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Review

Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium

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Abstract. The vascular endothelium plays a crucial role in regulating normal blood vessel physiology. The gene products responsible are commonly expressed exclusively, or preferentially, in this cell type. However, despite the importance of regulated gene expression in the vascular endothelium, relatively little is known about the mechanisms that restrict endothelial-specific gene expression to this cell type. While significant progress has been made towards understanding the regulation of endothelial genes through cis/trans paradigms, it has become apparent that additional mechanisms must also be operative. For example, chromatin-based mechanisms, including cell-specific DNA methylation patterns and post-translational histone modifications, have recently been demonstrated to play important roles in the cell-specific expression of endothelial nitric oxide synthase (eNOS). This review investigates the involvement of epigenetic regulatory mechanisms in vascular endothelial cell-specific gene expression using eNOS as a prototypical model, and will address the possible contributions of these pathways to diseases of the vasculature.

Key words. Chromatin; epigenetics; endothelium; gene regulation; histone code; endothelial nitric oxide synthase; sONE; cell-specific.

Introduction

The vascular endothelium is one of the first differentiated cell types to appear in the early embryo, and disruption of genes involved in the development of the primitive vascular tree are lethal [1–3]. The endothelial cell layer of blood vessels represents a crucial interface between the components of the blood and the rest of the body. However, this single layer of cells does not simply provide a physical barrier to regulate the movement of materials from the blood into tissues and visa versa, but is actively involved in maintaining homeostasis, by controlling such diverse processes as coagulation, blood vessel tone and vascular remodeling. To accomplish these functions, gene expression patterns are tightly regulated in endothelial cells. The expression of many of the key genes involved in these processes is unique, or highly restricted, to the endothelium. Yet, surprisingly, the molecular mechanisms underlying how this endothelial-specific pattern of gene expression is established and maintained are poorly understood. Endothelial nitric oxide synthase (eNOS) is a well-characterized endothelial-specific gene. In this review we will examine the mechanisms responsible for endothelial-specific gene expression using eNOS as a model.

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Endothelial nitric oxide synthase

The importance of eNOS to the maintenance of blood vessel homeostasis is highlighted by the phenotype of eNOS

null mice. While not lethal, targeted ablation of eNOS in the mouse has revealed that this gene is critically important in the control of blood vessel tone [4] and remodeling [5], hemostasis [6], angiogenesis [7] and the mobilization of endothelial progenitor cells [8]. eNOS is responsible for the production of the majority of the nitric oxide (NO) in endothelial cells. NO possesses several anti-inflammatory properties, including the ability to reduce pro-inflammatory NF-κB activity [9] and the adherence of white blood cells and platelets to the blood vessel wall [10, 11]. NO also inhibits the proliferation of the underlying layer of vascular smooth muscle cells (VSMCs) [12].

While eNOS plays a crucial role in blood vessel biology, it is not required for the development of the primitive vasculature. Compared with other endothelial genes such as VE-cadherin, the vascular endothelial growth factor (VEGF) receptors Flt-1/VEGFR-1 and Flk-1/KDR/VEGFR-2, and the angiopoeitin receptors Tie1 and Tie2/TEK, which are all expressed prior to embryonic day 8.5 [13, 14], eNOS is a late endothelial marker. Onset of eNOS occurs after the establishment of unidirectional blood flow in mice, at embryonic day 9.5 [our unpublished observations]. Robust expression of eNOS in the endothelium continues into adulthood, with expression of eNOS being highly restricted to the endothelial cell layer of medium- to large-sized arterial blood vessels. The constitutive expression of eNOS in mature endothelial cells is attenuated upon exposure to pathological stimuli. For example, tumor necrosis factor-α (TNF-α) [15, 16], hypoxia [17–19] and oxidized low-density lipoprotein [20] are all known to decrease eNOS mRNA levels. *In vivo*, eNOS RNA levels are also decreased in endothelial cells that overlie advanced atherosclerotic plaques [21]. While post-transcriptional mechanisms contribute to the decrease in eNOS RNA in these models of endothelial activation, changes in the transcription of the gene also play a prominent role. For example TNF-α [22, 23] and hypoxia [17] have been shown to decrease transcription of the eNOS gene. While the mechanisms responsible for decreased eNOS transcription in pathology are not known, the mechanisms responsible for the constitutive expression of the gene are likely compromised. Therefore, understanding how endothelial genes such as eNOS are constitutively regulated in a cell-specific manner may aid in the study of gene regulation during pathology.

Endothelial-specific gene expression – classic paradigms

Many endothelial-specific genes are constitutively expressed in endothelial cells of the adult, including eNOS, von Willebrand Factor (vWF), VE-cadherin, intracellular cell adhesion molecule-2 (ICAM-2), Tie1, Tie2, Flt-1/VEGF-R1 and Flk-1/VEGF-R2. Others are specifically induced primarily in endothelial cells, including E-selectin and vascular cell adhesion molecule-1 (VCAM-1), among others. In contrast to other cell types such as skeletal muscle or adipocytes, there are no known 'master regulators' of gene expression, such as MyoD [24] or PPAR-γ [25], respectively, that are specifically expressed only in endothelial cells. Several transcription factors have recently been shown to be preferentially expressed in differentiating endothelial progenitor cells and mature endothelial cells, and are thought to orchestrate the expression of a wide number of endothelial genes. For example, HoxA9 has been shown to regulate the expression of eNOS, Flk-1/VEGF-R2 and VE-cadherin during endothelial cell maturation [26] and KLF2 has been shown to positively regulate the expression of eNOS while preventing inflammatory-gene expression such as VCAM-1 and E-selectin in mature endothelial cells [27, 28]. However, the ability of these factors to act as 'master' regulators of endothelial-specific gene expression has not been documented.

In contrast to muscle-specific promoters, the majority of which contain consensus binding sites for serum response factor, ((CC(A/T)$_6$GG) [29], also known as the CArG box), endothelial cells do not have a common cis element that can bind endothelial-specific factors. There are, however, some commonalities that are shared among genes that are highly enriched in the vascular endothelium, including binding sites for Ets family members, GATA, Sp1, AP-1 and Octamer transcription factors. However, the transcription factors that bind these sites are by no means restricted in expression to the endothelium. It therefore stands to reason that either a specific combination or level of these factors is only found in endothelial cells, that unique biochemical modifications of these factors ensue in this cell type, that unknown endothelial-specific master regulators exist or that distinct modes of regulation apart from classic cis/trans paradigms are operative. We will first summarize the classic paradigms that have been used to explain the regulation of some of the better-characterized endothelial-specific genes. These classic paradigms have provided important information regarding the basal expression of endothelial genes.

