The Evolving Role of MicroRNAs in Endothelial Cell Dysfunction in Response to Infection

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Abstract

The microRNAs are short noncoding RNA molecules responsible for translational repression and silencing of target genes via binding to the mRNA. They are found in all eukaryotic cells and play a critical role in virtually all physiological processes, including within the cardiovascular system where they influence cellular development, differentiation, cardiovascular function, hemostasis, and programmed cell death. Dysregulated microRNA expression is associated with several conditions ranging from cancer and autoimmune disease to infection. Progressively, it has become increasingly clear that microRNAs are important components of the host response to microbes. The cardiovascular system, coupled with cells of the innate immune system, provide the initial interaction and first response to microbial infection, respectively. This review presents the current state of knowledge regarding the role of microRNAs with emphasis on their role in controlling endothelial cell function.

Keywords
- vascular endothelial cell
- microRNA
- infection
- sepsis

The vascular endothelium is a large heterogeneous organ that forms the innermost lining of the vasculature.[1] It is a highly metabolically active monocell layer that protects against injury and regulates systemic blood flow and tissue perfusion.[2] In addition to regulating systemic blood flow, the endothelium plays a key role in maintaining barrier integrity which controls the movement of fluid, ions, and other macromolecules between circulating blood and surrounding tissues.[3] This is achieved by tight control and coordinated opening and closure of endothelial–endothelial cell connections.[4] These junctional connections are mediated by homophilic interactions which form a zipper-like structure at the endothelial cell border.[5] Adherens and tight junction proteins ensure tight endothelial cell–cell attachment (►**Fig. 1**). Adherens junctions are mediated by vascular endothelial-cadherin, which is linked to critical intracellular proteins, such as p120-catenin, α-catenin, and β-catenin.[6] Tight junctions are mediated by members of the claudin family, which in turn recruit other "facilitators," such as occludin, junctional adhesion molecule, and zona occludens-1 to solidify the attachment.[7]

Following vessel injury, tissue damage, or infection, the endothelium regulates recruitment and extravasation of proinflammatory leukocytes and platelets through the release of a myriad of chemokines and cytokines that trigger the upregulation of cell adhesion molecules.[8] Both platelets and leukocytes roll along the endothelium in a tightly regulated fashion.[9] The selectin family of adhesion molecules plays a key role in this event. For example, E-selectin is synthesized by cytokine-activated endothelial cells, and P-selectin is released from Weibel–Palade bodies and expressed on the endothelial cell surface.[10] Upon stationary adhesion to endothelial cells, leukocytes downregulate the selectin-based adhesion and upregulate β 2 integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), which in turn promote firm adhesion to intercellular adhesion molecule 1 (ICAM-1) expressed on endothelial cells. Once this process is complete, the leukocyte rapidly emigrates into the surrounding tissue. Weibel–Palade bodies also release von Willebrand factor (VWF), which forms large and ultralarge-VWF multimers on the endothelial cell surface, which results in platelet recruitment via the GPIb–V–IX complex in the presence of shear stress.[11] Intraplatelet activation triggers the fibrinogen-mediated cross-linking of activated platelets via GPIIb/IIIa (integrin αIIbβ3), to finally generate a stable aggregate for arresting blood loss.[12,13] Aggregate formation is solidified when activated endothelial cells express tissue factor (TF) on its surface. TF acts as a cofactor for factor (F) VII proteolysis, by

