediated by the lysine-specific demethylase 1 (LSD1) while JARID1B/KDM5B catalyzes H3K4me2 and H3K4me3 demethylation [125]. In addition to its H3K4me1 and H3K4me2 demethylase activity, LSD1 is also known to remove H3K9me1 and H3K9me2 repressive marks. LSD1 has been shown to suppress angiogenesis and metastasis in breast cancer by inducing H3K4 demethylation and repressing the chemokine CCL14 in coordination with JARID1B/KDM5B [132]. Furthermore, LSD1decreases eNOS expression and the NO relaxation pathway during a high salt diet [133]. Interestingly, pharmacological inhibition of H3K4 methylation with methylthioadenosine in ECs decreases eNOS expression and prevents the HDAC-inhibitor-induced eNOS expression in VSMCs [37]. These results suggest that H3K4 methylation is important for angiogenesis.

Taken together, these studies suggest that the regulation of H3K4 methylation through MLL1 and LSD1 is important for EC function (Fig. 5). Therefore, modulating the function of these enzymes by epigenetic drugs could represent a potentially useful means for improving the regenerative function of endothelial progenitors for stem cell therapy.

H3K9 methylation in the endothelial lineage

H3K9 methylation is triggered by a family of methyltransferases comprising G9A/EHMT2, GLP/EHMT1, SETDB1, SETDB2, SUV39H1 and SUV39H3 [134]. While G9A and GLP are responsible for H3K9 mono- and di-methylation, SETDB1, SETDB2, SUV39H1 and SUV39H3 catalyze the formation of H3K9me2 and H3K9me3 marks. H3K9 demethylation is performed by members of the Jumonji-domain-containing demethylases (JMJD), including KDM4A/JMJD2A/JHDM3A, KDM4B/JMJD2B, KDM4C/JMJD2C/GASC1, KDM4D/JMJD2D that demethylate H3K9me3/2 as well as KDM3A/JHDM2A, KDM3B/JHDM2B and JMJD1C that demethylate

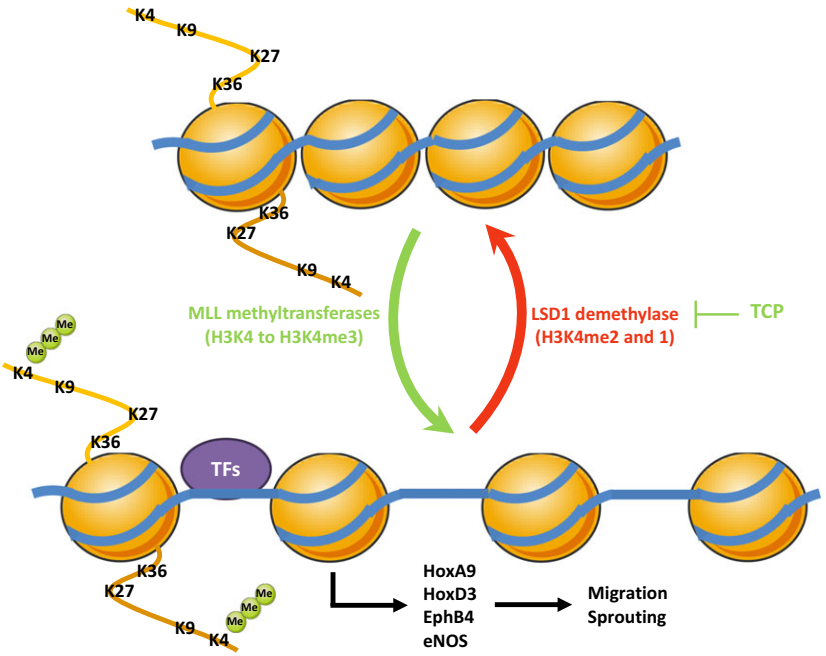

Fig. 5. Schematic representation of gene regulation and function through H3K4 methylation in ECs. MLL H3K4 methyltransferase activity promotes endothelial gene expression and endothelial function while LSD1 demethylase activity decreases the expression of endothelial genes.

