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SPOTLIGHT REVIEW

NO points to epigenetics in vascular development

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Abstract Our understanding of epigenetic mechanisms important for embryonic vascular development and cardiovascular differentiation is still in its infancy. Although molecular analyses, including massive genome sequencing and/or *in vitro*/*in vivo* targeting of specific gene sets, has led to the identification of multiple factors involved in stemness maintenance or in the early processes of embryonic layers specification, very little is known about the epigenetic commitment to cardiovascular lineages. The object of this review will be to outline the state of the art in this field and trace the perspective therapeutic consequences of studies aimed at elucidating fundamental epigenetic networks. Special attention will be paid to the emerging role of nitric oxide in this field.

Keywords Epigenetics • Vascular development • Nitric oxide

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1. Introduction

Nitric oxide (NO) is an important gaseous molecule that is synthesized in virtually every living cell[1,2] by three different nitric oxide synthases, namely endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Arginine is the substrate for NO synthesis, but the synthases require oxygen and other co-factors, including tetrahydrobiopterin and calmodulin, for efficient production of NO. NO concentration influences its effects. At 100 nanomolar (nM) or less it typically activates cyclic guanosine monophosphate (cGMP) synthesis and the protein kinase G (PKG) or ERK pathways. At higher concentrations, encompassing the 300–800 nM range, other factors/signalling pathways become activated including the AKT pathway, the hypoxia inducible factor-1α (HIF-1α), and p53 which is stabilized. At micro and millimolar concentrations, nitrosation, and oxidative processes prevail.[3] In these conditions quite different effects may be achieved ranging from cell proliferation to survival and senescence. However, as a general paradigm, we may assume that lower NO levels are physiological, whereas high concentrations may be lethal to the cell. The relevance of NO in cardiovascular physiology and physiopathology is largely demonstrated by the occurrence of severe dysfunctions and diseases associated with its reduced availability. Diminished NO levels have been, in fact, associated with atherosclerosis, cardiac infarction, and diabetes, to cite some the most common occurrences.[4,5] In mice knock-out for NO synthases, several cardiovascular accidents may occur as a consequence of severe NO deficiency, suggesting the cardiovascular system as the most important NO target organ.[6] Intriguingly, most of the NO effects have been recently ascribed to its regulatory function on gene expression. Specifically, an exquisite negative effect of NO on gene expression has been reported in endothelial cells after a series of microarray analysis.[7,8] In other cell types, including neurons and hepatocytes, NO has been found to positively regulate gene expression through the activation of neurotrophins, proliferation-associated, or functional genes.[9,10] All in all, these observations confirm the importance of NO as an important modulator of cellular function and provide consistent basis for its role in epigenetics.

The US National Institutes of Health, proposing their epigenomics initiative, recently stated that 'epigenetics refers to both heritable changes in gene activity and expression and to stable, long term, alterations in the transcriptional potential of a cell that are not necessarily heritable' (source: nihroadmap.nih.gov/epigenomics/). Indeed, epigenetics is a very rapidly evolving field covering, in our view, not only inheritable non-genomic chromatin-associated histone modifications but, nowadays, also those mechanisms that control transient changes in response to sudden environmental modifications, with rapid functional consequences on gene expression and/or cell metabolism, beyond covalent modifications of histones and non-histone proteins.[11–13]

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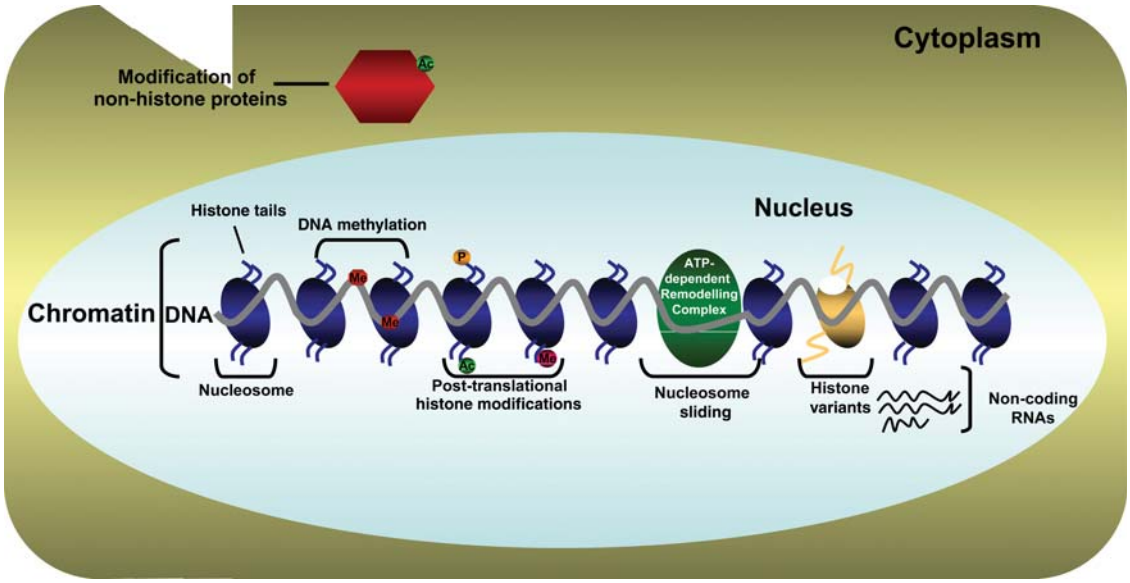