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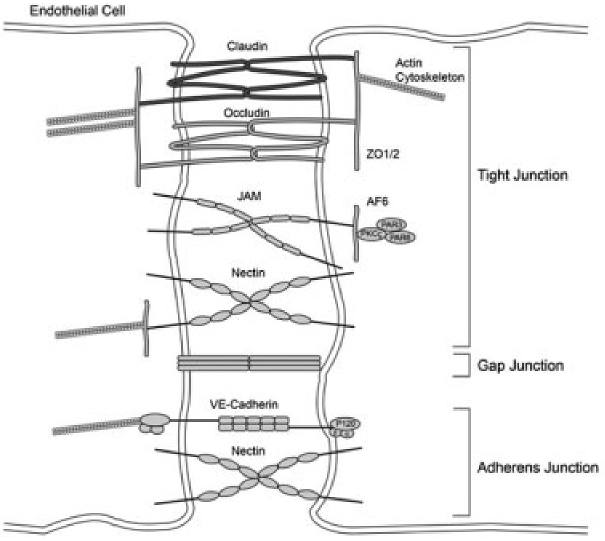

Fig. 1 The composition of tight junction and adherens junction complexes. Tetramembrane spanning proteins claudins and occludins bind their intracellular binding partners including the ZO family or directly to the actin cytoskeleton in the case of occludin. JAMs traverse the membrane just once and are also capable of binding the ZO-1 adaptor protein. Adherens junctions are largely mediated by VE-cadherin, a protein that binds to the p120 complex. The nectin molecule behaves as an integral member of both the tight and adherens junctions by binding to its intracellular binding partner AF6. AF6, afadin 6; JAMs, junctional adhesion molecules; VE-cadherin, vascular endothelial-cadherin; ZO, zonula occludens.

forming a TF/FVIIa complex which proteolytically cleaves FIX/FX to FIXa/FXa, so promoting the subsequent steps leading to thrombin generation.[14–18] Thrombin converts fibrinogen to fibrin and promotes fibrin cross-linking coupled with aggregated platelets to form a stable blood clot on the endothelial surface, thus attenuating blood loss from injured sites.[16,18]

Dysregulation of normal endothelial cell functions has been implicated in several conditions, including atherosclerosis, diabetes, tumor metastasization, inflammatory disease, and infection.[19] However, the processes involved in such dysregulation are incompletely understood, although are known to involve the signal transduction pathways responsible for modifying the transcriptional regulation of gene expression allowing posttranscriptional and translational modifications within the endothelial cell.[20,21] MicroRNAs (miRNAs) are critical regulators of gene expression and their role in regulating endothelial cell gene expression in response to insult or injury remains largely unexplored.[22] Here, we describe the role of miRNAs induced upon endothelial cell infection and their role in either causing or mitigating dysregulation of endothelial cell function as it relates to hemostasis and other essential homeostatic processes.

miRNA Biogenesis and Function

miRNA are short (~18–22 nucleotides) posttranscriptional and translational repressors of gene expression.[23] miRNA bind messenger RNA (mRNA) with partial or complete Watson and Crick base pairing.[24] Briefly, miRNA are transcribed in a mono- or polycistronic fashion throughout the genome by RNA polymerases, thereby forming large primary-miRNA transcripts (pri-miRNA) (►**Fig. 2**).[25–27] Pri-miRNA is further processed by the microprocessor, a complex comprised of Drosha and its double-stranded binding partner DiGeorge syndrome critical region gene 8. This microprocessor produces an approximately 70-nt stem and loop precursor-miRNA (pre-miRNA) from the pri-miRNA which harbors mismatches along a duplex.[28] Alternatively, miRNA sequences located within the extronic region are processed by the spliceosome to form miRTrons, which also serve as precursors for the genesis of mature miRNA.[29,30] Pre-miRNA translocates from nucleus to cytoplasm coupled to exportin-5/RanGTP, which releases pre-miRNA once the enzyme Ran-GTPase catalyzes the breakdown of guanosine triphosphate to guanosine diphosphate + Pi.[31,32] Pre-miRNA is further processed by Dicer and its double-stranded binding partner and Argonaute (Ago) family members I to IV, to form mature double-stranded miRNA with a two nucleotide overhang on both its 3′ and 5′ ends.[33] A single strand (3′ or 5′) is loaded onto the RNA-induced silencing complex (RISC), a large multiprotein complex comprising