H3K9me2/1 [135]. Interestingly, a study examining VEGFA expression and H3K9 methylation in HEK293 cells showed that it is possible to induce H3K9 methylation and subsequent repression of VEGFA by using engineered zinc-finger TFs (ZFPs) coupled with G9A and SUV39H1 methyltransferase domains [136]. These data suggest that H3K9 methylation might be sufficient to repress VEGFA gene expression. However, the exact role of H3K9 methyltransferases and demethylases during EC differentiation or function remains to be established. Finally, even though H3K9 demethylation could potentially be used to enhance endothelial function, it should be noted that the levels of H3K9me3/2 are already low on the eNOS gene promoter in ECs and endothelial progenitors [60] suggesting that H3K9 demethylation may not further improve EC function and/or endothelial differentiation (Fig. 6).

H3K27 methylation in the endothelial lineage

Another type of repressive histone mark is H3K27 methylation. This modification is mediated by the enhancer of zeste 2 (EZH2) enzyme that is part of the polycomb repressive complex 2 (PRC2). PRC2 is composed of polycomb group (PcG) proteins known to repress differentiation genes and maintain stemness through the deposition of repressive H3K27me3 marks during development. EZH2 is often overexpressed in cancer cells and its inhibition decreases tumor growth suggesting that EZH2 often acts as an oncogene [137–139]. Interestingly, EZH2 silencing in tumor cells induces re-expression of endothelial genes such as ephrin receptor B2 (EPHB2) and allows tumor cells to form a capillary-like structure on Matrigel, which suggest that EZH2 is a negative regulator of EC differentiation in non-ECs [140]. In contrast, in ECs VEGFA increases EZH2 expression, resulting in EZH2 binding and subsequent H3K27 tri-methylation of the gene coding for the angiogenesis inhibitor vasohibin1 (VASH1), thereby causing VASH1 downregulation and subsequent angiogenesis. In addition, EZH2 silencing in tumor-associated ECs decreases angiogenesis through VASH1 re-expression, leading to decreased cancer growth [141]. Consistent with these results, Smits *et al.* showed that decreased expression of miR-101, a repressor of EZH2 expression, in ECs results in increased blood vessel formation [142]. Interestingly, while EZH2 silencing in ECs reduces some of the cells' angiogenic properties (e.g. capillary-like structure formation) it also increases other angiogenic properties such as adhesion and migration [143], suggesting that EZH2 inhibition could potentially be useful to improve the function of endothelial progenitors for stem cell therapy. Furthermore, Dreger *et al.* [143] used whole-genome mRNA expression arrays to identify an entire set of genes implicated in cell–cell communication and adhesion (e.g. TGFA, FGF1, EFN and WNT/FZD signaling pathway and CDH13) that are being overexpressed upon EZH2 downregulation.