Figure 1 General mechanisms of epigenetics. DNA methylation, post-translational modification of the histone tails, nucleosome sliding, and deposition of histone variants are the principal mechanisms regulating chromatin structure and, consequently, gene transcription. Small non-coding RNAs and post-translation modification of non-histone proteins have recently been found to be other important epigenetic regulatory mechanisms (modified from[147]). Ac, acetylation; Me, methylation.

The study of epigenetics then, encompasses all those extra- intracellular messages able to introduce, in addition to DNA methylation and RNA-based mechanisms, structural modifications of histones, and non-histone proteins including acetylation, methylation, ubiquitination, sumoylation, neddylation, isomeration, and others, by means of a large number of dedicated modifying enzymes (*Figure 1*). Chromatin changes may become inheritable and transmissible to descendants avoiding the burden of primary DNA sequence modification.[14,15] It is currently unknown whether epigenetically modified non-histone proteins may also be a source of 'inheritable' signals.

Chromatin modifications, both in terms of DNA and histone proteins, represent the best-known consequence of the activation of epigenetic mechanisms. Chromatin consists in an organized sequence of nucleosomes made of two series of histone H2A, H2B, H3, and H4 wrapped by 147 base pairs of genomic DNA. The projections of histone tails from this core make histones susceptible to modification by different families of enzymes.[16]

DNA methylation represents a fundamental epigenetic mechanism regulating genomic activity and it is required for the proper development of mouse and human embryos. It consists in the addition of methyl groups to the dinucleotide CpG in the context of the so-called CpG islands by the DNA methyltransferases (Dnmts) family of enzymes. This process changes the biophysical properties of the DNA, impairing its recognition by some proteins while allowing the binding of others with the biological consequence of transcription repression.[17] Indeed, specific proteins, as the methyl-binding domain proteins (MBDs) and the methyl CpG-binding protein 2 (MeCP2), recognize methylated DNA, inhibiting transcription by creating a repressive chromatin structure not accessible to the basal transcriptional machinery, which contains, besides methylated CpGs, modified histone proteins. The modification of histones at specific amino acid residues is the triggering event determining the association of chromatin-binding proteins with specific regions to direct the structural change between the close (transcriptionally silent) and open (transcriptionally competent) chromatin state.

One striking feature of histone modifications is their reversible nature. Different families of enzymes add or remove chemical groups from histones, allowing the fine tuning of gene transcription.[18] Lysine (K) and arginine (R) methylation, K-acetylation, and serine/tyrosine (S/Y) phosphorylation are the most well-characterized histone modifications. Usually methylated CpG islands recruit K-histone methyltransferases (HMTases)[19,20] that methylate K9 on histones H3. This modification increases histone H3 affinity for the DNA, creating a highly packed chromatin structure, the so-called constitutive heterochromatin, not accessible to any transcription factor and/or proteins of the basal transcriptional machinery (represented by RNAPolII and associated proteins). However, the transcriptional repressive function of histone methylation is strongly influenced by residues position and may vary according to the abundance of methylated residues and their degree of methylation (from mono- to tri-methylation).

Opposite to methylation with a transcription repressive function, typically occurring on histone H3 at K9 and K27, methylation of residues K4 and K79 on the same histone may result in a positive transcription effect often fostered by the presence of other methylated residues, such as Rs on histone H4, which may in turn be targeted by negative transcription regulators leading to methylation of H4 K20.[21,22] Conversely, acetylation of lysines by histone acetyltrasnsferases (HATs)[23] and phosphorylation of serine and tyrosine residues on histone tails by nuclear kinases, such as Msk[24] and RSK2,[25] neutralizes the histone positive charge, decreases their affinity for the DNA, unwinds chromatin, and creates the conditions for access of those specific regions by the basal transcriptional machinery. Therefore, acetylation is usually associated with transcriptional activation.[16] Its counterpart, histone deacetylation, by histone deacetylases (HDACs),[23] has the opposite transcriptional effect. Beside their

transcriptional properties, the pattern of histone modification defining the so-called 'histone code' may have alternative functions including those aimed at regulating histone incorporation into chromatin during S phase (histone H4 K4 and K12 acetylation) or chromatin condensation during mitosis (histone H2A and H3 phosphorylation).[26] Several families of ATP-hydrolyzers, which break each of the 14 histone–DNA contacts, are responsible for these processes.[27] This 'simple' hydrolytic reaction accounts for: (i) nucleosome sliding to a new position; and (ii) ejection or displacement of the histone octamer.[28] The ATP-remodelers also remove H2A–H2B dimers, destabilizing the nucleosome,[29] and replace it with H2A histone variants.[30] In conclusion, all these modifications account for the combinatorial regulation of chromatin structure and function related to its processing during replication or differentiation directed events.[31]