Characterization of the eNOS promoter

The eNOS gene *(NOS3)* is composed of 26 exons spanning approximately 21 (kb) of genomic DNA on chromosome 7q35–36 [30]. The 4052-nucleotide (nt) eNOS mRNA is constitutively expressed in endothelial cells and is a very stable mRNA [31]. eNOS expression has been documented in a handful of non-endothelial cell types [8, 32–35]. For example, eNOS-derived NO in the hippocampus plays a role in long-term potentiation of neurons [33,

36]. However, vascular endothelial cells are the primary eNOS-expressing cell type and the regulation of eNOS expression in this cell type is the best characterized to date. eNOS is comparable in endothelial cell specificity to other endothelial markers such as CD31/PECAM and vWF, while mRNAs known to be even more restricted to the vascular endothelium are uncommon but include Flt-1/VEGF-R1, Flk-1/VEGF-R2, Tie1 and Tie2 [37]. Analysis of genomic regions that correspond to 5′ regulatory regions of the *NOS3* gene revealed binding sites for a variety of trans factors in the setting of a TATA-less promoter, including Sp1, Ets, GATA, NF-1, AP-1, shear-stress response elements and sterol regulatory elements [38]. Cis elements involved in the basal transcription of the eNOS gene were localized to two positive regulatory domains (PRD I and II) located at –104/–95 and –144/–115 relative to transcription initiation, respectively [39]. Ets family members, Sp1, variants of Sp3, MAZ and YY1 were shown to be involved in the regulation of eNOS through these two domains [39]. Both negative and positive protein-DNA and protein-protein interactions were identified at PRD I and II, suggesting that the basal expression of eNOS is highly regulated by precise stereospecific interactions of transcription factors. The functional importance of a GATA site (–230) upstream of these regions in the basal expression of eNOS has also been argued [40].

Characterization of an eNOS enhancer

DNase hypersensitivity (DHS) mapping can be used to identify the locations of proximal and distal regions that are involved in gene regulation. Using such a strategy, Laummonier et al. [41] found several DHSs located upstream of the human eNOS transcriptional start site. Further characterization of a DHS 5 kb upstream of the eNOS transcriptional start site revealed that this site acts as an enhancer of eNOS expression in endothelial cells. A 269-bp element located at –4.9 kb could increase the activity of the eNOS proximal promoter in endothelial cells, independent of the distance from the promoter and the orientation of the enhancer element, thus fulfilling the criteria of a classic enhancer element [41]. Importantly, this region shares significant sequence homology between mouse and humans. Characterization of this element revealed that MZF-like, AP-2, Sp1-related and Ets-related factors are implicated in the formation of nucleoprotein complexes [41].

Regulation of endothelial-specific genes – cis/trans paradigms

The eNOS promoter shares many features with other endothelial-specific promoters. These genes tend to share Ets, GATA, Sp1, AP-1 and Octamer cis regulatory elements that are required for their basal expression (table 1).

Ets family members

The identification of Ets-binding sites in the promoters of several endothelial genes has prompted the study of the involvement of this family of transcription factors in endothelial-specific gene expression. Several distinct Ets family members are constitutively expressed in endothelial cells, including Ets-1, Ets-2, Fli-1, Elf-1, Nerf2 and Erg-1, among others [42]. Ets proteins bind to a consensus sequence (GGAA/T) through the 85-amino-acid Ets-binding domain [43]. Important functional roles for Ets proteins have been identified in the basal and inducible expression of several endothelial genes (table 1). An Ets-

Table 1. Transcription factors known to regulate endothelial gene expression.

Transcription factor	Genes regulated
Ets family members	eNOS promoter (Ets-1, Elf-1) [39] and enhancer (Erg) [41] Flk-1/VEGF-R2 (Ets-1) [47] Flt-1/VEGF-R1 (Ets-1/2, Erg) [46] ICAM-2 (Erg) [191] Tie1 (Nerf2, Ets-1/2) [48] Tie2 (Nerf2, Elf-1, PEA3) [50, 52, 104] Utrophin-B (Ets-1/2, PU.1) [192] vWF (Ets-1/2, Erg) [193] VE-Cadherin (Ets-1, Erg) [49, 71]
GATA-2	Endothelin-1 (ET-1) [57] eNOS [40, 58] Flk-1/VEGF-R2 [47] ICAM-2 [61] P-Selectin [63] PECAM-1 [62] Utrophin-B [192] vWF (GATA-2 and GATA-6) [94, 144]
Sp family members	eNOS promoter (Sp1/3) [39, 40, 44] and enhancer (Sp1) [41] Flk-1/VEGF-R2 (Sp1) [69] ICAM-2 (Sp1) [61] VE-Cadherin (Sp1/3) [71]
AP-1	Endothelin-1 (ET-1) [75] eNOS [30, 74] Utrophin-B [192] Notch4 [78]
Octamer proteins	Tie1 [48, 81] Tie2 [194] vWF [83]
HoxA9	eNOS [26] Flk-1/VEGF-R2 [26] VE-Cadherin [26]
KLF2	eNOS [27, 28] Thrombomodulin [27] Flk-1/VEGF-R2 (negative regulation) [195]

binding site was identified in the eNOS proximal promoter within positive regulatory domain II (PRD II) (–144/–115) [39]. While the expression of Ets-1 transactivated the eNOS promoter/reporter construct in an Sp1-dependent fashion in cells which do not express Ets factors, namely *Drosophila* Schneider SL2 cells, an even greater effect was observed by expressing threshold amounts of Ets-1 together with Sp1 and Sp3 [39]. This effect required the presence of PRD II. This suggests that a multiprotein complex, including Ets-1, Sp1 and Sp3, forms on this element to positively regulate the eNOS promoter. Chromatin immunoprecipitation (ChIP) experiments have confirmed that Sp1, Sp3 and Ets-1 bind to the native eNOS promoter in endothelial cells [44]. Though these factors bind to the same genomic region in VSMCs, ChIP assays revealed significantly decreased amounts [44]. The Ets-binding site in PRD II at –132/–122 (5′-ACAGGAACAA-3′) was almost identical to a known Elf-1 interaction site in the HIV-2 LTR enhancer (5′-ACAGGAACAG-3′) [45]. Indeed Elf-1, an Ets family member, could also bind to PRD II and facilitated increased eNOS promoter activity in endothelial cells [39].

Ets factors have also been shown to regulate the expression of Flt-1/VEGF-R1 [46], Flk-1/VEGF-R2 [47], Tie1 [48] and VE-cadherin [49] (table 1). Multiple Ets sites were also found in the promoter of the Tie2 gene: one cluster in exon 1 and one cluster in an intronic enhancer [50]. These sites cooperatively regulated the expression of a Tie2 promoter/reporter transgene in the endothelium of mice, suggesting that interactions among multiple Ets sites are important for the expression of this gene [51]. A role for Ets factors in the regulation of endothelial genes in the developing vasculature has also been suggested [52, 53], and Ets proteins are potently regulated by angiogenic and inflammatory signaling pathways [54, 55]. While several Ets factors are highly expressed in endothelial cells, they are by no means endothelial specific. For example, VSMCs express basal amounts of Ets-1 and cellular activation can further increase Ets-1 expression [56]. Therefore, Ets factors may contribute to the expression of endothelial genes, but they cannot explain the cell-specific expression of these genes.

GATA

GATA sites are also prominent in endothelial promoters, and several of these sites have been shown to have functional importance to the basal expression of these genes (table 1). While there is no endothelial-specific GATA factor, GATA-2 is present in the greatest abundance in endothelial cells [57]. GATA-2 binds to the eNOS promoter at a consensus GATA site (–230) [40]. While some groups have revealed a modest effect of mutating this site in transient transfection analyses of eNOS promoter/reporter assays in endothelial cells [40, 58], others have failed to demonstrate a functional effect [39]. This site has been shown to be functionally important in the regulation of eNOS expression in airway epithelium, one of the non-endothelial cell types that express eNOS [58]. GATA factors are known to cooperate with Sp1 in gene regulation [59, 60]. Mutating the GATA site together with the Sp1 site in PRD I (–104/–95) did not have an additional repressive effect on transiently transfected promoter/reporters [40]. Importantly, the involvement of GATA-2 in chromatin-based regulation of eNOS is not known.