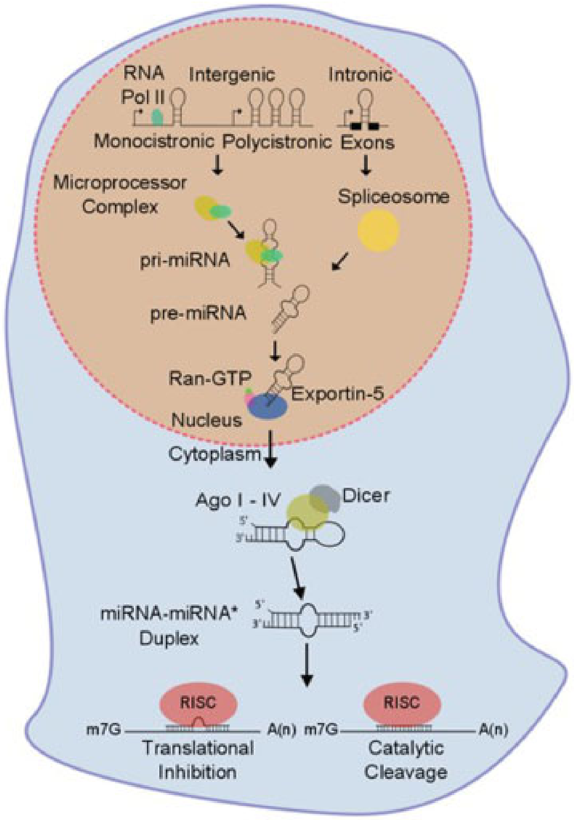

Fig. 2 miRNA are initially transcribed by RNA polymerases in the nucleus as large primary-miRNA transcripts and subsequently processed to pre-miRNA as described above. Pre-miRNA translocates to the cytoplasm where an enzymatic complex composed Dicer and its double-stranded binding partner process pre-miRNA to mature miRNA transcripts. A single strand (-3p or -5p) is loaded onto and guides the RISC to its respective mRNA targets facilitating spatiotemporal regulation of gene expression. miRNA, microRNA; pre-miRNA, precursor-miRNA; RISC, RNA-induced silencing complex; RNA, ribonucleic acid.

predominantly Ago 2.[34–36] The miRNA guides RISC to mRNA, where it binds to mRNA at positions 2 to 8, commonly referred to as "the miRNA seed sequence." The degree of complementarity between the miRNA and its target mRNA dictates the effect a miRNA elicits upon the target whereby high complementarity between miRNA and mRNA results in catalytic cleavage of mRNA by RISC.[37–39] Low complementarity does not bring RISC close enough to cleave the mRNA, but it prevents transfer RNA delivery of amino acids and thus prevents ribosome synthesis of polypeptide chain.[40,41]

Endothelial Cell Regulation by MiRNAs

The importance of endothelial regulation by miRNA is indicated by the vast endothelial dysfunction induced in response to Dicer inactivation.[42,43] miRNA (miR)-126, in particular, being specific to all vascular endothelial cell beds, is a major regulator of endothelial function, including processes, such as endothelial proliferation, permeability, and apoptosis.[44] Wang et al, demonstrated that targeted deletion of miR-126 leads to reduced Sprouty-related, EVH1 domain-containing protein 1 expression, a negative regulator of angiogenesis, ultimately leading to reduced vascular integrity. This implicates the involvement of miR-126 in controlling the process of angiogenesis.[44] miR-126 has also been implicated in regulating endothelial permeability by interacting with vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1 under the influence of miR17–3p.[45] Other miRNAs associated with maintenance of junctional protein complex, angiogenesis, and apoptosis include miR-221/222 and miR-21. Both these miRNAs are thought to be antiproliferative, while miR-21 has been demonstrated to negatively regulate proapoptotic proteins, such as phosphatase and tensin homolog and B-cell lymphoma 2.[46–49] Interestingly, miR-21 has been shown to modulate expression of Rho-GTPases, essential proteins for actin organization, in human umbilical vein endothelial cells (HUVECs). Specifically, miR-21 has been shown to target RhoB expression in these endothelial cells, which affect vital processes, such as cell adhesion and actin organization.