Small RNAs-dependent chromatin remodelling is a rapidly expanding field of investigation. Usually, these small antisense RNAs interfere with gene transcription (small interfering RNAs, siRNAs, and short hairpin RNAs, shRNAs) creating a duplex with homologous DNA target regions in order to impair the binding of transcriptional complexes. Examples of siRNA-mediated gene silencing include the gene promoter of the progesterone receptor,[32] the huntingtin protein,[33] and eNOS.[34] Intriguingly shRNAs allow long-term transcriptional repression by recruiting onto the neighbouring chromatin domain Dnmts, HDACs, and HMTases.[35] For a detailed description of RNA-based epigenetic mechanisms refer to Malecova and Morris.[36]

A further level of complexity is represented by the structural organization of the chromatin in loops sticking to docking sites on the nuclear envelope. For example, the progeroid phenotype is caused by mutations in the nuclear membrane constituent LaminA (LamA) or in its processing enzyme Zmpste-24, which correlates with chromatin disorganization and transcriptional alterations. This leads to the establishment of an aged epigenetic pattern.[37] In the cardiovascular system, the dysregulation of histone acetylation/deacetylation processes is an established cause of cardiac hypertrophy,[38] and inhibitors of HATs have been proved useful to prevent heart failure (HF) in a rat animal model.[39] These examples underlie the relevance of the epigenetic processes also in human diseases, opening new fields of investigations with possible therapeutic implications.

In this review, we will focus on those epigenetic events related to embryonic development with special attention to the epigenetics of vascular precursors. It must be anticipated, however, that the epigenetic mechanisms important in vascular development remain, at present, largely uncharacterized.[11]

2. Embryonic stem cells as a model for early embryonic epigenetics

The formation of the embryonal vasculature goes through different phases, the earliest of which are determined by the mesoderm derivation of vascular precursors or haemangioblasts (*Figure 2*, for more information see ref.[40,41]). It has been well established that the expression of the vascular endothelial growth factor (VEGF) and its receptor FLK1 is one if the most critical gain-of-function events determining the phenotype of blood and vascular progenitors.[42–44] Relevant to the scope of this review is the evidence that the mesoderm is now accepted as the most important embryonic source of early progenitor cells of endothelial, smooth muscle, and cardiac origin.[41,45–47]

In spite of the criticism that the differentiation process taking place in '*in vitro*' cultured embryonic stem cell (ESC) does not properly reflect the differentiation process ongoing during mouse or human development, this cell system still remains a good and suitable model to molecularly dissect the early differentiation machinery. The ESC system, in fact, has been largely used to mimic *in vitro* the early phases of the haemato- angio-differentiation process and to investigate the implicated molecular processes.[40,45,48,49] Although human ESC (hESC)[50] and other types of pluripotent cells, including the inducible pluripotent stem cells (iPSs),[51] have been recently made available, the mouse ESC (mESC) must be considered the best characterized.[50,52] The recent views regarding the epigenetics of mESC self-renewal and differentiation shed new light on the role of histone modifications and the activity of specific epigenetic enzymes, including members of the HDAC family or the Polycomb group (PcG), either important for stemness maintenance than for progression towards the early multi-layer embryo-like structure.[11,53–59]

mESC are cultured in an undifferentiated state characterized by the presence of a large amount of nucleosome-free chromatin. High levels of histone H3 lysine 4 trimethylation (H3K4me3) and histone H4 acetylation (H4ac) are typically present in mESC transcriptionally active regions.[56,60,61] Remarkably, as a peculiar feature of undifferentiated mESC, the presence of histone H3 trimethylated lysine 27 (H3K27me3),[56,60,61] a repressive transcription mark, has also been found associated to chromatin domains containing the H3K4me3 modification. The presence of bivalent (positive/negative) modifications is believed to provide a dual function: the counterbalance of high levels of transcriptional activity and the priming of genomic regions poised to be transcriptionally activated or silenced during the subsequent steps of differentiation.[56,57]