Several endothelial genes possess functional GATA sites in their core promoters (table 1), most of which have been demonstrated to bind GATA-2. Examples include endothelin-1 (ET-1) [57], Flk-1/VEGF-R2 [47], ICAM-2 [61], PECAM-2 [62], ET-1 [57] and P-selectin [63]. A role for GATA-6 has also been suggested in the regulation of vWF [64].

Sp1

Several members of the Sp family, belonging to a family of zinc-finger (His_2Cys_2) domain proteins, are ubiquitously expressed, including Sp1 and Sp3 [65]. These transcription factors bind to GC-rich promoter regions known as GC boxes. The basal expression of eNOS is highly dependent on a high affinity Sp1-binding site (5′-GGGGCGGGGC-3′) present in PRD I at –104 to –95 [39, 40, 66]. Mutation of this site greatly diminishes eNOS promoter activity. Using an electromobility shift assay (EMSA), both Sp1 and multiple variants of Sp3 were found to bind to this site [39]. The eNOS core promoter also contains a low-affinity Sp1 site (5′-CCTCCC-3′) at –146 to –141 that is located in PRD II. This site was also found to be functionally important for eNOS expression, and could bind both Sp1 and Sp3 [39].

Four variants of the Sp3 transcription factor are produced from different translational start sites. Each differs in their N-terminal extensions [67], with the two long isoforms containing two glutamine-rich domains, while the shorter isoforms contain only one [67]. The shorter isoforms have been suggested to inhibit or have no effect on transcription, while the full-length proteins are activators of transcription [67, 68]. Indeed, the addition of full-length Sp3 in the presence of half-maximal amounts of Sp1 in *Drosophila* cells increased eNOS activity [39]. Detailed analysis of the role of the shorter versions of Sp3 in controlling eNOS promoter activity revealed that NH_2-deleted Sp3 did not activate transcription by itself, but had an activating effect in the presence of Sp1, full-length Sp3 and Ets-1. When limiting amounts of NH_2-deleted Sp3 alone were used, repression of activity was observed, perhaps suggesting that this shorter version of Sp3 can interfere with intermolecular interactions at PRD I and II

[39]. Such intermolecular interactions among protein complexes involving PRD I and II are highly relevant to eNOS transcriptional activity. The strongest activation of the eNOS promoter was achieved with limiting amounts of Sp1, full-length Sp3 and Ets-1, suggesting a complex interplay between these factors [39].

Sp1 sites are also functionally important in the basal expression of other endothelial genes (table 1), including Flk-1/VEGF-R2 [69], VCAM-1 [70], VE-cadherin [71] and ICAM-2 [61].

AP-1

Initial characterization of the 5′-flanking region of the human eNOS gene revealed putative binding sites for AP-1 (TGASTCA) at –1530 and –661 [30]. AP-1 has subsequently been implicated in the up-regulation of eNOS transcription by cyclosporine A [72], hypoxia [73] and shear stress [74]. AP-1 has also been implicated in the expression of ET-1 in endothelial cells [75], where it cooperates with GATA-2 to maintain basal expression. Notch4, which is restricted to arterial endothelial cells during embryogenesis and in the adult vasculature [76], cooperates with Notch1, a ubiquitous Notch protein, to control vascular morphogenesis [77]. The cell-specific expression of Notch4 is dependent upon preferential localization of AP-1 to the promoter [78]. In this example, treatment of non-endothelial cells with angiogenic factors resulted in the functional activation of AP-1 and transcriptional induction of Notch4 [78], implying a crucial role for AP-1 in the cell-specific expression of this gene.

Octamer

Octamer transcription factors are members of the POU protein family that bind to the sequence ATGCAAAT [79]. Oct-1 is a ubiquitously expressed member of this family [80] that has been shown to bind to the Tie1 and Tie2 promoters and direct robust expression in the vascular endothelium [48, 81, 82]. Consistent with a role for Oct-1 in directing the endothelial expression of these genes, Boutet et al. [81] revealed that mutations to an Octamer-binding site resulted in non-endothelial expression of the normally endothelial-specific Tie1 promoter/reporter transgene in mice. This suggests that this element may be involved in cell-specific regulation. Oct-1 has also been shown to negatively regulate the expression of endothelial genes such as vWF [83] and VCAM-1 [84].

Endothelial genes are regulated by transcription factors that are not endothelial specific

As outlined above, classic cis/trans paradigms have primarily invoked Ets family members, GATA-2, Sp1, AP-1 and Octamer transcription factors as an explanation for the basal expression of constitutively expressed genes in the vascular endothelium. These models have been insightful, but they are obviously not complete since these factors are not restricted in expression to the vascular endothelium. For example, Sp1 and Oct-1 are ubiquitously expressed transcription factors, as are several members of the Ets family. While levels of GATA-2 are high in endothelial cells, GATA-2 is also expressed in hematopoetic (erythroid, mast, megakaryocyte) [85], and neuronal cell types [86]. Because of the limitations of this model, novel endothelial-specific regulators of endothelial genes have been sought.

Identification of novel transcriptional regulators of endothelial genes

To date, master transcriptional regulators have not been characterized for the vascular endothelium. The binding of transcription factors to promoters does not necessarily directly depend on known cis DNA elements. Transcription factors can be tethered to the promoter by other factors and can also bind to non-consensus or unknown elements. Wang et al. [87] used a series of methods to determine the proteins that are functionally engaged at the vWF promoter in endothelial cells. They performed extensive EMSAs with seven overlapping probes corresponding to the endothelial-specific region +155 to +247. Upon characterization of protein complexes binding to these probes they were able to map a 6 nt element (+164 to +169) that formed an endothelial-specific complex. Mutation of this element inhibited promoter activity in endothelial cells. By utilizing mass spectrometry and Western blotting, they concluded that a histone H1-like protein regulated the vWF promoter in endothelial cells. The involvement of this regulatory protein in endothelial-specific gene expression could not have been predicted from the promoter sequence alone. These findings suggest that novel endothelial-specific transcriptional activators may exist that do not bind to known consensus binding sites.

Another powerful technique for the identification of novel endothelial gene regulators is the use of microarray platforms. cDNA and oligonucleotide microarrays have been used to identify transcriptional regulators involved in gene regulation in endothelial cells exposed to prolonged, steady laminar flow [88]. These studies revealed that KLF2, a Kruppel-like transcription factor, is potently up-regulated by flow *in vitro*, and is preferentially expressed in the endothelium of blood vessels [88]. While KLF2 expression is increased by flow, detectable basal levels of KLF2 are found in endothelial cells grown under static conditions. Basal levels of KLF2 are decreased following the administration of pro-inflammatory cytokines [28, 88], which are known to result in endothelial dysfunction, in part through decreased eNOS transcrip-

tion [22, 23]. Indeed, reduction of KLF2 levels in endothelial cells using RNA interference caused a decrease in the expression of anti-thrombotic genes such as eNOS and thrombomodulin, while amplifying the TNF-α-induced expression of pro-coagulant genes, such as tissue factor [27]. In addition, over-expression of KLF2 increased eNOS and thrombomodulin expression and inhibited the activation of VCAM-1 and E-selectin in response to cytokines [27]. KLF2 therefore appears to be involved in the regulation of genes that maintain the anti-thrombotic and anti-inflammatory properties of the endothelium. KLF2 may be a 'master' regulator of some, but by no means all, endothelial genes.

