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Mini Review

Genome- and epigenome-wide analysis of endothelial cell activation and inflammation

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Activation and dysfunction of the endothelium underlie many vascular disorders including atherosclerosis, tumor growth, and inflammation. Endothelial cell activation is mediated by many different extracellular signals, which results in overlapping, yet, distinct patterns of gene expression. Comparative ChIP-seqs with either STAT6, GATA2, or NFATc1 antibody between endothelial cells and erythroids or lymphoids revealed that each transcription factor bound the consensus recognition motif genome-widely, but the bound regions showed exclusive cell type specificity and strong correlation to the each cell's crucial function. By using the ChIP-seqs with epigenetic histone modification in endothelium, constitutively expressed GATA2 and chronic IL-4-stimulated STAT6 binding regions were detected at both proximal and distal promoters. In contrast, VEGF-stimulated NFATc1 preferentially bound to the proximal promoters, the majority of which were pre-opened chromatin due to responding to the acute VEGF activation signal. This review is to combine our recent genome/ epigenome wide ChIP-seqs information and the related literatures as well as to summarize the crucial roles of tight regulation by the endothelial cell activation that is pathologically leading to tumor growth and inflammation.

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Introduction

The endothelium is highly malleable cell layer that is constantly responding to changes within the extracellular environment and responding in ways that are usually beneficial, but at times, harmful to the organism. Many extracellular mediators including the growth factors, cytokines, and shear stress activate gene transcription in endothelial cells, resulting in changes in hemostatic balance, increased leukocyte adhesion, loss of barrier function, increased permeability, migration, proliferation, and successive angiogenesis[1]. The tight control of these processes is essential for homeostasis. Endothelial cell activation, if excessive, over-sustained, or spatially and temporally misplaced, may result in vasculopathic diseases such as pathological an-

giogenesis, atherosclerosis, and inflammation. Thus, an understanding of the molecular pathways leading to endothelial activation may provide novel insights into therapeutic targets.

Moreover, recent technological advancement now allows transcription factor binding regions to be defined genome-widely and the epigenetic histone code to be unlocked. For example, modifications associated with tri-methylated histone 3 lysine 4 (H3K4me3) only occurred in the proximal active promoter. While mono-methylated histone 3 lysine 4 (H3K4me1)- and tri-methylated histone 3 lysine 27 (H3K27me3)-modifications are defined as distal active enhancer marks[2, 3]. Thus, in this mini review, I will summarize the recent reports performing chromatin immunoprecipitation with deep sequencing (ChIP-seq) to determine genome-wide occupancy of transcription factors in endothelial cells. Comparison of epigenetic markers and the transcriptional regulation would lead to the detailed understanding of the molecular mechanisms of endothelial cell activation.