Besides specific chromatin marks, the undifferentiated state of mESC is characterized by a series of transcription factors which are necessary for stemness maintenance. Among these, Oct4 and Nanog[62–64] are perhaps the best characterized, and recent evidence demonstrated their co-existence in repressive transcription complexes made of other transcription factors and the HDACs 1 and 2, important members of the HDAC class I family.[63,64] This finding, providing more information about the mechanism of stemness maintenance, also underlies the importance of specific epigenetic enzymes in this process. In this regard, PcG and the Trithorax group complexes are important epigenetic regulators of mESC differentiation being, respectively, implicated in the H3K27me3 and H3K4me3 modification and playing a crucial role in transcription control during the undifferentiated state or at the transition to lineage-associated gene expression.[60,65,66] HATs, such as p300 or regulators of DNA methylation as the TET protein family, emerged recently as additional classes of epigenetic enzymes important for the regulation of stemness and the initiation of mESC commitment.[67–69] Many other epigenetic factors or epigenetically regulated processes, including histone arginine methylases, cell–cell positioning and cytokines secretion, are implicated in mESC self-renewal, pluripotency, and differentiation, as detailed in several reviews.[14,54,59,65,70–72]

The presence of an open chromatin conformation and the co-existence of an active transcriptional repression mechanism is a remarkable feature of pluripotent mESC. The transition from self-renewal to differentiation occurs *in vivo* upon environmental cues and *in vitro* after leukaemia-inhibitory factor (LIF) withdrawal. Oscillation of stemness factors and waves of chromatin conformational