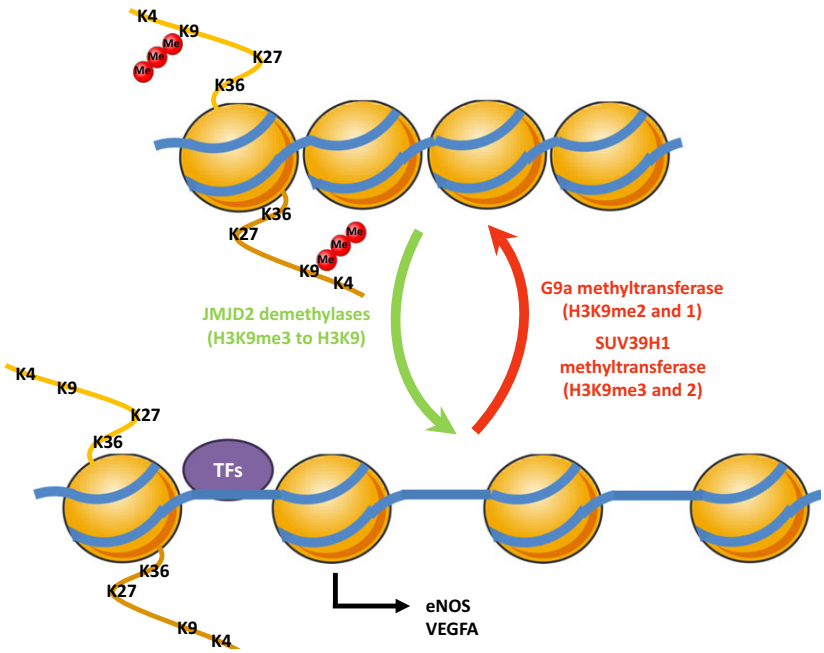

Fig. 6. Schematic representation of gene regulation and function through H3K9 methylation in ECs. G9a and SUV9H1 methyltransferase activities are sufficient to induce H3K9 tri-methylation at the VEGFA promoter as well as downregulation of this gene.

EZH2 silencing followed by H3K27me3 chromatin immunoprecipitation analysis confirmed a decrease in H3K27me3 methylation on selected EZH2 target gene promoters in agreement with their increased expression level [143]. Therefore, these last results argue for an anti-angiogenic role of EZH2 in ECs as its binding H3K27me3 repressive catalytic activity and gene repression have been linked to major factors inducing angiogenesis (FGF1, WNT/FZD signaling pathway and CDH13). A very recent study [144] highlighted the role of EZH2 in ischemic vascular repair. Indeed, the authors found that knockdown or pharmacological inhibition of EZH2 increased EC migration and capillary-like network formation *in vitro* both in normoxia and hypoxia although the effect was more pronounced in hypoxia. Consistent with these data, EZH2 was found to be bound to eNOS and BDNF promoters where it mediates H3K27 tri-methylation in normoxia and hypoxia [144]. Focusing on the EC response to hypoxia the authors found that EZH2 expression and activity is enhanced in hypoxia both *in vitro* and *in vivo* in a mouse model of hind-limb ischemia.

In summary, contrasting results have been obtained regarding the role of EZH2 in the endothelial lineage. Indeed EZH2 has been shown to repress angiogenic gene expression in non-ECs [140], non-pathological ECs [143] and in response to hypoxia and ischemic injury [144]. In contrast, EZH2 promotes angiogenesis in tumor-associated or activated ECs through the inhibition of anti-angiogenic genes such as VASH1 [141]. Altogether, these results suggest that the function of EZH2 is highly dependent on the cellular context.

Counteracting EZH2/PRC2 activity, KDM6A/UTX and KDM6B/JMJD3 enzymes are responsible for removing the repressive H3K27me3 and H3K27me2 marks thereby inducing the expression of target genes. Inactivating mutations in UTX have been identified in several cancers suggesting that it can act as a tumor suppressor in specific cellular contexts [145]. In the endothelial lineage, JMJD3 and UTX have been shown to demethylate H3K27me3 during differentiation leading to the activation of Hox genes [146]. Since Hox genes play important roles during endothelial differentiation, this suggests that JMJD3 and UTX may also be involved in regulating this process. Interestingly, UTX is part of a protein complex that also contains the H3K4 methyltransferase MLL3/4 suggesting the concomitant removal of the repressive H3K27me3 histone mark and introduction of the H3K4me1 and/or H3K4me3 activating marks [126].

Modulating H3K27 methylation through increasing UTX demethylase activity and/or inhibiting EZH2 methyltransferase activity represents a potentially useful strategy to enhance the expression of 'poised' genes (e.g. FGF1, WNT/FZD signaling pathway, CDH13, and Hox family of TFs) in ECFCs to improve their pro-angiogenic vascular repair properties (Fig. 7).