IL-4/STAT6-mediated endothelial cell activation and inflammation

Interleukin (IL)-4 is a 20 kDa pleiotropic cytokine expressed by T helper 2 (Th2) lymphocytes, eosinophils, basophils, and mast cells (reviewed in 4)). It has been shown that IL-4 is necessary to stabilize the Th2 phenotype and promote IgE synthesis (reviewed in 5)). IL-4 has been implicated in the pathogenesis of atherosclerosis and allergic asthma[6]. Signaling of IL-4 in endothelial cells occurs via a heterodimeric IL-4 receptor (IL-4R), consisting of IL-4Rα and IL-13Rα subunits[7]. Activation of the receptor results in janus kinase (JAK)-1/2-dependent tyrosine phosphorylation and subsequent dimerization of Signal Transducer and Activation of Transcription (STAT)-6, which then translocates to the nucleus and binds to consensus sequences (TTCN$_{3\text{-}4}$GAA) found within promoters of IL-4-regulated target genes[8, 9]. Previous studies in endothelial cells have demonstrated that IL-4 induces the expression of CXCL-8, inducible nitric oxide[10], urokinase-type plasminogen activator[11], vascular endothelial growth factor (VEGF)[10], P-selectin[12], Monocyte chemotactic protein-1[13], CCL26[14], IL-6[15], 15-lipoxygenase[16], and osteoprotegerin[17]. In addition, previous studies have shown that IL-4 upregulates the expression of vascular cell adhesion molecule (VCAM)-1 in endothelial cells[9, 18]. In contrast, IL-4 does not lead to the increased expression of intercellular adhesion molecule (ICAM)-1[19, 20] and has a variable effect on E-selectin expression[10, 20, 21]. Since the mechanisms underlying IL-4-mediated endothelial activations are poorly understood, we recently performed a genome-wide survey for IL-4-mediated STAT6 binding from ChIP-seq in endothelial cells[22]. Through the combination of DNA microarrays and ChIP-seq at the same time points, the majority of IL-4-responsive genes were shown to be STAT6-dependent and associated with direct STAT6 binding to their promoters. Noteworthy, most of IL-4-mediated sustained induced genes were truly reflected by the stable STAT6 binding on their promoters. Only 4% of STAT6 bound to the proximal promoter region categorized in 5'-flanking region (up to 5kbp) and 5'-untranslated region (UTR). In contrast, half of STAT6 binding sites were located in the distal promoter region, which is between -25 to -5kbp 5'-flanking region and +10kbp downstream region (Fig.1A, *left*). Next, we wished to determine the association between genome-wide STAT6 bindings and the target gene expression. To that end, total 17,444 significant working array probes via IL-4 stimulation were sorted by the induction/ reduction ratio, which were compared to the STAT6 ChIP enrichment level corresponding to the gene of each array probe. As shown in Fig.1A, highly significant gene set enrichment (GSEA) score was shown. Especially, treating with IL-4 for 16 h (*yellow*) revealed the greatest enrichment score (Fig.1A, *right*), suggesting that stable STAT6 bindings commonly reflect the target gene upregulation. As well as STAT6 binding of IL-4 inducible genes mostly mentioned above, our ChIP-seq led to the new finding of the epigenetically coordinated VCAM-1 enhancer, which locates -16 kbp upsetream of the VCAM-1 transcriptional start site[22]. Although there are other STAT-consensus elements on the VCAM-1 locus revealing the STAT6 enrichment, they are unstable. The -16 kbp upstream enhancer region indicating the stabilized STAT6 associations until 24 hours after the IL-4 treatment, where co-enriched by the enhancer histone marking; p300, H4Ac and H3K4me1, but not the proximal promoter marking; H3K4me3. Interestingly, IL-4 mediated STAT6 binding regions were already fixed by the cell-type. When we compared IL-4-treated CD4$^+$-T cells[23] to endothelial cells, only two genes; SOCS1 and elongation factor for polymerase II, were commonly induced by STAT6. More than eighty other IL-4 induced genes showed the separated STAT6 binding to each cells. Many endothelial-induced genes were categorized with inflammation; whereas T-cells-induced