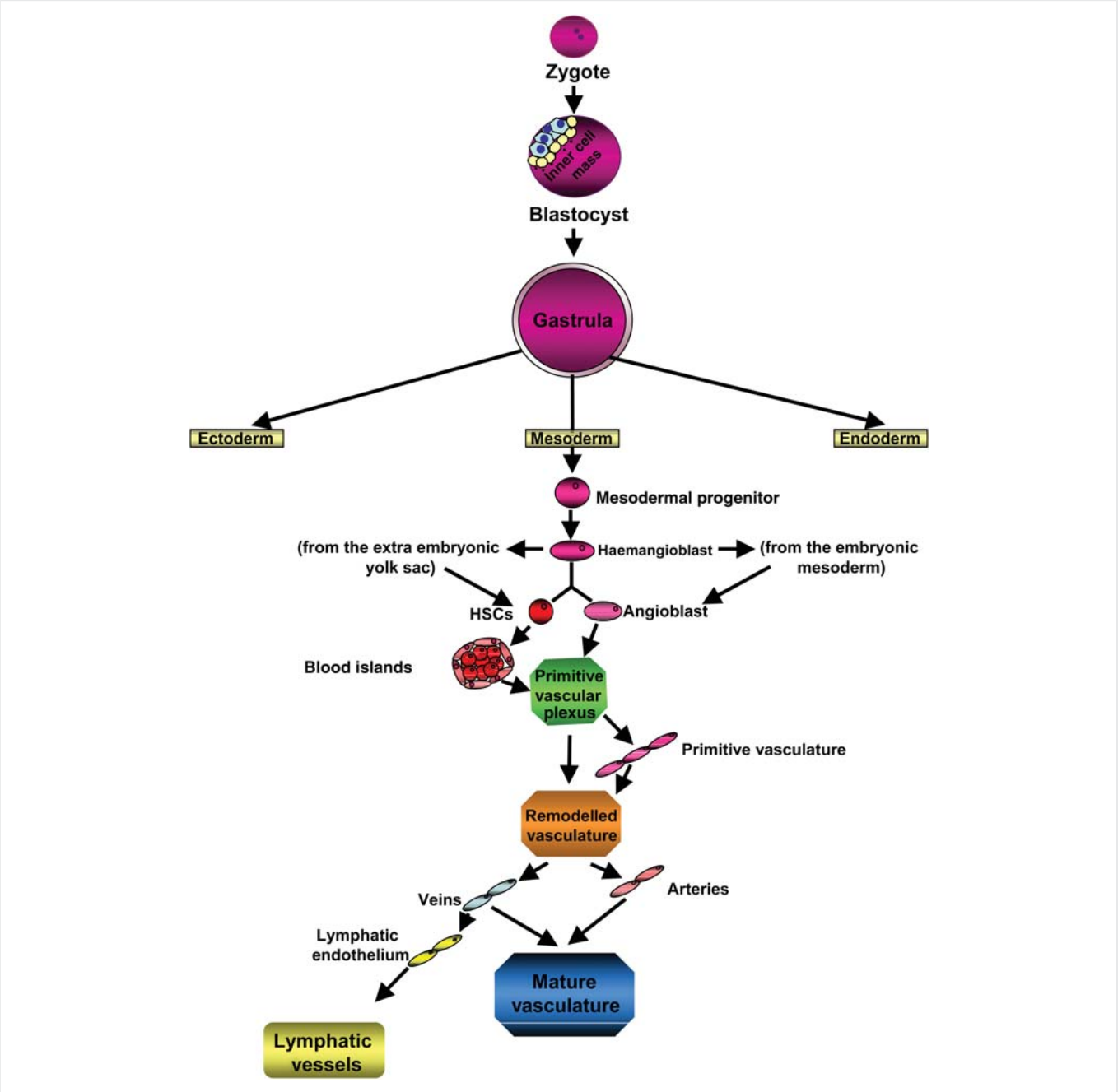

Figure 2 Vascular differentiation of ESCs. After gastrulation and formation of the three embryonic layers (ectoderm, mesoderm, and endoderm), haemangioblasts arise from the mesodermal progenitors of both the embryonic mesoderm and the extra embryonic yolk sac. Haemangioblasts of the yolk sac give rise to haematopoietic stem cells first and later to the early blood islands. Haemangioblasts arising from the embryonic mesoderm give rise to aggregates which join the yolk sac-derived blood islands to origin the primitive vascular plexus and a primitive vasculature. After a remodelling process, arterial, and venous endothelial cells differentiate to organize and consolidate the mature vasculature. From the venous ECs, lymphatic endothelial cells arise to give rise to the specialized lymphatic vessels. HSC, haematopoietic stem cell; EC, endothelial cell.

chances are among the very first events which follow LIF deprivation.[44,51,70] From this perspective, the mESC biological system has represented so far a unique source of information about the very early epigenetic events leading to embryonic commitment, although the number of studies focusing on this topic are still limited.[53,67,73–75] Nevertheless, our current understanding of mESC epigenetics let us to envisage that important contributions unravelling the mechanism of early vascular development are near to be discovered. The most recent observations emerged in this specialized field will be object of discussion in the next paragraphs.

3. Epigenetics of mesoderm specification

The permanent repression of self-renewal and other non-specific genes and the activation of early, tissue-specific molecular markers/

effectors represent a common mechanism of specification of early germ layers. During development, the early mesoderm is marked by the T-box (Tbx) transcription factor Brachyury.[76,77] In mESCs, many mesodermal gene promoters (e.g. GATA[78] and Tbx family members,[76,77,79] Mixl1,[80] and Brachyury[76,77]) are characterized by bivalent chromatin domains, bearing simultaneously transcription positive and negative histone modifications, and by the presence of Oct4, Sox2, and Nanog, suggesting that these stem factors may control these bivalent epigenetic marks and the expression of mesodermal-specific genes. Although the precise molecular mechanism controlling the balance of bivalent marks at gene-specific promoters is not yet known,[81–84] it is conceivable that, upon external cues, de-methylation of repressive H3K27me3 occurs, while the activating H3K4me3 is maintained at specific mesodermal chromatin domains. These chromatin regions are defined 'poised' for transcription.[85] Remarkably, chromatin within poised, non-mesodermal gene promoter regions, becomes permanently silenced as the opposite event occurs (e.g. H3K4 tri-methylation is lost, while H3K27-methylated residues are accumulated). Further, DNA and H3K9 methylation also account for the permanent repression of non-mesodermal genes transcription. Although the withdrawal of LIF represents the first signal triggering differentiation of mESCs into the three germ layers, the mechanism by which a particular cell population within the context of embryoid bodies (EBs) decide to differentiate into a specific cell lineage is not fully understood. It has been suggested that EB three-dimensional structure provides a proper environment which allows the onset of spatio-temporal events closely related to those occurring *in vivo* in the developing embryo.[52] Moreover, it is possible that a specific cell fate may be determined by the so-called 'gene dosage' of self-renewal transcription factors once a specific differentiation process is activated. The intensity of gene dosage, in fact, may determine the balance of epigenetic bivalent marks making the cell ready to transcribe a specific gene. A demonstration of this hypothesis is represented by the stemness-associated transcription factor Oct3/4, whose differential expression levels may induce either mesoderm differentiation[86] or dedifferentiation to trophectoderm. However, we cannot invoke only transcription factors involvement as the main mechanism regulating tissue specification. Chromatin remodelling enzymes, such as histone methyltransferases and demethylases, may respond to extra cellular signals, altering the balance of histone marks. Indeed, the expression of the Polycomb complex subunits Ezh2 and Eed decreases during differentiation and may contribute to the resolution of the bivalent domains.[87] The removal of histone methylation marks represents, in fact, a critical event for the resolution of the bivalent domains during the specification of any cell lineage. Recently, several reports pointed out the importance of H3K27 demethylases UTX and Jumomji domain-containing family of demethylases (Jmjd3) and that of several other members of the Jmjd as well as that of the LSD1 enzyme which catalyses H3K4 demethylation.[88,89] UTX and Jmjd3 associate with MLL complexes,[90] suggesting that removal of the H3K27me3 mark and maintenance of the H3K4me3 in genes that become activated during development are coordinated events.[75]