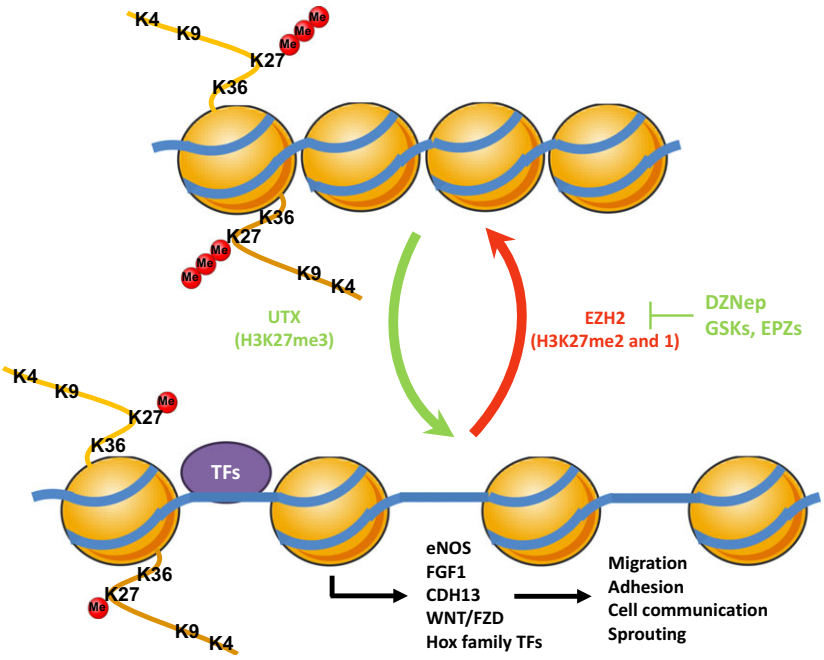

Fig. 7. Schematic representation of gene regulation and function through H3K27 methylation in ECs. H3K27 methylation regulates an entire set of genes implicated in EC differentiation (eNOS), migration (WNT/FZD, EphB2), adhesion and cell communication (CDH13, FGF1) through EZH2 activity in ECs. Inhibiting or downregulating EZH2 leads to H3K27me3 demethylation through UTX activity, enhancing EC functions.

H3K36 methylation in the endothelial lineage

The SETD2/HYPB histone methyltransferase is responsible for catalyzing the addition of H3K36me3 active marks. The gene encoding HYPB/SETD2 (HSPC069) has been initially isolated from hematopoietic progenitors [147]. Mice knockout for HYPB/SETD2 display impaired H3K36me3 (but not H3K36me1 and H3K36me2) leading to embryonic lethality between embryonic day 10.5 and 11.5 due to embryo, yolk sac and placental vascular defects. Specifically, capillaries of HYPB/SETD2$^{-/-}$ mice were found to be abnormally dilated due to lack of remodeling into large blood vessels or intricate networks. Gene expression profiling in HYPB/SETD2$^{-/-}$ yolk sacs further confirmed altered expression of genes involved in vascular remodeling [148]. *In vitro* experiments with HYPB/SETD2$^{-/-}$ ESC-derived embryonic bodies that recapitulate the formation of vascular plexus during embryogenesis also revealed impaired vessel formation even though the cells express the endothelial marker CD31 [148]. Consistent with these findings, knocking down HYPB/SETD2 in ECs resulted in reduced H3K36me3, altered migration due to disorganized actin stress fibers and decreased capillary-like formation *in vitro*. Furthermore, it led to a decrease in ANGPTL3, ANGPTL6 expression and increased c-JUN expression consistent with the altered expression pattern observed in HYPB/SETD2$^{-/-}$ yolk sacs [148]. In conclusion these data have shown that the HYPB/SETD2 H3K36me3 methyltransferase is required for vascular development, EC differentiation and function. While H3K36 demethylases have been identified (e.g. JMJD2a and JMJD2b that demethylate H3K36me3and H3K36me2; JHDM1a and JHDM1b that demethylate H3K36me2 and H3K36me1) [135], a potential role for these enzymes in vascular development or endothelial function has not been described.