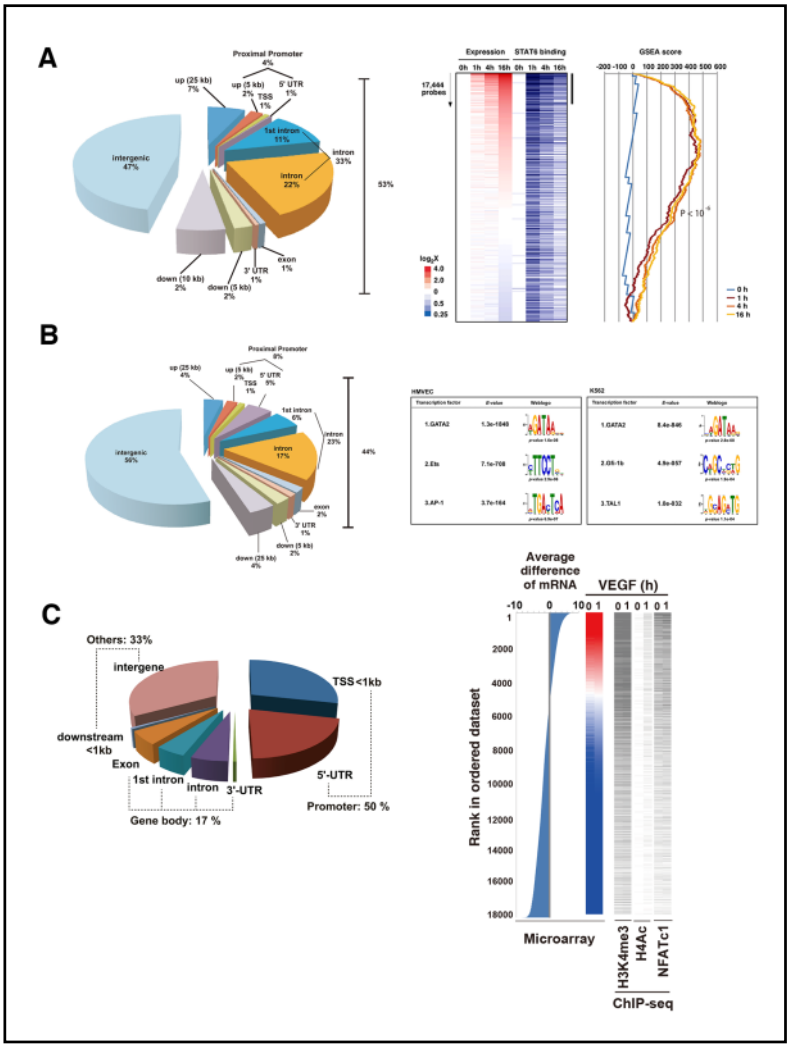

Fig.1 Genome-wide ChIP-seq analysis of STAT6, GATA2, and NFATc1 in primary cultured endothelial cells

(A) *Left*, distribution of IL-4-mediated STAT6 binding sites in the proximal promoter (within 5kb upstream or at the 5' untranscribed region from the transcriptional start site (TSS), exon, intron, and intergenic regions. *Right*, IL-4 responsive genes at 1, 4, and 16 hours sorted according to the induction ratio at 16 hours. The vertical black bar indicates a group of highly induced IL-4 responsive genes at 16 hours that are enriched in STAT6 binding. A graphic representation of the GSEA enrichment score is shown on the right. (B) *Left*, distribution of GATA2 binding sites that were classified according to the distance from the TSS of known genes. *Right*, determination of the sequence recognized GATA2 in human microvascular endothelial cells (HMVEC) and K562 cells. *E-value* and *P-value* mean the probability-enriched sequences obtained from ChIP-seq are matched to the displayed Weblogo, and known consensus motifs by chance, respectively. (C) *Left*, distribution of VEGF-treated NFATc1 binding sites that were classified according to the distance from the TSS of known genes. *Right panel* shows heat map from average difference of gene expression. *Red* or *blue* means induced or reduced genes via VEGF, respectively. Enriched signals ($p<10^{-30}$) around known genes were indicated by gray bar from ChIP-seqs with NFATc1, H3K4me3, and H4Ac.

genes were correlated with Th2 development such as GATA3[23]. Further experiments would uncover when and why STAT6 accessible binding regions were fixed via the hematopoietic and vascular cells developmental stage.