Studies by Chi et al. [89] also used microarray analyses to identify key regulators of endothelial genes. By examining the transcriptional profile of 53 cultured large-vessel and microvessel human endothelial cells isolated from different tissues and from arterial or venous blood vessels, they identified Hey2 as a key regulator of arterial gene expression patterns. Hey2 is a homolog of the zebrafish gene *gridlock*, mutation of which results in defective vascular patterning of the dorsal aorta in zebrafish [90]. Importantly, when Hey2 was over-expressed in venous endothelial cells, characteristic arterial genes were expressed. These data were taken to indicate that Hey2 is a key regulator of arterial gene expression [89].

Another method for identifying important endothelial transcriptional regulators is to perform genetic screens of embryonic stem (ES) cells using retroviral entrapment vectors to identify genes required for vascular development [91]. Using this technique, Xiong et al. [91] found that vascular endothelial zinc finger-1 (Vezf1) was coexpressed along with Flk-1/VEGF-R2 during the differentiation of ES cells into embryoid bodies. Transgenic mice generated with these 'entrapped' ES cells revealed that this gene was expressed during the differentiation of angioblasts into endothelial cells, and expression remained restricted to endothelial cells in the adult. Mice deficient in Vezf1 displayed vascular developmental defects, including abnormalities of endothelial cell adhesion and tight junction formation [92]. It has been suggested that Vezf may be a 'master regulator' of endothelial genes, but so far a role for this factor has only been shown for ET-1 in mature endothelial cells [93]. Aitsebaomo et al. [93] ruled out a role for Vezf in the regulation of the Flk-1/VEGF-R2 gene, at least in transient transfections involving the proximal promoter. Because Vezf is highly expressed in endothelial cells, determining the endothelial genes that are targets of this factor during development and in the adult is an important future aim.

Despite the identification of several novel regulators of endothelial genes, no 'master' regulator has yet been identified. Transcription factors that are highly restricted in expression to endothelial cells, such as KLF2, Hey2 and Vezf-1, may cooperate together with more ubiquitously expressed factors such as Sp1, GATA-2 and Ets factors to control the expression of endothelial genes. However, this model of endothelial-specific gene regulation is poorly understood.

Endothelial-specific promoters: episomal promoter/reporter studies

The promoters of several genes that are highly expressed in endothelial cells relative to other cell types *in vivo* have been studied in detail in an attempt to understand the basis for endothelial-specific expression. Most studies have utilized transiently transfected promoter/reporter systems to study the transcriptional regulation of these genes. The majority of the endothelial promoters studied to date are highly active predominantly in endothelial cells, and have little activity in other cell types. For example, portions of 5′ regulatory sequences of the Tie2 [50], ICAM-2 [61], vWF [94], Flt-1/VEGF-R1 [95], Flk-1/VEGF-R2 [96, 97] and the VE-cadherin [98] genes were all selectively active in endothelial cell types. Because the factors that regulate these genes are not in most cases endothelial-specific, perhaps the relative abundance of these factors, or the presence of alternatively spliced factors or post-translational modifications of the factors themselves, are crucial to the cell-specific regulation of these genes. In contrast, despite the highly endothelial-specific expression of eNOS, the promoter was found to be highly active in transient transfection assays performed in a wide variety of cell types, including those that do not express native eNOS RNA [44, 99].

While transient transfection analyses have been instrumental in defining the cis/trans regulatory mechanisms controls these genes, the systems are artificial since they do not involve the native chromatin configuration. In addition, vascular-bed-specific extracellular signaling pathways, including shear-stress-induced signaling, play an important role in regulating endothelial genes [100]. These pathways are, for the most part, absent from such *in vitro* culture systems. For these reasons, *in vivo* systems must be employed to study gene function.

Endothelial-specific promoters: chromosomally integrated transgenes

While the majority of endothelial promoter are cell restricted in transient transfections, the construction of transgenic mice with chromosomally integrated promoter/reporter constructs has revealed that these short regions of 5′ regulatory regions are often insufficient to recapitulate the expression profile of the native gene. An added complexity in the regulation of a transgene is the random chromosomal integration that occurs, as well as the variability in the number of copies of the transgene that become incorporated. The chromatin surrounding

the integration site of the transgene can impede the activity of the promoter of interest. Several endothelial-specific regulatory regions have been shown to direct expression of reporters in the endothelium of transgenic mice potently. For example, a mere 0.33 kb (–202/+44) of the ICAM2 promoter allows for endothelial-specific expression in most vascular beds [61], as does 2.5 kb (–2486/+24) of the VE-cadherin promoter [98]. However, initial studies using Tie2 promoter fragments, even up to 7.2 kb in size, did not result in endothelial-specific expression in adult mice [101]. Studies using up to 4.4 kb (–4100/+299) of the Flk-1/VEGF-R2 promoter were also unsuccessful [102]. Furthermore, 0.49 kb (–487/+246) of 5′-flanking vWF sequences and exon 1 resulted in expression in only a subpopulation of endothelial cells in the yolk sac and in the brain of adult mice [103]. This expression profile did not match that of native vWF mRNA. An important facet of gene regulation in a chromatin context is the action of enhancer elements, which can modulate promoter activity at a considerable distance. For both the Tie2 and Flk-1 genes, enhancers were found to be important for directing endothelial gene expression. Schlaeger et al. [104], studying the regulation of Tie2 transgenes found that 2.1 kb (–1800/+318) of the 5′-flanking genomic sequence and portions of exon 1, together with a 10 kb fragment of the 5′ end of intron 1 were necessary to direct expression of a reporter in vascular endothelial cells in development and in adulthood. Subsequently, a mere 1.7 kb fragment of intron 1 was found to be sufficient for expressionism in endothelial cells [105]. Likewise, a 0.94 kb (–640/+299) region of the Flk-1 promoter together with a 510-bp (+3437/+3947) region of the first intron allowed for endothelial-cell-specific expression [102].

eNOS promoter/reporter transgenes have also provided crucial information about the regulation of endothelial genes *in vivo.* Guillot et al. [106], using 1.6 kbp (–1600/+22) of the 5′-flanking region of the human eNOS gene driving the expression of LacZ demonstrated that while the eNOS promoter was active in micro- and macro-vessel endothelial cells of the heart, brain and skeletal muscle, and in a subpopulation of endothelial cells of the coronary artery and aorta, and in pulmonary arteries in one founder, there was a lack of promoter activity in the endothelium of blood vessels in the liver, kidney or spleen. Because this region of the eNOS promoter was insufficient to recapitulate the expression profile of the native eNOS gene and expression of the reporter was variable across founders, this particular transgene may have been influenced by the local chromatin structure of the integration site. Indeed, this group also targeted the same eNOS sequences to the *Hprt* locus on the X chromosome, a region of the genome that is thought to be permissive to transcription, and found that the variability between founders was eliminated, and the transgene was active in a wider spectrum of endothelial cells, including the endothelium of blood vessels in the heart, brain, kidney, lung, spleen and skeletal muscle [100]. This expression pattern is similar to that of the native eNOS gene. Considering that an endothelial-specific enhancer [41] may exist distal (–4.9 kb) to the region used by Guillot et al. [106], there may be genomic regions in the 5′-flanking region of eNOS that are responsible for modifying the structure of the chromatin in which the transgene resides. Using a larger region of 5′-flanking eNOS sequences (–5200/+28 of the mouse eNOS promoter), Teichert et al. [107] revealed that expression mirrored that of the native gene, with robust expression evident in the medium – and large-sized vessels of the heart, lung, kidney, liver, spleen and brain. Expression was also found to be uniform in multiple founders, independent of integration site in the genome, and robust even at the single-copy level. In addition, expression of the transgene was developmentally regulated in a manner similar to the native eNOS gene [our unpublished results]. Importantly, this transgene contained regions homologous to the human enhancer, suggesting that an eNOS enhancer is operative in mice as well as in humans.