Recent studies indicate a significant role of vascular inflammation in the development of venous thrombus development.[50] Importantly, activation of the inflammatory response is largely influenced by miRNA regulation of pathways including nuclear factor-kappa B (NF-κB), activator protein 1, and mitogen-activated protein kinase/early growth response protein 1 signaling.[51] miR-146 has a critical role in endothelium activation in response to inflammation.[51–53] Upon endothelial exposure to interleukin (IL)-1β and tumor necrosis factor (TNF)-α, miR-146 is significantly increased, thus preventing endothelium activation by binding to human antigen R, a signaling molecule required for activating endothelial cells through endothelial NOS (eNOS) expression.[51] Although miR-10a, miR-17–5p, miR-31, and miR-181b[54–56] are implicated in a positive-feedback loop pertaining to anti-inflammatory cytokine production (IL-4, IL-10, IL-13, and interferon-α), Rajput et al suggested that enhancing miR-146 in the vasculature may confer a broader anti-inflammatory role. Conversely, Olivieri et al identified miR-146a as a potential biomarker for the senescence-associated proinflammatory status of vascular cells, including HUVECs and human coronary artery endothelial cells.[57,58] In addition to miR-146, Sun et al later assessed the role of miR-181b and found that it effectively regulated the NF-κB pathway by inhibiting importin-α3, an essential protein for induction of VCAM-1 and E-selectin.[54] Collectively, these studies indicate that specific miRNAs may contribute to regulating the vascular response to inflammatory mediators. This may, therefore, imply a role for miRNAs in modulating thrombus development under inflammatory conditions, but further studies will be required to explore this possibility.

Endothelial barrier function is essential for the vascular integrity and preventing procoagulant blood-borne mediator contact of the extravascular tissue, thus ultimately preventing unnecessary thrombin generation or platelet activation. Interestingly, recent publications have highlighted the critical nature of miRNA in maintaining the semipermeable nature of the endothelial monolayer, largely through regulation of tight junction proteins.[59,60] miR-150 upregulated Tie2 in a mouse model of sepsis, indirectly leading to downregulation of claudin-5 and increasing vascular permeability.[61,62] Using miR-150 (−/−) mice and endothelial cells (in vitro analysis), Rajput et al observed a persistent increase in angiopoietin-2 levels, thus resulting in irreversible loss of vascular permeability. Upregulation of miR-150 restored junctional reannealing and reestablished barrier function both in vivo and in vitro. The mRNA transcript of vital junction protein zonula occludens-1 (ZO-1) was shown to be a direct target of many miRNAs, including miR-98, miR-18a, and miR-105 in other cell types, and therefore ZO-1 may be the target miRNA regulation within the endothelium.[63–65]

Given the earlier mentioned vast endothelial dysregulation following Dicer inactivation, it is not surprising that miRNA may be connected to cardiovascular disease and other inflammatory conditions characterized by vascular defects. Notably, however, individual miRNA dysregulation does not tend to cause lethality in vivo.[66] This may be due to the redundancy found within this vast network of miRNA signaling. Although miRNA networks behave collectively as a critical regulatory system, with stated exceptions, many miRNAs were not found to exert a substantial negative effect.

Endothelial Cell Dysregulation by miRNAs Following Infection

As described, miRNA dysregulation in the endothelium is likely a substantial trigger for endothelial dysfunction and barrier degradation. Thus, there is ground for linking endothelial miRNA dysregulation to barrier degradation and functional degradation of the endothelium observed under acute inflammatory conditions, including sepsis.