Thrombin/ GATA2-mediated endothelial cell activation and inflammation

Thrombin is a multifunctional serine protease that is involved not only in mediating the cleavage of fibrinogen to fibrin in the coagulation cascade but also in activating a variety of cell types, including platelets and endothelial cells. Thrombin signaling in the endothelium may result in a multitude of phenotypic changes including alterations in cell shape, permeability, vasomotor tone, migration, angiogenesis, hemostasis, and leukocyte trafficking leading to the inflammation (reviewed in 24)). Thrombin signaling in the endothelium is mediated by a family of 7-transmembrane G-protein coupled receptors, termed protease activated receptors (PAR)[25]. Currently, four members of the PAR family have been identified, PAR-1 to -4. Of the various PAR family members, PAR-1 is the predominant thrombin receptor in endothelial cells[26]. Once PAR-1 is activated, it is coupled to a family of hetero-trimeric G proteins, consisting of a α subunit and a $\beta\gamma$ dimer. The G-proteins are, in turn, linked to a number of signal intermediates that include, but are not limited to, MAPK, protein kinase C, calcineurin, PI3K, and Akt. Thrombin primes the transcriptional networks in endothelium. Most famous factor is NF-κB that predominantly leads the inflammatory genes upregulation[24]. Besides the NF-κB, GATA DNA-binding activity and /or GATA mRNA expression has shown to be controlled in response to a thrombin signaling[27]. For example, thrombin stimulated VCAM-1 expression was mediated by GATA2 increased bindings on the promoter

through the GATA2 phosphorylation via thrombin-PKC-ζ cascade[28].

GATA family members have evolutionally conserved six proteins, of which GATA2, 3, and 6 are selectively expressed in endothelium[29]. GATA2 is uniformly expressed in all endothelial cells[30], whereas GATA3 is predominantly expressed and functions in endothelial cells derived from large vessels. GATA6 was reported to induce the expression via inflammatory stimuli in endothelium[31]. Because targeted disruption of *gata2*, not gata3 or 6, gene in mice resulted in embryonic lethality between embryonic day 9.5-11.5 due to the defects in primitive hematopoiesis and hemogenesis[32], GATA2 is well studied in primary cultured endothelial cells. GATA2 is recognized as an important regulator of endothelial selective gene expression including endothelium-1, platelet/endothelial cell adhesion molecule (PECAM)-1, endothelial-nitric oxide synthase (e-NOS), von Willebrand factor (vWF), down syndrome critical region (DSCR)-1, VCAM-1, VEGFR2, and GATA2 itself[27, 33-40]. Although these findings have suggested that GATA2 plays a crucial role in the gene expression profile in vascular endothelial cells, the molecular mechanism by which GATA2 controls many endothelial specifically expressed genes remains largely unknown. Thus, we carried out recently ChIP-seq to determine genome wide occupancy of GATA2 in endothelial cells and compared it to the respective gene expression profiles to understand GATA2 function on a genome-wide scale[41]. Up to 44% GATA2-binding regions were positioned around 10kbp from each transcript. Among them, only 8% were in the proximal promoter categorized within 6kbp 5'-flanking region and 5'-UTR. Also, 33% of GATA2 binding area was located in intron (Fig.1B, *left*). Collectively, these data suggest that the defined GATA2-binding regions were not selectively located within the proximal promoter of each gene, rather they are scattered widely in the whole genome. In addition, comparing epigenetic markers and chromatin conformation between endothelial cells and erythroids, we identified GATA2 binding regions were pre-determined, perhaps via the epigenome state, and that exclusively trans-activate nearly complete tissue specific genes expression. Similar to our GATA2 ChIP-seq in microvascular endothelial cells, Linnemann, et.al. reported later the GATA2 ChIP-seq in large vessel-derived umbilical vein endothelial cells[42]. It is interesting to see the consistent data representation between two reports, suggesting that comprehensive genome-wide screening methods are non-biased and great ways throughout the genome-wide data normalization. In addition, both reports denoted that AP-1 was co-enriched by the GATA2 ChIP-seq[41, 42]. AP-1 is a pivotal regulator of inflammatory gene, and AP-1 target genes in which GATA2 overlapped with c-JUN and c-FOS induce IL-8, CXCL2 and GM-CSF[42]. GATA2 conferred maximal phosphorylation of chromatin bound c-JUN at Ser-73, which stimulated AP-1-dependent inflammatory genes transactivation[42]. Taken together with the fact that AP-1 is a key regulator of inflammation, it is suggested that GATA-AP-1 regulatory network is a common mode of orchestrating inflammatory processes in endothelium.