The remodelling of the chromatin structure by chromatin-associated non-histone proteins is also probably involved in the induction of mesoderm gene expression during differentiation. Recently, it has been demonstrated that the high mobility group (HMG) protein superfamily, such as HMG2A, is necessary for cardiogenesis. By interacting with Smad transcription factors, HMGA2, in fact, synergistically stimulates the transcription of the cardiogenic Nkx2.5.[91]

The silencing of non-mesodermal genes is as critical as the activation of mesoderm specific genes for the proper establishment of cell lineages. For example, LSD1,[89] by associating with the CoREST complex,[92] demethylates H3K4 within neural gene promoters, ensuring the repression of neural-specific genes during non-neural specification. However, other epigenetic events, like microRNAs,[93] participate in ensuring that non-mesodermal and self-renewal factors are silenced during specification. A detailed description of microRNAs involvement in mesoderm specification is beyond the scope of the present manuscript and will be addressed elsewhere.

4. Molecular events during early embryonic vascular development

Vascular structures derive from the extra- and intra-embryonic mesoderm. The interaction between mesoderm and endoderm, however, has been also found important providing instructive signals to the haemangioblast, the common blood and vascular precursor, whose transient appearance precedes the intra-embryonic endothelial and mural cell maturation and vessel formation.[94] *In vivo*, upstream from the vascular differentiation programme, lays the acquisition of the mesendodermal and mesodermal features. Experiments carried out in differentiating mESCs suggest that, as during embryo development, mesoderm cells are committed to the blood lineage prior to the occurrence of cardiovascular commitment. A role in this process is covered by the transforming growth factor (TGF) β superfamily, including TGFβ, nodal, and bone morphogenetic proteins (BMPs), the fibroblast growth factor (FGF), and the Wnt families.[95–97] The activation of the canonical Wnt-dependent signalling pathway[95] is one of the earliest molecular event leading to mesoderm- and endoderm-associated gene expression which includes Eomesodermin (Eomes),[77] Brachyury (T),[77] Mix-like homeodomain 1,[98,99] and GATA6.[98,99] Other transcription factors, soluble molecules and membrane receptors are involved in the mesoderm patterning towards the specification of vascular cells including Wnt/Frizzled, Delta/notch, BMPs, TGFβ, platelet-derived growth factor, FGF, Scl, Runx-1, Ets and other molecules whose concerted action have been shown to play a fundamental role.[96,100] More recently some microRNAs[101] have also been implicated.[102,103] The concerted action of this molecular regulators determines the appearance of FLK1+ mesodermal/vascular precursors, the endothelial specification, and the progression of vasculogenesis.[41,46] Interestingly, two FLK-1+ populations seem to emerge in a timely regulated fashion from an original Brachyury positive population. The earliest corresponds to the haemangioblast, which co-expresses Flk1 and Brachyury[104] and contributes to the formation of the primitive erithroid progenitors and the more mature CD34+ cells.[105] The second FLK1+ population is represented by cardiovascular progenitors able to generate cardiac, endothelial, and vascular smooth muscle cells (vSMCs).[106]

The regulation of FLK expression is complex and dependent on ill-defined transcription mechanisms.[68,74] A body of evidence indicates that the HIF family has a role in this process suggesting that environmental cues, such as the oxygen gradient, may also play an important function during vascular development.[107–110] Other lines of evidence shows that the activity of HATs has a relevant role in mesoderm specification[67,111] and FLK transcription.[68] Noteworthy, HATs and