Epigenetic drugs as a key tool to enhance the regenerative potential of endothelial stem/progenitor cells for vascular repair

A recent study from our laboratory [19] provided proof-of-principle that epigenetic drugs can be used to enhance the function of ECFCs prior to their transplantation for vascular repair. While we have shown that HDAC inhibitors increase the kinetics of ECFC migration, other epigenetic drugs could potentially ameliorate distinct cell properties (e.g. proliferation, secretion of pro-angiogenic factors) that will further improve the vascular repair potential of these therapeutically important cells. One of the main advantages of epigenetic drugs is that their action is reversible. As such, these drugs can potentially be used to provide a functional 'boost' prior to transplantation of stem/progenitor cells without the additional risk of genetic modification. In the following, we provide an overview of existing epigenetic drugs and their potential to be used in epigenetic therapies for endothelial regeneration/vascular repair.

DNMT inhibitors

DNMT inhibitors are divided into two classes: the nucleoside-derived class of inhibitors that can incorporate into DNA and the non-nucleoside class of inhibitors. Among the nucleoside-derived inhibitors, two have been approved by the Food and Drugs Administration for the treatment of myelodysplastic syndromes, i.e. 5-azacytidine (Vidaza®; Celgene Corporation, Summit, NJ, USA) and decitabine (Dacogen®; Eisai Co. Ltd., Tokyo, Japan). While at low dose these drugs act through inhibition of DNA methylation, at high dose they lead to covalent binding of DNMTs, inhibition of DNA synthesis and cell death. 5-azacytidine was approved first for myelodysplastic syndromes treatment at low dose while decitabine was approved later in 2006 [149,150]. 5-azacytidine treatment, alone or in combination with the pan-HDAC inhibitor TSA, has also been shown to induce eNOS promoter demethylation and the subsequent increase in eNOS mRNA expression in several non-EC types [37,40]. Furthermore, 5-azacytidine increases the expression of several DNA methylated endothelial genes including CD31 and vWF but does not change the expression of DNA unmethylated endothelial gene VEGFR2 in aortic VSMCs and none of the previously cited genes in ECs [57]. Altogether, these results show that 5-azacytidine has the ability to induce endothelial gene expression and endothelial differentiation through DNA demethylation in non-ECs. While nucleoside-derived DNMT inhibitors are highly toxic, and therefore poorly adapted to clinical applications [151], non-nucleoside-derived DNMT inhibitors have been identified, including the SGI-110 compound that is currently in clinical trial as a potential treatment for myelodysplastic syndromes [151]. However, so far, SGI-110 has not been tested in ECs.

HDAC inhibitors

TSA is a potent pan-HDAC inhibitor with a predominant activity towards class I and II HDACs [152]. A number of studies have described the effect(s) of TSA

treatment on endothelial stem/progenitor cells with conflicting results. For instance, early studies have shown that TSA decreases the proliferation and migration of ECs, and inhibits differentiation of endothelial progenitors derived from bone marrow and peripheral blood, through a decrease in HoxA9 expression [30,153]. Furthermore, TSA treatment led to decreased eNOS expression that correlated with reduced endothelial functions in vasorelaxation and angiogenesis [154]. Finally a recent report described broad histone deacetylation and loss of p300 binding upon TSA treatment of human aortic ECs [152]. Importantly, these studies have used relatively high concentrations of TSA (ranging from 0.5 μM to 2.5 μM), and the observed phenotypes (i.e. histone deacetylation and decreased gene expression) represent the exact opposite of the increase in histone acetylation and increase in transcription that would be expected after HDAC inhibition, suggesting that the observed anti-angiogenic response results mostly from indirect effects of the TSA treatment. Indeed, when used at a lower concentration (e.g. 100 $ng{\cdot}mL^{-1}$, i.e. 0.3 μM), TSA has a clear pro-angiogenic effect, leading to increased eNOS expression in ECs [155] as well as increased histone acetylation and increased expression of endothelial TAL1 target genes (CDH5, EFNB2, SOX7, HoxA9) in ECFCs, which results in faster migration of these cells and enhanced vascular repair [19]. Altogether, these findings highlight the importance of establishing the right dosage of epigenetic drugs for endothelial stem cell therapy.