GATA2 is uniformly expressed not only in endothelial cells but also in erythroid cells. However, each GATA2 protein bound sharply on different regions. Erythroid cells expressing GATA2 preferentially bound to the genes that regulate the hematopoietic differentiation such as GATA1, Gfi-1b, and NF-E2. On the other hand, endothelium-expressing GATA2 preferentially bound to the endothelial specific or functionally important genes for endothelial cell activation. Thus, in spite of the fact that GATA2 is co-expressed in blood cells and endothelial cells, GATA2-bound regions were mutually and exclusively pre-determined between two cell types.

Moreover, GATA2-ChIP-seq indicated the co-enrichment of ETS recognition motif in endothelial cells (Fig.1B, *right*). ETS-related proteins, ERG and FLI1, have recently been reported well about the indispensable function of endothelial cell differentiation[43]. ETV6 is also reported that it is required for hematopoiesis and maintenance of the vascular network[44, 45]. These reports may give important hints to why and how GATA2 regulates the tissue specificity in expressed each cell type. In addition, surprisingly, abrogation of the GATA2 expression in endothelium demonstrated not only reduction of the endothelial specific markers but also induction of the endothelial-mesenchymal cell transition (EndMT) promoting gene expression by using the genome-wide array screening[41]. Taken all together, these results have provided new insights into the cooperation between endothelial-expressed GATA2 binding and epigenetic modification, resulting in the determination of endothelial cell specific gene expression and inflammation.

VEGF/NFAT-mediated endothelial cell activation and inflammation

VEGF is a well-known endothelial cell specific mitogen

and chemotactic agent involved in microvascular permeability and endothelial cell survival. The VEGF receptors have been shown to activate several signaling pathways including PKC, PI3K, AKT, MAPK, and Ca^{2+}-calcineurin[46, 47]. Calcineurin signaling activates Nuclear Factor of Activated T cells (NFAT) transcription factors. NFAT was first identified from the extracts of activated T lymphocytes but recent studies have demonstrated that NFAT not only functions for immune response in leukocytes but also regulates bone homeostasis, cardiac development, tumor progression, and pathogenesis of Down syndrome[48]. VEGF treatment of endothelial cells caused NFAT nuclear localization. In endothelial cells, many putative NFAT target genes have been described including tissue factor, IL-8, E-selectin, Cox-2, and ATP2A3[47, 49, 50]. The balance of the NFAT activity is important for proper endothelial activation. We recently have demonstrated that DSCR-1, an NFAT primary target, is critical for the formation of auto-inhibitory loop via the DSCR-1 suppression of NFAT activator calcineurin[47]. In contrast, Egr-3, another NFAT target, positively transmits VEGF-mediated angiogenesis and pro-inflammation[51]. Overexpression of DSCR-1 completely attenuates NFAT activation resulting in the downregulation of VEGF-mediated angiogenesis and tumor growth[47]. Null mutation of DSCR-1 leads to NFAT hyper-activation, which also causes primary tumor growth suppression via endothelial cell destabilization[52].