Endothelial-specific gene expression – new paradigms

Chromatin structure regulates transcription

The presence of a cis element in the proximal 5′ regulatory region of a gene does not necessitate an interaction with the corresponding trans factor *in vivo*. There are many mechanisms that regulate factor binding, including the amount of the factor present in the cell type, differential subcellular localization of the trans factor [i.e. sterol-response-element-binding proteins (SREBPs), NF-κB], the presence of factors that compete for the same binding site, and post-translational modifications of the factor. A more recent theme is the importance of the structure of the chromatin encompassing the binding site. Chromatin structure can be influenced in a variety of ways. DNA methylation, which occurs on cytosine residues primarily in the context of a CpG site, can prevent the binding of trans factors. This is true for some (i.e. HIF1α [108], AP-2 [109] and c-Myc [110]), but not all (i.e. Sp1 [111], NF1 [112]) transcription factors with a CpG in their recognition site. DNA methylation can also influence the physical structure of DNA and can mediate changes in higher-order chromatin structure [113, 114]. The compaction of chromatin is determined in large part by the post-translational modifications of the unstructured N terminus of histones H3 and H4. These histone tails can be modified by acetylation, methylation, phosphorylation, ubiquitylation, ADP-ribosylation and sumoylation, among others. Because individual histone tails can be ‘decorated’ with a

variety of covalent tags, and because the presence of particular modifications can influence the binding of chromatin regulatory proteins, as well as components of the transcriptional machinery, a histone code is thought to exist that allows the cell to 'read' whether a portion of chromatin should be transcriptionally active or inactive [115–117].

Acetylation of lysine residues located on the N-terminal tails of H3 and H4 have been the most comprehensively studied. In general, acetylated histones are located at the promoters of transcriptionally active genes [118]. Lysine methylation has also been extensively studied and has been shown to have divergent effects on transcription. While methylation of lysine 4 of histone H3 is a signature of transcriptionally active chromatin [119], particularly at the promoter and 5′-coding region of genes [118, 120], lysine 9 methylation of histone H3 is a silencing signal that facilitates the recruitment of heterochromatin protein 1 [121, 122].

Transcription factors regulate transcription via chromatin-remodeling activity

Importantly, trans factor function is not only influenced by chromatin structure, but the factors themselves can regulate chromatin structure via interaction with a variety of chromatin-modulating enzymes, including DNA methyltransferases, histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and nucleosomal remodeling proteins. The regulation of muscle-specific gene expression is illustrative of the role of chromatin modifiers in transcription. The control of smooth-muscle-specific genes is controlled by common CArG elements that bind serum response factor (SRF). Myocardin binds to SRF and regulates the expression of muscle-specific genes during muscle development. In undifferentiated muscle, myocardin interacts with HDACs to negatively regulate transcription, but during differentiation, myocardin switches interaction partners from HDACs to p300, a HAT [123]. Analysis of the smooth-muscle-specific gene SM22 during differentiation has revealed that histone acetylation increases at the promoter of this gene. By altering the coactivator/corepressor interactions of transcription factors such as myocardin by treatment with drugs that inhibit the activity of HDACs, genes that are normally repressed in undifferentiated cells can be expressed, and cells are forced to differentiate [124].

In contrast to the regulation of muscle-specific genes, the control of endothelial gene expression is not by a single cis element but, rather, a combination of factors are required for expression, and the activity of the majority of these factors is not cell specific. As mentioned previously, models involving Ets family members, GATA, Sp1, AP-1 and Octamer proteins have been used to define endothelial gene expression. Not surprisingly these factors have been shown to be associated with various chromatin-modifying enzymes (table 2). For example, both Ets-1 and Ets-2 have been shown to associate with p300 and the cAMP-response-element-binding protein (CBP), proteins known to have HAT activity [125]. This interaction with HATs can be dynamically regulated through phosphorylation of Ets proteins, which are targets of several signaling cascades, including MAPK [126]. Ets family members also associate with HDACs [127–129], and with the histone H3 lysine-9-specific methyltransferase ESET (ERG-associated protein with a SET domain) [130, 131]. Sp1 also associates with both HAT [132, 133] and HDAC [99, 134, 135] activity. Similarly, GATA factors have been implicated in the recruitment of HATs [136, 137] or HDACs [138, 139] to promoters or enhancers. Of interest for eNOS transcription, YY1 has been shown to associate with HAT [140], HDAC [141] and HMT activity [142]. Thus, the presence or absence of a particular trans factor at a promoter is not enough information to be able to conclude whether this interaction will positively or negatively affect transcription. Rather, the concerted action of transcription factors together with coactivators/corepressors determines the activity of a promoter.

Table 2: Coactivator and corepressor proteins known to associate with transcription factors involved in endothelial gene regulation.

Transcription factor	Coactivator interaction	Corepressor interaction
Ets family members	p300/CBP (Ets-1/2, PEA3) [125, 196, 197]	mSin3A/HDAC1 (Elk-1) [127] HDAC1 (PU.1) [129] ESET/mSin3A/B complex (Erg) [130, 131]
GATA-2	p300 [136] CBP [198]	HDAC3,5 [138]
Sp1	p300/PCAF [132, 133]	HDAC1 [99, 134, 135]
AP-1	p300/CBP [199]	HDAC3 [200]
Octamer proteins		TSA-sensitive HDAC(s) [201]
KLF2	p300/CBP [28]	

An endothelial gene that is regulated by the mechanisms described above, namely the differential recruitment of coactivators or corepressors by the same trans factor, is vWF. The vWF promoter is only active in cells of the endothelial and megakaryocyte lineage. The locations of activating elements, which promote expression in endothelial cells, and repressing elements, which repress expression in non-endothelial cells, have been mapped using transient transfections of linker-scanner mutants. The vWF promoter is positively regulated by GATA and Ets factors and negatively regulated by Oct1 and NF1. Additionally, two NFY-binding sites were identified in the vWF promoter, which have opposing effects on transcription. One of the sites located at –18 is a consensus (CCAAT) binding site and acts as an activator element, while the other is a non-consensus site located between +226 to +234 which acts as a repressor of non-endothelial expression of vWF [143]. Peng and Jahroudi [144] demonstrated that both sites could bind NFY, but the site at +226 to +234 recruited HDAC1 and 2 to this region to repress transcription in non-endothelial cells [144]. This repression was correlated with a decrease in the acetylation of histone H4 at this region. It was also hypothesized that NFY-associated HDACs may deacetylate GATA-6 in non-endothelial cells.

Similar mechanisms may also regulate the cell-specific expression of eNOS. Sp1 is an important activator of eNOS transcription, but Sp1 is also known to inhibit transcription through its interaction with HDAC1 [134, 145]. In the context of the native eNOS locus, Sp1 has been shown to bind with high affinity at the eNOS promoter in endothelial cells compared to non-endothelial cells [44]. However, the limited binding of Sp1 that occurs in non-endothelial cells may be functionally important in repressing transcription in these cell types. Gan et al. [99] demonstrated that Sp1 can interact with the eNOS promoter of non-endothelial cells and can also interact with HDAC1, suggesting that HDAC1 may repress eNOS transcription. Indeed, ChIP assays revealed that HDAC1 is bound to the eNOS promoter in non-endothelial cell types [146].