Vascular endothelial cells represent a major target for bacterial attachment. Many studies have now demonstrated that bacterial binding to the vascular endothelium triggers a series of events contributing to the pathophysiology of sepsis. For example, following bacterial binding, VWF is released from endothelial Weibel–Palade bodies, thus contributing to rapid platelet translocation and thrombus formation.[67] A coordinated decrease of circulating anticoagulant factors together with increased cell surface shedding of thrombomodulin leads to a decrease of activated protein C (APC) generation. APC, in particular, plays a critical role in maintaining hemostasis, since reduced values of protein C (PC)/APC predispose to thrombosis. Collectively, this coagulopathy culminates in disseminated intravascular coagulation (DIC).[68] TF is an important underlying trigger for DIC, and is expressed on, and released from, stimulated leukocytes, monocytes, and circulating microparticles.[69] Loss of endothelial barrier integrity due to reduced expression of junction proteins between the endothelial cells results in fluid leakage into the extravascular space, thus leading to life-threatening edema in septic patients. Vascular endothelial cells are both active participants in, and regulators of, inflammatory processes, thus directing the innate immune response to infections. Notably, there is significant evidence accumulating that all of these processes are at least partially controlled by a significant change in endothelial miRNA expression profile following infection. These dysregulated miRNAs can affect the endothelial cell itself (intrinsically) or extrinsically modulate the immune/inflammatory response by releasing miRNAs encapsulated in extracellular vesicles.[70]

Intrinsic Endothelial miRNA Dysregulation Following Infection and Inflammation

Intrinsic dysregulation of miRNA in the endothelium is currently being uncovered as an essential stage in the pathogenesis of sepsis, although prior knowledge in this area is noticeably limited. Upon infection, the miRNA profile of endothelium is altered, worsening the condition of the host, and so allowing the pathogen to further manipulate the internal cellular environment (►**Table 1**).[71] For example, miR-23b is a central regulator of the inflammatory pathway.[68] Upon exposure to lipopolysaccharide (LPS), a common method for experimentally inducing a sepsis-like environment, miR-23b was significantly downregulated in a two-dimensional model using a cultured inhibitor of DNA binding/differentiation-3 human VECs. In the absence of LPS, miR-23b naturally targets proinflammatory cytokine gene expression, such as NF-κB, TNFα, and IL-6, to inhibit the inflammatory response. Therefore, downregulation of miR-23b within cultured cells leads to overexpression of proinflammatory cytokines and ultimately to the excessive inflammatory response associated with sepsis.[72] The presence of infectious organisms can alter the miRNA expression patterns of endothelial cells.[73] This may lead to suppression of inflammatory endothelial regulators resulting in sustained production of the aforementioned proinflammatory cytokines (i.e., TNFα).[54]

miR-210 expression in endothelial cells has been implicated in chronic vascular diseases characterized by endothelial dysfunction, including atherosclerosis. Known to contribute in plaque formation, miR-210 was later discovered to play a specific role in endothelial apoptosis by directly targeting pyruvate dehydrogenase kinase 1 in an atherosclerosis setting. Upon induction of atherosclerosis in mice, Liu et al recently found that apoptosis was notably increased in CD31+ endothelial cells compared with healthy mice. Increased apoptosis was found to correlate with the increase of miR-210 and apoptosis was lessened upon inhibition of this miRNA.[79]

Extrinsic Endothelial miRNA Dysregulation in Vascular Disease

Recent studies indicate that endothelial miRNA expression can be regulated by extrinsic factors, specifically extravascular vesicles (EVs). There are several morphologically distinct classes of EVs, including exosomes, microparticles, and apoptotic bodies. Apoptosis (programmed cell death) is a nonrandom process; the biological material is selectively packaged into large apoptotic bodies (1–5 µm in diameter) before being released into the extracellular environment.[80,81] Microparticles, commonly