Other HDAC inhibitors that have been tested on ECs include the pan-HDAC inhibitors VPA, vorinostat (also called SAHA), panobinostat (also called LBH589) as well as the HDAC1/3-specific synthetic benzamide-derived inhibitor entinostat (also called MS-275) [30,156,157]. Specifically, it was shown that VPA (500 μM) inhibits angiogenesis *in vitro* and *in vivo* [158,159], decreases EPC proliferation, differentiation and migration [30,153] and increases the resistance of ECs to apoptosis [159]. Furthermore, similarly to high concentrations of TSA, vorinostat (SAHA, 2 μM) decreases the binding of p300 to several gene promoters, leading to histone deacetylation and alterations in gene expression [152]. Finally, entinostat (MS-275, 10 μM) treatment results in impaired differentiation of endothelial progenitors [30], while panobinostat (LBH589, 100 nM) impairs angiogenesis-related functions of multiple myeloma ECs [157]. It should be noted, however, that lower doses of VPA, SAHA, MS-275 and LBH589 have not been tested and may increase endothelial differentiation and/or function, similarly to low doses of TSA treatment.

While TSA is among the most potent HDAC inhibitors (IC_{50} of 2 nM), it is also highly toxic and as such has been ruled out as a potential therapeutic compound. Other HDAC inhibitors such as SAHA and MS-275 display reduced side effects but are also less efficient with an IC_{50} of 10 nM and 510 μM, respectively [160]. In fact, the HDAC inhibitor with the highest potential as a therapeutic agent may be panobinostat (LBH589) which combines a low IC_{50} (5 nM) with a relatively long half-life, reduced toxicity and a growing body of clinical experience [160].

LSD1 inhibitors

The antidepressant drug *trans*-2-phenylcyclopropylamine (also called tranylcypromine, TCP or PCPA) is a potent (IC_{50} < 2 μM) and irreversible LSD1 inhibitor [161]. Initially used in the clinical treatment of mood and anxiety disorders, TCP also demonstrates a number of attractive properties that include (a) inducing cytotoxicity in ovarian cancer cell lines [162], (b) inducing differentiation and reducing engraftment of non-APL acute myeloid leukemia (AML) cells (in combination with ATRA) [163] and (c) inducing fetal hemoglobin expression in adult erythroid cells (i.e. a potential treatment for hemoglobinopathies) [164]. While TCP is yet to be tested on ECs, this drug is already approved for clinical use and as such represents an attractive candidate to enhance the function of endothelial progenitors in vascular repair given that LSD1 is a potential inhibitor of angiogenesis [132]. Furthermore, a number of additional LSD1 inhibitors (with both reversible and irreversible activities) have been developed and are currently being tested for cancer treatment, including GSK9552 for small cell lung carcinoma and ORY-1001 for the treatment of relapsed acute leukemia [151]. All these newly developed LSD1 inhibitors remain to be tested on ECs.