Cell-specific modifications to chromatin structure

Because transcription factors associate with a variety of chromatin-modifying enzymes, it is not surprising that chromatin structure at promoter and 5′-proximal regions of genes plays a crucial role in transcriptional activity. However, differential post-translational histone modifications of promoter and 5′-coding regions between expressing and non-expressing cell types is a newer concept for the control of cell-specific genes [147–149]. As described above, this mechanism has been implicated in the regulation of vWF [64]. Recently, Notch4, which is preferentially expressed in vascular endothelial cells, was also shown to have differential histone acetylation patterns in endothelial and non-endothelial cell types [78]. Wu et al. [78] found that the promoter and 5′ regulatory regions of the gene were enriched in acetylated histones H3 and H4. These same regions were also enriched in lysine 4 methylation of histone H3, a mark of transcriptionally active chromatin. AP-1 was implicated in the control of the cell-specific expression of the gene, as non-expressing cells could be forced to express Notch4 by administering growth factors that promoted AP-1 expression [78]. However, the functional role of histone acetylation or lysine 4 methylation was not addressed in this study. Edelstein et al. [150] also investigated the role of histone modifications in the endothelial-specific induction of E-selectin by cytokine stimulation. They found that the promoter and 5′-coding region of the gene was hyperacetylated following treatment of endothelial cells with the pro-inflammatory cytokine, TNF-α. The nucleosomes encompassing the promoter were also remodeled upon treatment. However, in VSMCs, which do not have increased E-selectin levels following TNF-α treatment, there was a lack of histone acetylation and nucleosomal remodeling. Edelstein et al. [150] noted that following TNF-α treatment, NF-κB was recruited to the E-selectin promoter along with acetyltransferases such as p300. The finding that the E-selectin promoter is preferentially acetylated by NF-κB-dependent p300 in endothelial cells could be attributed to the prior finding that E-selectin promoter DNA is differentially methylated in endothelial cells compared to non-endothelial cells. DNA methylation is known to inhibit the binding of NF-κB to the E-selectin promoter [151].

The observations regarding the regulation of vWF, Notch4 and E-selectin expression in endothelial cells is suggestive of an important role for chromatin structure in the determination of endothelial-specific gene expression. Several other observations have also led to this conclusion. As mentioned previously, several transgenes incorporating segments of endothelial-specific promoters suffer from position effects. Such variegated expression occurs due to the random nature of integration into the genome and the influence of neighboring, repressive chromatin. In their native context, endothelial genes likely contain cis elements required to direct appropriate chromatin remodeling. This may allow for transcription factors, which are not restricted in expression to endothelial cells, to obtain access to cis regulatory elements only in endothelial cells. Prior findings by Patterson et al. [69] support this premise. They demonstrated that while Sp1 could bind to Flk-1/VEGF-R2 promoter sequences in EMSAs using extracts from both endothelial and non-endothelial cell types, *in vivo* footprinting experiments revealed that an Sp1 footprint was present at the promoter only in endothelial cell types. This suggests that the binding of Sp1

on naked DNA fragments differs from the binding activity on the native chromatin.

Involvement of chromatin-based mechanisms in the regulation of eNOS cell specificity

Several observations regarding the regulation of the eNOS gene have also suggested that chromatin-based mechanisms must play a prominent role in the restriction of eNOS RNA to endothelial cells. As mentioned previously, the cis elements located in the proximal promoter that are responsible for the basal expression of eNOS have been identified, and many of the trans factors (i.e. Sp1, variants of Sp3, Ets-1, Elf-1, YY1, MAZ and GATA-2) that interact with these sites have been identified. In transient transfection analyses, eNOS proximal 5′ regulatory regions directed expression in both endothelial and non-endothelial cell types [44], suggesting that mechanisms must restrict expression of eNOS to endothelial cells in the setting of the native chromatin

Because the eNOS promoter behaves differently when it is located in episomes compared to chromosomal integration, Chan et al. [44] sought to determine the chromatin structure of the native eNOS gene in endothelial versus non-endothelial cell types. DNA methylation is a potent regulator of chromatin structure [113, 114]. By determining the methylation status of the CpGs in the eNOS promoter by high-resolution sodium bisulfite genomic sequencing, Chan et al. [44] found that DNA methylation was responsible, in part, for the repression of eNOS in non-endothelial cells. While such mechanisms have been suggested for other cell-specific genes, such as Maspin [152], this was the first report of a constitutively expressed endothelial gene that is controlled by this mechanism. A wide variety of non-endothelial cells, in both the *in vitro* and *in vivo* setting, were densely methylated at the proximal promoter, while endothelial cells were devoid of methylation in these same regions. This differentially methylated region extended from –361 to the start site of transcription, a region that encompasses PRD I and II. Considering that eNOS promoter/reporter transgenes require distal sequences to achieve endothelial specificity *in vivo* [107], including a putative enhancer element [41], the DNA methylation of this region was also analyzed. Levels of methylation were very low in both endothelial and non-endothelial cells [44], suggesting that the very highly localized differentially methylated region is limited to proximal promoter sequences. Importantly, DNA methylation could restrict the ability of Sp1, Sp3 and Ets-1 to transactivate the eNOS promoter. This was demonstrated in both transient transfection assays and at the native locus, since Sp1, Sp3 and Ets-1 binding was reduced in non-endothelial cells even though the total amount of these factors was similar between these cell types [44]. DNA methylation was functionally important, as the repressive effect of DNA methylation could be negated by treatment of non-endothelial cells with the DNA methyltransferase inhibitor, 5-azacytidine [44]. This finding has subsequently been confirmed by others [99].

DNA methylation can inhibit transcription by directly blocking the binding of transcription factors or by influencing chromatin compaction. Using EMSAs it was shown that methylation alone did not importantly affect protein complex formation on eNOS promoter sequences, at least on naked DNA templates [146]. These data suggested that modulation of chromatin structure must be an important, additional repressive mechanism. An important mediator of chromatin compaction of methylated DNA is MeCP2, a methyl-CpG-binding protein. Indeed, MeCP2 further repressed the activity of methylated promoter/reporters in transient transfection analyses [44] and was greatly enriched at the native eNOS promoter in non-endothelial cells, where there were high levels of methylation [146]. Since MeCP2 is known to recruit HDACs that can modulate chromatin structure by deacetylating the N terminus of histones H3 and H4, regulation of eNOS by post-translational modifications of histones at eNOS promoter sequences might also be important in controlling the cell-specific expression of eNOS. Indeed, we [146] and others [99], found that by inhibiting HDAC activity, eNOS could be expressed in normally non-expressing cell types. This effect on eNOS expression was accompanied by an increase in the acetylation of histones H3 and H4 at the eNOS promoter [99, 146].

Gene expression is now appreciated to be controlled at the chromatin level by a myriad of post-translational modifications of core promoter histones, primarily located on the N-terminus of histones H3 and H4. Fish et al. [146] found that a unique histone code is present at the eNOS core promoter: lysine 9 acetylation of histone H3 and lysine 8 and 12 acetylation of histone H4 [146]. How this code communicates with the transcriptional apparatus is not yet fully known. It is known, however, that H3 lysine 9 acetylation is required for TFIID recruitment, and that H4 lysine 8 acetylation is responsible for the recruitment of nucleosomal remodeling proteins [117]. H4 lysine 12 acetylation, which was originally thought to be a repressive mark [153], has been associated with the activation of several genes [150, 154]. Lysine 12 acetylation of H4 is also known to create a high-affinity binding site for Brd2 [155], a protein that can act as a scaffold for multi-protein transcriptional complexes [156]. How this endothelial-specific code is established during development and read by the transcriptional machinery is an important future direction that will aid in our understanding of how endothelial genes are regulated.