Table 1 Targets and functions of intrinsic endothelial miRNA

miRNA	Target gene(s)	Endothelial cell function	References
miR23b	*TNFα*	Prevents overexpression of proinflammatory cytokines	72
miR27a	*TNFα* *IL6*	Prevents overexpression of proinflammatory cytokines	74
miR181b	*ZO-1* *VE-Cadherin*	Regulates endothelial junctions and endothelial differentiation	75
miR181a	*KLF6*	Maintains blood-brain barrier	76
miR126–5p	*DLK1*	Promotes endothelial proliferation	44
miR17∼92 cluster	*miR19a*	Regulates WNT pathway	77
miR19a	*FZD4/LRP6*	Regulates the WNT pathway	77
miR98	*FIH-1*	Regulates vascular permeability	63
miR107	*ZO1*	Regulates endothelial junctions	78
miR210	*PDK1*	Regulates endothelial apoptosis	79
miR150	*Tie2*	Regulates blood-brain barrier permeability	62

Abbreviations: DLK1, delta-like 1 homolog; FIH-1, factor inhibiting HIF-1; FZD4, frizzled 4; IL-6, interleukin 6; KLF6, Krüppel -like factor 6; LRP6, low-density lipoprotein receptor-related protein 6; miR, microRNA; PDK1, 3-phosphoinositide-dependent kinase 1; Tie2, tyrosine kinase with Ig and epidermal growth factor homology domain 2; TNFα, tumor necrosis factor-α; VE-cadherin, vascular endothelial-cadherin; WNT, wingless int-1; ZO-1, zonula occludens.

referred to as "ectosomes" or "shed microvesicles," are a class of cellular membrane-derived lipid vesicle ranging from 0.1 to 1 µm in diameter.[82] Their formation requires reorganization of phospholipid bilayer and cytoskeleton, thus ultimately facilitating the outward "blebbing" of plasma membrane.[83–86] Exosomes, on the other hand, are nanovesicles (30–120 nm in diameter), and their formation is a downstream process associated with the endosomal pathway—late endosomes.[87] Multivesicular bodies (MVBs) form when the limiting membrane of late endosomes invaginates inwards forming intraluminal vesicles (ILVs). The term exosome refers to ILVs released into the extracellular milieu when MVBs fuse with the plasma membrane.

It is well documented that EVs are elevated in acute and chronic inflammatory diseases while circulating EV load increases in parallel with the severity of the disease.[88,89] Vascular endothelial cells are believed to be one of the major cell types contributing to EV load in vivo.[90] The endothelium can alter gene expression patterns of a plethora of cell types through transport of EV encapsulated miRNAs (EV-miRNAs).[91,92] Curtis et al reported that the potent proinflammatory cytokine TNFα promoted the release of endothelial-derived extracellular vesicles (EC-EVs) in human aortic endothelial cells.[93] Furthermore, Yamamoto et al showed that inflammatory miRNAs, released from EC-EVs, mediated an inflammatory response in pericytes, whose primary functions involve modulation of endothelial cell phenotypes and intracellular signaling pathways.[94,95] Shear responsive transcription factor Krüppel-like factor 2 (KFL2) binds to the miR-143/145 cluster and promotes its expression. Hergenreider et al demonstrated that endothelial cells transduced with KFL2 released EVs enriched in miR-143/45, which was effective to reduce atherosclerotic lesion formation in the aorta of ApoE$^{-/-}$ mice.[96] Upon TNFα stimulation, Zhang et al showed that HUVECs shed microparticles harboring proangiogenic miRNAs (miR-106b, miR-25, miR-92, and miR-21) in the extracellular milieu in vitro.[97] Shed microparticles from activated or apoptotic endothelial cells transfer miR-222 to neighboring endothelial cells, thus reducing the expression of ICAM-1 and abating monocyte adherence in vitro and in vivo (ApoE$^{-/-}$ mice).[98] Jansen et al reported that miR-126-enriched EC-MPs promoted vascular repair under hyperglycemic conditions, while miR-126 knockdown limited reendothelialization in vivo.[99] EC-EVs enriched in anti-inflammatory miRNAs (miR-10, miR-126, miR-146, miR-147, and miR-181b) repressed NF-κB signaling pathways by targeting components including *IRAK4* in monocytes.[100] Repression of NF-κB signaling has several adverse effects on the endothelium, including downregulation of E-selectins (which are needed for leukocyte adhesion) and suppression of proinflammatory cytokine release (i.e., TNFα and IL-1β).[101,102] Although the role of EC-EVs has been investigated in inflammatory conditions involving the endothelium, the selective transport of EVs by endothelial cells during infection has yet to be explored. While EC-EV released miRNA are understood to play a distinct role in endothelial function, how miRNAs derived from EC-EVs contribute to the characteristic loss of coordination leading to development of sepsis remains unclear (►**Table 2**).[75–78,103–107]