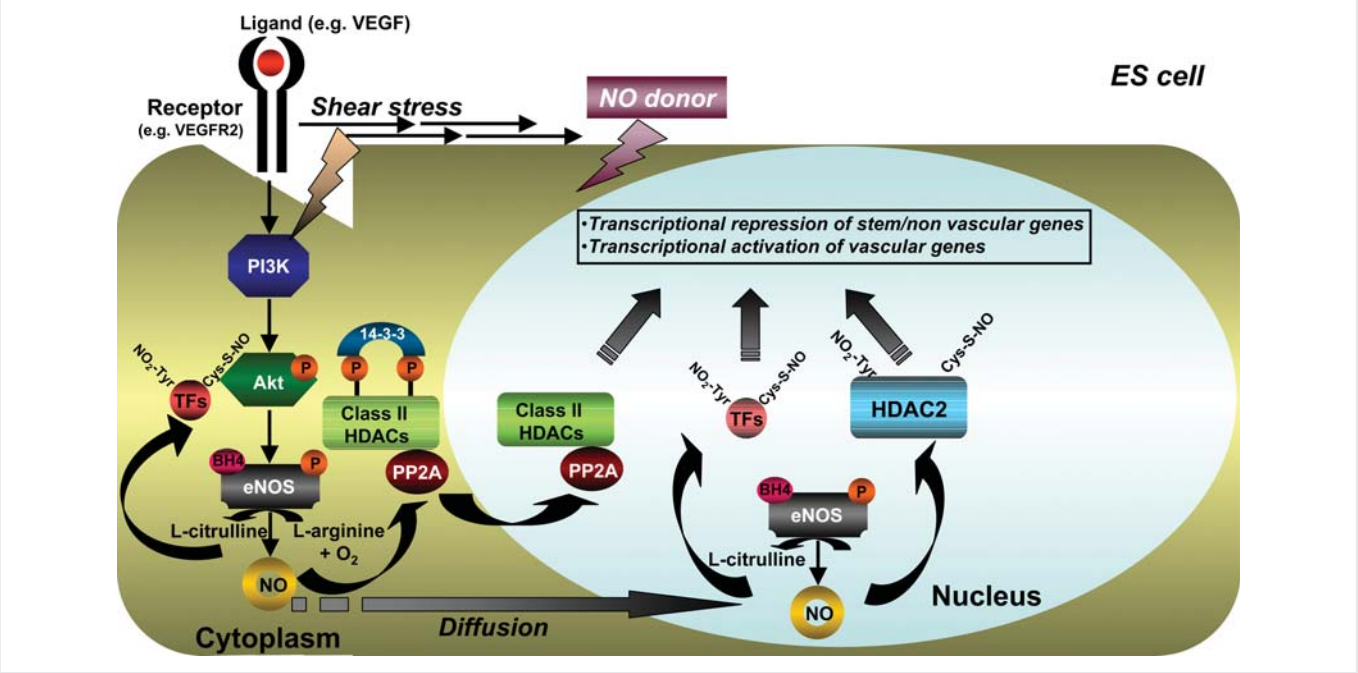

Figure 3 A model for NO-dependent epigenetic effect during ESC vascular differentiation. NO may be produced both by ligand-activated receptors and environmental cues (e.g. shear stress), which activate the PI3K/Akt pathway leading to eNOS phosphorylation. Cytosolic NO, in turn, induces class II HDACs nuclear translocation *via* PP2A activation and post-translational modification (mainly tyrosine nitration and *S*-nitrosylation) of transcription factors. NO may exert its function in the nucleus after diffusion from the cytosol. Further, it may be directly produced by the nuclear eNOS (ref). In the nuclear compartment, NO post-translationally modify HDAC2 and transcription factors. Altogether, these processes lead both to the repression of stem and non-mesodermal genes and to the activation of vascular genes. Tyr-nitration, tyrosine nitration; BH4, tetrahydrobiopterin.

HDACs are associated with HIF modulating its function, suggesting therefore that epigenetic regulators possibly activated by environmental signals are very important in the early commitment to mesoderm and during vascular differentiation.[112,113]

5. Epigenetics of vascular development

It is unclear whether specific epigenetic events are required for vascular development. It is however of recent observation that the alteration of epigenetic molecules such as the expression of HDAC7 altered the development of the normal vasculature probably trough an abnormal remodelling of the extracellular matrix.[63,114–116] Other epigenetic events involving HDAC function are triggered by shear stress[53,117] which is required for later angiogenic processes possibly occurring at the embryo/foetal boundary in the presence of a beating heart. Several lines of evidence support the role of HDACs in vascular development indicating that they could be crucial during the embryonic differentiation of vascular endothelial precursors as well as during regenerative processes occurring in adult individuals. Acetylases also play important although less characterized functions. Acetylation of histone at specific loci seems to control vascular gene expression in endothelial and smooth muscle cells.[8,117,118] The differentiation of vSMCs, in fact, depends on the activity of transcription factors known to modify chromatin structure at promoter level by increasing the local concentration of p300[67,116,119–121] which in turn locally modifies the 'histone code'[16] allowing an open chromatin configuration and transcription of lineage specific genes. The role of other epigenetic modifications or enzymes in the regulation of early and late phases of the vascular development is currently unknown. It is envisaged however that, due to the complexity of the cardiovascular system control network, further studies will be necessary to clarify the important contribution of environmental stimuli leading to vascular-specific combinatorial histone tail modifications,[118,122] that of non-histone proteins and the role of epigenetic enzymes other than HAT and HDACs.[72]