Lysine 4 methylation of histone H3 has recently been found to be enriched at the promoters of active genes [118–120]. This mark has also been suggested to play a role in the induction of gene transcription [150, 157]. However, the importance of this mark to cell-specific expression is unknown. Fish et al. [146] demonstrated that lysine 4 methylation was enriched at the eNOS proximal promoter in endothelial cells compared to non-endothelial cells. Lysine 4 methylation was also essential for the constitutive expression of eNOS in endothelial cells, as inhibition of lysine 4 methylation inhibited eNOS transcription and decreased steady-state levels of eNOS RNA. In addition, lysine 4 methylation was necessary for the inducible expression of eNOS in non-expressing cell types by HDAC inhibition [146]. Recently, lysine 4 methylation of histone H3 was also found to be enriched at the Notch4 promoter in endothelial cell types [78], suggesting that this may be a common mechanism of cell-specific expression.

Recent findings, described above, have allowed us to propose a model for the molecular mechanisms controlling eNOS cell-specific expression (fig. 1). In non-endothelial cell types, DNA methylation, methyl-binding domain proteins such as MeCP2 and HDACs regulate post-translational histone modifications, that together create a repressive chromatin environment at the eNOS promoter. This chromatin structure is thought to impede the accessibility of the eNOS promoter to ubiquitous transcription factors such as Sp1, Sp3 and Ets-1 in non-endothelial cell types. On the other hand, the eNOS promoter in endothelial cells is devoid of DNA methylation and the histones encompassing the promoter and 5′-coding regions are decorated with marks of active chromatin (i.e. acetylation, lysine 4 methylation of histone H3) that allow binding of the transcriptional apparatus. This endothelial-specific histone code is likely maintained by HATs and lysine-4-specific HMTs, the identity of which is unknown.

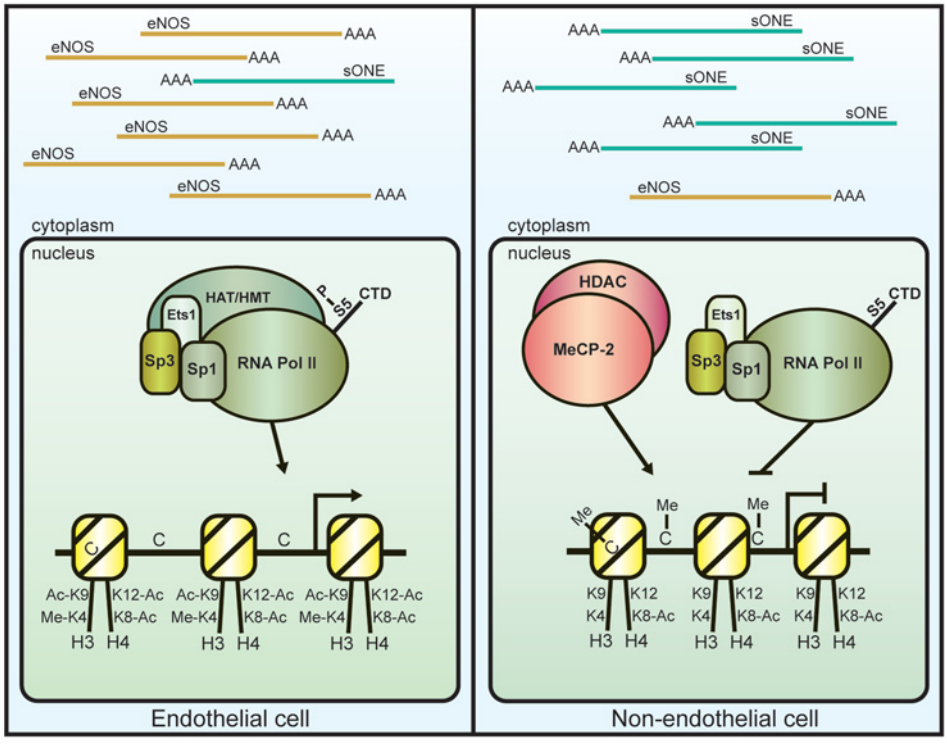

Figure 1. Proposed model for the endothelial-specific expression of endothelial nitric oxide synthase (eNOS). Transcription of the eNOS/*NOS3* gene is regulated by transcription factors such as Sp1, Sp3 and Ets-1, which are not endothelial-specific. While present in both endothelial and non-endothelial cell types, Sp1, Sp3 and Ets-1 are differentially recruited to the eNOS proximal promoter, suggesting that chromatin structure plays an important role in promoter accessibility. eNOS promoter DNA is highly methylated in non-endothelial cell types and is devoid of methylation in endothelial cells. This leads to preferential recruitment of the methyl-binding domain protein MeCP2 and HDAC activity in non-endothelial cell types. An endothelial-specific histone code is maintained in endothelial cells, likely through the recruitment of HATs and lysine 4-specific HMTs. This code consists of high levels of di- and tri-methylation of histone H3 at lysine 4, and high levels of acetylation at lysine 9 of histone H3 and lysine 8 and 12 of histone H4 at the eNOS proximal promoter and 5′-proximal coding regions. RNA polymerase II is preferentially recruited to the eNOS proximal promoter in endothelial cells and is hyperphosphorylated at serine 5 of the heptapeptad repeat of the C-terminal domain (CTD). While not shown, the cis antisense gene, sONE/*NOS3AS*, negatively regulates eNOS steady-state RNA levels by post-transcriptional mechanisms. Thus, high levels of eNOS are present in endothelial cells, whereas levels of sONE accumulate in non-endothelial cells.

Regulation of eNOS by an overlapping cis antisense gene

A perplexing observation during the initial dissection of the transcriptional mechanisms regulating eNOS expression was that while nuclear run-on signals, a measure of transcriptional activity of genomic regions, were higher in endothelial cells than in non-endothelial cells in the middle portion of the eNOS gene, signals at the 3′ end of the gene were very similar between cell types [our unpublished results]. This observation, together with the screening of cDNA libraries using 3′ eNOS probes and analysis of expressed sequence tag databases resulted in the identification of an overlapping transcriptional unit, *NOS3AS*, also known as sONE or APG9-like2, which overlaps with eNOS transcribed sequences in a tail-to-tail orientation [158, 159]. The major sONE mRNA species found in human cells is 2.9 kb, and spans 6.5 kb of genomic DNA. While sONE contains an open reading frame of 363 amino acids, the structure of the sONE mRNA is unusual in that it contains a stop codon in exon 8 of a 12-exon gene. mRNAs with stop codons in exons other than the final exon are normally targeted for degradation by the nonsense-mediated decay pathway [160]. While it is unclear whether sONE protein is expressed in cells, or whether sONE plays a primarily non-coding role, forced expression of the sONE open reading frame in cells appears to play a role in the process of autophagy [159]. The region of overlapping transcription stretches from exon 23 to exon 26 of eNOS, corresponding to 182 amino acids of eNOS and includes the 3′ untranslated region (UTR). This is a large overlap compared to other natural cis antisense genes, which typically involve, on average, 139 bp of overlap, predominantly in the 3′ UTR [161, 162]. The spliced mRNA for sONE contains a 662-nt overlap with spliced eNOS mRNA sequences. Only 123 nt of this overlap correspond to eNOS 3′ UTR sequences. This large region of complementary sequences suggested that there might be a functional interaction between these two RNAs.