Most recently, we performed ChIP-seqs in the presence or absence of VEGF-inflammatory stimulus to determine genome-wide occupancy of NFATc1 in endothelial cells and compared these results with the respective gene expression profiles to understand NFAT function on a genome-wide scale. It is shown in Fig.1C, at the *left* that VEGF-mediated NFATc1 binding regions are classified by the known gene through genome wide. By combining this with ChIP-seq data examining epigenetic markers, NFATc1 preferentially bound to promoter regions, the majority of which were commonly located with proximal active promoter histone marks; H3K4me3. VEGF-stimulated gene groups (shown in *red*) were significantly correlated to the NFATc1 enrichment; positive count by the rank in ordered data set, which also showed the similar density compared to H3K4me3, but not H4Ac, in ChIP-seq (Fig.1C, *right*). These NFATc1 occupancy patterns were unique, considering our GATA2- and STAT6-mediated ChIP-seqs in endothelial cells. GATA2 and STAT6 predominantly occupied distal enhancer regions (please see Fig.1A and B). These differences might be based on the functional differences of transcription factors. NFAT is crucial for turning on the VEGF-switch, but GATA2 and STAT6 function in endothelial specificity and in chronic inflammation, at least in part, via changing the chromatin structure.

NFAT nuclear localization and subsequent target gene activation are tightly regulated by calcium/calcineurin, which is inhibited by the immune-suppressors, CsA or FK506. NFAT directly transactivates the genes in T cells, many of which genes function in inflammation or immune-responses[53]. Compared to T cells, the majority of NFAT activated genes in endothelial cells are unique, except Egr-3 and Cox2. The differential activation mechanism between endothelial cells and T cells might be based on the epigenetic microenvironment. Indeed, T cell specific induced genes, TNF-α, colony stimulating factor-1, IL-2, and IL-4 were fully covered with silencer histone marking and never enriched by active promoter marking in primary cultured endothelial cells. Specific genome-wide regulation of these cell types was similarly observed in IL-4-STAT6 or GATA2 mediated gene expression patterns in between endothelial cells and T cells or erythroid cells respectively.

Tight correlation between endothelial cell activation and inflammation

Endothelial cell activation primes various phenotypic changes from the inert endothelial cell layer. Especially, inflammatory stimulus causes the upregulation of VCAM-1, ICAM-1, and E-selectin. VCAM-1 and ICAM-1 are also expressed in other cell types including smooth muscle cells and fibroblasts, whereas E-selectin is an endothelial cell specific activated adhesion molecule[54]. VCAM-1 and ICAM-1 induction machinery are far different in endothelial cells. Although NF-κB and NFAT induce both molecules, for example, GATA2 and STAT6 only bind to and transactivate the VCAM-1 expression[22, 28]. Treatments of PI3K inhibitor, HDAC inhibitor, or HDAC3 knockdown abrogated TNF-α-or thrombin-stimulated VCAM-1 but not ICAM-1 expression[55]. The proteoglycan decorin treatment increased ICAM-1 and downregulated VCAM-1 expression after TNF-α stimulation[56]. Further studies on the detailed mechanisms would be needed to uncover selective adhesion molecules inhibitory methods.

Upregulation of these endothelial cell activation markers resulted in the rolling and the firm adhesion of lympho-