6. Epigenetic regulation of the nitric oxide signalling molecules

The soluble guanylate cyclase1 (sGC1) promoter has been recently described.[123] At present there is little or no information regarding its epigenetic regulation and that of other NO signalling molecules with the exception of eNOS which, on the contrary, has been thoroughly investigated. From an epigenetic point of view, it has been demonstrated that the eNOS proximal promoter is un-methylated in endothelial cells, but heavily methylated in non-endothelial cell types.[124] Further, Ets1, Sp1, and Sp3 transcription factors are recruited to the eNOS promoter in endothelial cells and not in vSMCs[124] while MeCP2 is preferentially recruited to eNOS promoter in vSMCs.[125] Of note, epigenetic processes have been found important also in the regulation of the other NO synthase isoforms. The methylation of K4 in histone H3 leads, in fact, to the expression of iNOS in chondrocytes[126] and the nNOS promoter is activated in neuronal cells by a nuclear factor kappa B (NFkB)-dependent chromatin remodelling mechanism.[127] This

experimental evidence highlights the importance of epigenetic mechanisms in the establishment of tissue-specific gene expression of NOS and may contribute to the epigenetic activities of NO.

7. Epigenetics of nitric oxide

During early embryonic life production of NO by eNOS will not occur until a valid shear stress will be generated by a beating heart.[128,129] It will occur well after the vascular bed it is formed and will contribute to its definitive maturation. It is currently unknown whether, in living mammalian embryos, NO and/or its derivatives may be present and playing active morphogenetic role before this stage. *In vitro*, mESC express detectable levels of nNOS and iNOS and, upon LIF deprivation, start to synthesize cGMP which mediates many of the NO-dependent metabolic effects.[130] Of note, a series of recent experiments demonstrated that mESC are very sensitive to NO donors.[53,63] Specifically, in the presence of various sources of NO, EBs developed more efficiently forming larger beating areas, indicating a pro-cardio-vasculogenic effect of this molecule.[131] In this regard, further evidence has pointed-out that shear stress, which elevates the intracellular level of NO in endothelial cells, as well as the direct exposure of adult endothelial or mESC to NO donors, determine the up-regulation of vascular genes including CD31, FLK/KDR, smooth muscle actin and the alpha-sarcomeric actin.[8,18,63,117]

Notably, the NO pathway mediates activation of the telomerase catalytic subunit (TERT) upon VEGF addition in an *in vivo* model of hind-limb ischaemia, during vascular and muscle regeneration.[132] Recently, TERT acquired a relevant role in angiogenesis, as mediator/effector of the VEGF-induced vascularization and capillarogenesis. Furthermore, in human umbilical vein endothelial cells (HUVECs), TERT transcription is regulated by NO/endothelial NOS signalling which, in combination with the oestrogen receptor pathway, contributes to chromatin remodelling, histone modification, and gene activation.[133–135]

Interestingly, other observations indicated that different post-translational modifications exert a strong regulatory control on epigenetic enzymes. Specifically, cysteine-*S*-nitrosylation represses class I HDAC2 function[136,137] and a similar effect to HDAC1 and 2 is obtained by alkylating agents.[138] An opposing NO effect has been reported for class II HDACs that were activated and nuclear localized in cells exposed to NO donors[8] (*Figure 3*). This event was found crucial for cellular differentiation in adult as well as mESC.[63,139] In the latter, the activity of class I and II HDACs including HDAC3, 4, and 7 could be associated with an important role in the regulation of gene expression. Although it has been reported that a member of the class II HDAC family, namely HDAC5, counteracts angiogenesis,[140] in the presence of a reduced class II HDAC content or activity the expression of vascular markers was significantly impaired.[63] These observations shed new light on epigenetics in the regulation of vascular differentiation and development. HDACs regulation emerges, in fact, as important in this biological process and suggests that different portions of the genome associated with the control of vascular development could be regulated by these enzymes. In this context a question arises about HDACs and the vascular regeneration processes to which vascular precursors may give an important contribution. Priming mESC with NO, in fact, activated a mesoderm/vascular differentiation programme which determined a significant improvement of tissue regeneration and vascular structures formation in an *in vivo* model of hind-limb ischaemia.[63] The evidence that the angiogenic process is significantly reduced by HDACs inhibitors[141–145] further supports the possibility that HDAC-dependent regulatory mechanisms are important in triggering this process.

8. Concluding remarks

The epigenetics of vascular development is still in its primordial age. Although remarkable progress has been made in recent years towards the comprehension of epigenetic signals and molecular mechanisms underlying embryonal differentiation and vascular lineage commitment, several questions remain unanswered. One of the most important is related to the identification of those chromatin regions active or inactive during embryonic vascular development and/or vascular regeneration in adult tissues. This information is of enormous relevance not only for its scientific value and the understanding of novel and important biological processes but for its potential implications in the definition of novel therapeutic strategies aimed at promoting angiogenesis and tissue repair. We believe, in fact, that the definition of environmentally driven signals and their effects on chromatin structure, gene expression and protein function will lead to the identification of degeneration/regeneration molecules whose activity could be controlled by specifically tailored epigenetically active drugs.[146]

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