Conclusion

The role of dysregulated miRNAs in the pathophysiology of immunothrombotic disease, particularly related to infection, is tantalizing but remains to be fully elucidated. In particular, recent evidence suggests that bacterial engagement with endothelial cells results in significant increase of EVs containing an enhanced number of miRNAs. In turn, these molecules help to aid inflammation and more impact directly upon associated coagulopathy. Evidently, dysregulation of normally

Table 2 Targets and functions of extrinsic miRNA released by endothelial cells under inflammatory conditions

miRNA	Target gene(s)	Endothelial cell function	References
miR-10	*IRAK4; TAK1; BTRC*	Suppression of NF-κB signaling pathway	100
miR-21	*RhoB*	Inhibits angiogenesis	49
miR-25	*KLF4*	Regulator of the inflammatory response	103
miR-92	*KFL2/4; SOCS5*	Regulation of the inflammatory response/ modulator of endothelial dysfunction	104
miR-106b	*PTEN*	Dampens TNFα-induced apoptosis	105
miR-126	*SPRED1*	Promotes vascular repair	99
miR-143/145	*Ssh1/2; Srgap1/2; Cdc42*	Cytoskeletal remodeling and modulation of actin dynamics	106
miR-146	*TRAF6; IRAK1/2*	Represses adhesion molecule expression and promotes eNOS production	51
miR-147	*ADAM15*	Regulates endothelial barrier function	107
miR-181b	*KPNA4*	Regulator of NF-κB inflammatory pathway	54

Abbreviations: ADAM15, a disintegrin and metalloproteinase domain-containing protein 15; BTRC, β-transducing repeat containing; Cdc42, cell division control protein 42; IRAK1, interleukin-1 receptor-associated kinase 1; IRAK4, interleukin-1 receptor-associated kinase 4; KLF4, Krüppel-like factor 4; KPNA4, karyopherin subunit alpha 4; PTEN, phosphatase and tensin homolog; RhoB, Ras homolog family member B; SOCS5, suppressor of cytokine signaling 5; SPRED1, Sprouty-related, EVH1 domain-containing protein 1; Srgap 1, SLIT-ROBO Rho GTPase activating protein 1; Ssh1, slingshot protein phosphatase 1; TAK1, transforming growth factor β-activated kinase 1; TRAF6, TNF receptor-associated factor 6.

tightly regulated miRNAs that sustain normal endothelial cell function may trigger serious downstream functional effects, and may contribute to modifications in vascular permeability leading to excessive edema, loss of endothelial cell proliferation, and endothelial interaction with blood-borne procoagulant factors to trigger thrombotic events. Also, dysregulated miRNAs released from endothelial cells via EVs are capable of instigating profound impacts on other circulating cells within the cardiovascular system. Such an outcome may lead to the excessive and sustained release of proinflammatory cytokines from recruited leukocytes, which were shown to contribute to the activated endothelial phenotype now associated with the development of the thrombotic disease. As current antibiotic treatment options for acute infection do not aim to modulate these dysregulated miRNAs specifically, novel therapeutics that reassert control on dysregulated miRNA expression profiles in endothelial cells may have significant benefit in countering cardiovascular disease.

Conflict of Interest
None.

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