EZH2 inhibitors

The *S*-adenosylhomocysteine hydrolase inhibitor 3-deazaneplanocin (DZNep) is the first described EZH2 inhibitor [165]. While DZNep treatment induces a global decrease in H3K27 methylation through degradation of EZH2 and other subunits of the PRC2 complex (e.g. SUZ12 and EED) [166], DZNep is not specific for EZH2 and can affect methylation of histone residues other than H3K27 [167]. Despite uncertainties in the molecular mechanism, DZNep has been used quite extensively in ECs. Specifically, it has been observed that DZNep treatment of EPCs leads to a

decrease in the H3K27me3 mark on the eNOS promoter together with a modest increase in eNOS transcripts [60]. Furthermore, DZNep treatment increases eNOS expression in CD34+ hematopoietic stem/progenitor cells [60]. Interestingly, a recent report showed that treatment with DZNep increases eNOS expression in ECs, and improves EC functionality (migration, angiogenesis) *in vitro* [144]. Furthermore, when DZNep is administered *in vivo* in a mouse model of limb ischemia, the authors have observed an increase in the mobilization of circulating pro-angiogenic hematopoietic cells as well as a significant improvement in angiogenesis, ischemic muscle revascularization and blood flow recovery [144]. While these results suggest that DZNep could improve vascular repair in the ischemic muscle, it is currently unclear whether the observed effects are mediated through DZNep action on ECs, muscle cells and/or other neighboring cells. Furthermore, since DZNep triggers a number of non-specific effects, it remains possible that the observed vascular repair may not be directly related to EZH2 inhibition. To better understand the potential of EZH2 inhibition to improve vascular repair, one could use other recently developed epigenetic drugs that are characterized by an increased specificity towards the enzymatic activity of EZH2, including the competitive EZH2 inhibitors GSK-126 (K_i = 0.5–3 nM; IC_{50} = 10 nM [168]) and GSK-343 (K_i = 1.2 nM; IC_{50} = 4 nM [169]) from GlaxoSmithKline as well as EPZ-5687 (K_i = 24 nM; IC_{50} = 80 nM [170]) from Epizyme. GSK-343 has been successfully used to decrease H3H27me3 without affecting EZH2 expression or other histone H3 methylation marks (H3K9me2, H3K9me3) in various cell lines [171–173]. Whilst these compounds have been developed for cancer treatment [168,169,171], they could potentially be re-purposed for enhancing the function of endothelial progenitors as a cell therapy in vascular diseases.

Epigenetic drugs combination

It has been noticed that the combination of several types of small molecules that target distinct, complementary epigenetic pathways (i.e. HDAC inhibitors, DNMT inhibitors, histone methyltransferases and demethylase inhibitors) can increase their respective efficiency in eliminating cancer cells. For instance, combining DNMT inhibitors with an LSD1 inhibitor