Because endothelial cells have high steady-state levels of eNOS mRNA and only low levels of sONE, and non-endothelial cells tend to have the opposite pattern of eNOS/sONE expression (fig. 1), sONE may play a role in the cell-specific expression of eNOS. Indeed, if sONE levels were decreased in non-endothelial cells by RNA interference, levels of eNOS increased [158]. Furthermore, treatment of endothelial cells with trichostatin A, an HDAC inhibitor, resulted in an increase in sONE RNA levels prior to a profound decrease in eNOS steady-state RNA levels, suggesting that sONE negatively regulates eNOS RNA [158].

Potential sense/antisense interactions are abundant in the human genome. It has been estimated that over 20% of human transcripts might form sense/antisense pairs [163]. Experimental validation of the function of sense/antisense interactions has only been demonstrated for a few genes [164, 165]. Interestingly, overlapping transcripts are often present at imprinted loci, where epigenetic mechanisms regulate parent-of-origin gene expression patterns [166]. The function of overlapping transcripts in establishing or maintaining imprinting is relatively unknown. However, the expression of the non-coding Air transcript is required for the silencing of Igf2r, Slc22a2 and Slc22a3, even though the Air transcript only overlaps with a portion of Igf2r. Truncations of Air, which decrease the amount of transcriptional overlap between Air and Igf2r, lead to a loss of repression of all three silenced genes [167]. This suggests that silencing generated by Air/Igf2r overlap can spread in cis. While the mechanism of Air-mediated repression has yet to be determined, it is intriguing that double-stranded RNAs have been demonstrated to direct epigenetic silencing of homologous DNA [168]. For example, bi-directional transcription across centromeric repeats in yeast is known to generate double-stranded RNA that can be processed into small interfering RNAs (siRNAs) by Dicer [168]. Centromeric siRNAs can then be incorporated into the RNA-induced transcriptional silencing complex (RITS) to direct histone H3 lysine 9 methylation at the centromere [169, 170]. The transfection of siRNAs complementary to human promoters has also been shown to result in DNA methylation and histone H3 lysine 9 methylation of the homologous genomic DNA [171, 172]. These findings imply that double-stranded RNA species can play a role in establishing and maintaining epigenetic marks. While there is no evidence that the eNOS/sONE locus is imprinted, a role for sONE in the epigenetic regulation of the eNOS promoter is intriguing, particularly since epigenetic silencing can spread in cis to regions that are not homologous to the RNA-RNA overlap [167].

The identification of an overlapping transcriptional unit with eNOS, and the involvement of this transcript in the post-transcriptional regulation of eNOS, implies that cell-specific gene expression may be regulated by both transcriptional and post-transcriptional mechanisms (fig. 1). Recently microRNAs, which are small RNA species that bind to the 3′ UTRs of target genes with imperfect complementarity, and regulate gene expression by altering RNA stability or inhibiting translation, have been implicated in cell- or tissue-specific expression [173, 174]. For example, over-expression of the brain-specific miR-124 in human cells shifted the global expression profile toward that of the brain, while over-expression of the muscle-specific miR-1 resulted in expression profiles characteristic of muscle cell types [175]. Over 500 microRNAs have been identified to date, most of which do not have assigned functions. Determining the function, if

any, of miRNAs in regulating the cell-specific expression of endothelial genes is an intriguing area for future work.

Conclusions and perspectives

The regulation of endothelial-specific gene expression is proving to be much more complicated than can be adequately explained by cis/trans paradigms. While several novel transcriptional regulators in endothelial cells have been identified recently, including KLF2, HoxA9, and Vezf1, their involvement in defining endothelial cell specificity is unclear. Experimental evidence reviewed here suggests that chromatin-based mechanisms must now be considered as a paradigm to explain endothelial-cell-specific gene expression. eNOS was the first endothelial-specific gene shown to be regulated by epigenetic mechanisms including proximal promoter DNA methylation and post-translational histone modifications [44, 99, 146]. As such, the eNOS gene represents a prototypical model that can be used to dissect the mechanisms responsible for the establishment and maintenance of gene expression in the vascular endothelium.

While recent progress has been made regarding the regulation of the cell specificity of eNOS, especially by chromatin-based mechanisms, many questions remain unanswered. For example, how are the cell-specific modifications of the eNOS promoter, including DNA methylation and post-translational modifications of histones, established and maintained? Endothelial cells are unique in their constant exposure to the physical forces of the circulation. Steady laminar flow is known to have profound effects on post-translational histone modifications [176–178]. Moreover, eNOS is potently regulated at the transcriptional level by shear [179, 180]. One could argue that since endothelial cells are uniquely exposed to the physical forces of the circulation, this stimulus might establish or reinforce endothelial-specific gene expression patterns. However, endothelial cells extracted from various vascular beds continue to express endothelial markers when grown *in vitro*, even in the absence of flow [89]. Thus, flow may reinforce endothelial gene expression *in vivo*, but other mechanisms must be present to direct the expression of endothelial-specific genes in the absence of flow. Since eNOS is first expressed at day 9.5, when unidirectional blood flow is already established, flow may play a very important role in establishing eNOS expression during development [our unpublished observations]. It has also been recently demonstrated that the application of flow can aid in the differentiation of ES cells toward an endothelial fate [177], suggesting that flow may play a role in endothelial specification. However, flow is likely to play a less predominant role in establishing the expression of other endothelial genes, especially considering that genes such as Flt1/VEGF-R1, Flk1/VEGF-R2, Tie1 and Tie2 are expressed before the establishment of circulatory flow. Understanding the intricate mechanisms involved in setting up and maintaining endothelial gene expression patterns is a key research priority for the future.

Defining the contribution of epigenetic mechanisms to the regulated expression of eNOS in mature endothelial cells will also be an important future goal. Several physiological stimuli are known to increase the expression of eNOS RNA by transcriptional mechanisms. These stimuli include transforming growth factor-β (TGF-β) [181], lysophosphatidylcholine [182, 183], laminar shear stress [180] and β-estradiol [184]. VEGF is also known to increase levels of eNOS RNA [185], but the involvement of transcription has not been studied in detail. While the transcriptional mechanisms have been defined for some of these stimuli, such as an increase in Sp1 binding to the core promoter of eNOS elicited by lysophosphatidylcholine [182] and β-estradiol [184], and binding of Smad2 to the eNOS promoter by TGF-β signaling [181], it will be of interest to determine whether chromatin-based mechanisms are also involved in the transcriptional induction of eNOS by these stimuli. A number of diverse pathological stimuli are also known to decrease eNOS expression by transcriptional mechanisms [17, 22, 23]. Interestingly, some of these stimuli are known to elicit changes in epigenetic pathways in endothelial cells. For example, hypoxia regulates the expression of HDACs in endothelial cells [186]. Particularly intriguing are recent reports that epigenetic mechanisms may be involved in the pathogenesis of atherosclerosis [187–190]. It is currently not known whether eNOS repression during endothelial activation involves epigenetic pathways. Because epigenetic modifications, whether elicited by DNA methylation or post-translational histone modifications, are for the most part reversible by drugs that inhibit these pathways, diseases of the cardiovascular system might be amenable to treatment with therapeutics targeted to epigenetic pathways.

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