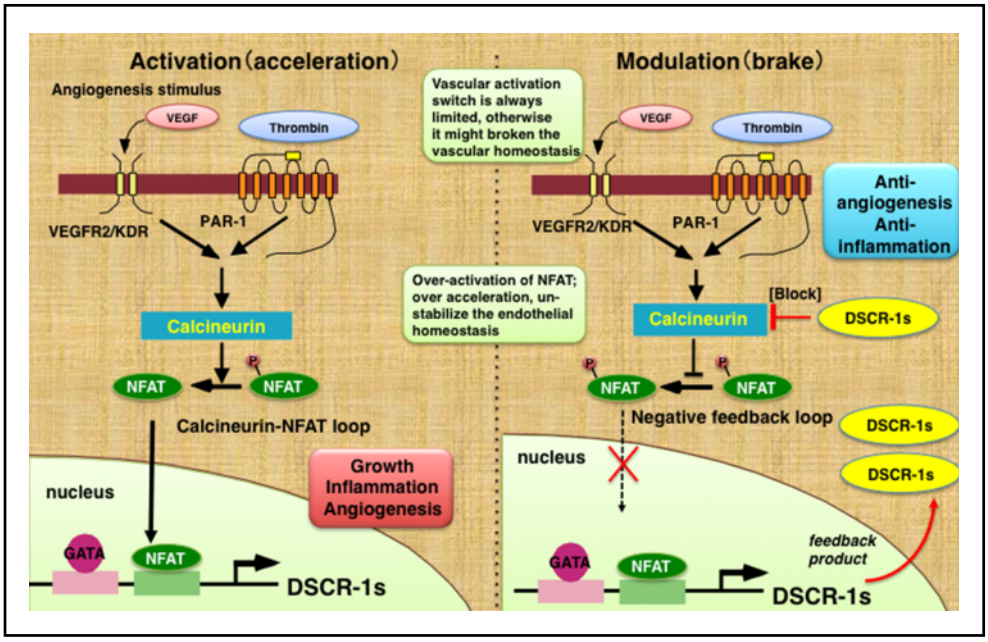

Fig.2 Schematic representation of the endothelial homeostasis, *acceleration vs. brake system* in endothelium

Angiogenetic and inflammatory stimulus such as VEGF and thrombin activates calcineurin following the NFAT nuclear localization, which leads endothelial cell activation. However, over-activation of NFAT causes destabilization of the endothelial cell homeostasis. Thus, NFAT transactivates the auto-inhibitory molecule; DSCR-1, which accurately modulates the calcineurin-NFAT signaling circuit.

cytes and monocytes. Those cells secreted inflammatory cytokines; such as IL-4, TNF-α, tissue factor, and VEGF, in turn resulting that the further stimulation of endothelial cells lead to transactivate the adhesion molecules expression. Over-sustained such a feed-forward loop causes the endothelial cells destabilization. Thus, negative feedback systems are definitely needed for endothelial cells to keep homeostasis. As well as NF-κB and I-κB/A20 loop, TGF-β/BMP and SMAD6/7, or STAT and SOCS system, our newly identified NFAT and DSCR-1 circuits are critical for proper endothelial function (Fig.2). Indeed, null mutation of *Dscr-1* in mice causes the partial embryonic lethality due to the hemorrhage in brain vessel[52]. The rest of viable mice have shown endothelial cell destabilization and apoptosis when they received septic shocks or inflammatory stimuli[36]. In contrast, stable expression of DSCR-1 profoundly down-regulated the inflammation-stimulated VCAM-1, ICAM-1, and E-selectin expression in endothelium[49]. Adenovirus-mediated DSCR-1 overexpression and endothelial cells specific DSCR-1 transgenic mice auto-inhibits the endothelial cell activation, resulting in the inhibition of the pathological angiogenesis-mediated tumor growth[57], and the protection against the septic shock-induced lethality[36]. Taken together, it would be helpful for the therapeutic use of anti-inflammation to utilize the new theory of "blocking the endothelial cell activation with the endogenous negative feedback molecules".

Conclusion

Endothelial cells are activated by various stimuli in the surrounding microenvironment, which leads to vascular inflammation and angiogenesis. The endothelial cell activation step occurs through many signaling cascade and the transcriptional regulation in genome-wide. There are several crucial transcription factors in each separate stimulus, but expression of the transcription factors usually is not limited to the endothelium. Even if there are no limitations on the tissue specificity of the expressions, each transcription factor-binding region is strictly pre-determined genome-widely in each cell-type. The histone marking supposed as the epigenetic modification during the cell differentiation stage would limit GATA2-, STAT6-, and NFATc1-occupied region in a cell type-specific manner. Further epigenetic study on the chromatin regulatory enzymes and the epig-

enome modifying complexes such as "pioneer factor" [58] in endothelial cells would uncover the molecular mechanisms of endothelial cell specificity and its function, and subsequently discover a new therapeutic ways against the vascular inflammation and pathological angiogenesis.

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Conflict of interests

None

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