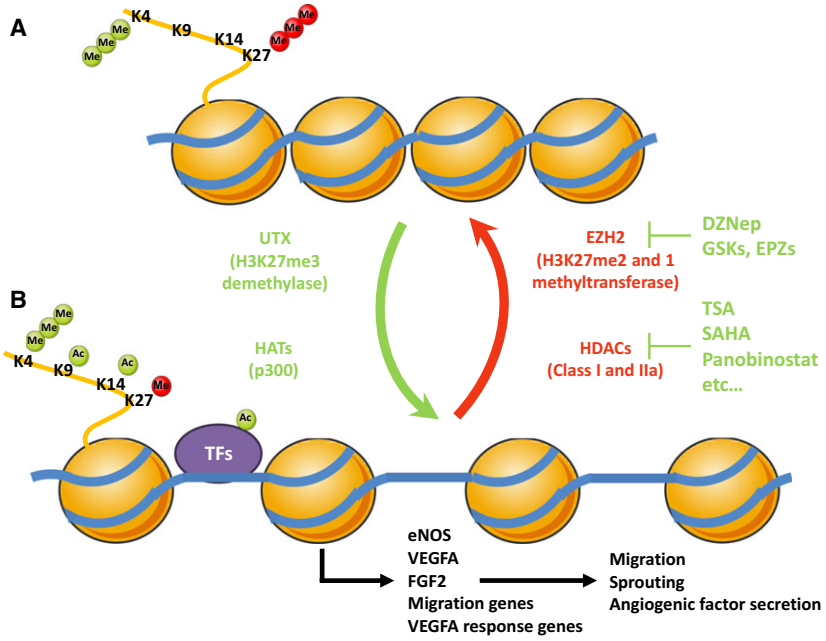

Fig. 8. Proposed model describing the effect of epigenetic drug combinations on endothelial progenitors with respect to endothelial gene expression and EC functions. (A) In endothelial progenitors, genes that are important for endothelial differentiation and EC functions are maintained in repressed/poised state (also called 'bivalent' state) characterized by lower levels of histone acetylation and by the co-localization of active (e.g. H3K4me3) and repressive (e.g. H3K27me3) histone marks. (B) The combined inhibition of HDACs (e.g. HDAC1) and H3K27 methyltransferases (e.g. EZH2) is predicted to release endothelial poised genes from their bivalent status, leading to increased transcription through inhibition of H3K27 methylation and increase in histone acetylation. This, in turn, could potentially lead to enhanced migration, adhesion, capillary-like network formation and/or pro-angiogenic cytokine expression.

significantly improves the growth inhibitory effect of these drugs on several cancer cell lines including bladder, colorectal and leukemia [174]. Furthermore, it has recently been demonstrated that the pan-HDAC inhibitor panobinostat and the novel LSD1 inhibitor SP2509 are synergistically lethal against cultured and primary AML blasts and improve the survival of a murine xenograft model of human AML [175]. Whilst it remains to be determined whether combining epigenetic drugs will also enhance the regenerative potential of endothelial stem/progenitor cells, several studies have suggested that this may be the case. Indeed, it has been shown that combining TSA and 5-azacytidine treatment increases the expression of eNOS [37,57], CDH5, vWF and CD31 endothelial markers in non-endothelial aortic VSMCs [57]. Furthermore, in endothelial progenitors, single drug treatments with TSA and DZNep did not change eNOS expression whereas a combination of these drugs was able to increase eNOS expression through simultaneous demethylation and acetylation of H3K27 at the proximal promoter [60]. Altogether, these findings highlight the combination of epigenetic drugs as a promising therapy to improve the vascular repair function of endothelial progenitors.

Conclusion

Even with our greater understanding of EPCs and ECFCs and emerging interest in their capacity for post-ischemic vascular repair, the lack of identification of a master gene regulator driving EC differentiation combined with our lack of refined knowledge concerning the epigenome of these cells has hampered their use as an efficient tool for cell based clinical therapy. Despite accumulating evidence regarding the regulation of these cells by epigenetic cofactors (HDAC inhibitors, DNMT inhibitors, histone modification inhibitors and combined treatments) progress towards clinical application using modified endothelial stem/progenitor cell mediated therapy has been slow. Therefore, characterizing the endothelial stem/progenitor epigenome remains a high priority for ongoing research that will capitalize on the well known pharmacokinetics and pharmacodynamics of epigenetic drugs (i.e. HDAC inhibitors) that have been extensively studied with regard to clinical absorption, distribution, metabolism and extraction properties in human subjects undergoing cancer treatments. Recent evidence suggests that combining epigenetic drugs that target different epigenetic pathways is the most promising strategy to enhance the function of EPCs and ECFCs for vascular repair. Indeed, we expect that *ex vivo* treatment of endothelial progenitors will provide a transient open chromatin conformation that is more permissive for TF binding (Fig. 8), thereby enhancing the capability of endothelial progenitors to respond to extracellular signals provided by the environment (e.g. hypoxia) after transplantation. Epigenetic drugs present the advantage of providing a transient change in chromatin structure resulting in a short-term therapeutic benefit required only during the vascular repair process. We suggest that modulating epigenetic modifications that are more stable (e.g. DNA methylation) with small molecules presents an increased risk of potentially harmful effects such as exacerbated angiogenesis that could result in the formation of leaky blood vessels (e.g. tumor vascularization) and/or the initiation of a tumorigenic program.

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Author contributions

S.F., C.G.P., D.D.A. and M.B. wrote the manuscript.

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