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Non-coding RNAs in cerebral endothelial pathophysiology: Emerging roles in stroke

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Abstract

Cerebral vascular endothelial cells form the major element of the blood-brain barrier (BBB) and constitute the primary interface between circulating blood and brain parenchyma. The structural and functional changes in cerebral endothelium during cerebral ischemia are well known to result in BBB disruption, vascular inflammation, edema, and angiogenesis. These complex pathological processes directly contribute to brain infarction, neurological deficits, and post-stroke neurovascular remodeling. Ischemic endothelial dysfunction appears to be tightly controlled by multiple gene signaling networks. Non-coding RNAs (ncRNAs) are functional RNA molecules that are generally not translated into proteins but can actively regulate the expression and function of many thousands of protein-coding genes by different mechanisms. Various classes of ncRNAs, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), small nucleolar RNAs (snoRNAs) and piwi-interacting RNAs (piRNAs), are highly expressed in the cerebrovascular endothelium where they serve as critical mediators to maintain normal cerebral vascular functions. Dysregulation of ncRNA activities has been closely linked to the pathophysiology of cerebral vascular endothelium and neurologic functional disorders in the brain's response to ischemic stimuli. In this review, we summarize recent advancements of these ncRNA mediators in the brain vasculature, highlighting the specific roles of endothelial miRNAs in stroke.

Keywords

non-coding RNAs; microRNA; cerebral vascular endothelial cells; blood-brain barrier; angiogenesis; ischemic stroke

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1. Introduction

Ischemic stroke generally causes vascular and neuronal damage, both of which affect the extent of ischemic injury and stroke outcome. Cerebral vascular endothelial cells are a major component of the brain microvasculature, and maintenance of healthy brain endothelium is essential to normal cerebrovascular physiology. One of the key functions of the cerebral endothelium is to form the BBB and play a dominant role in maintaining the BBB integrity and cerebral homeostasis in physiological conditions (Hawkins and Davis, 2005; Sandoval and Witt, 2008). There is increasing evidence showing that ischemia-induced cerebral endothelial injury, endothelial inflammation, and subsequent impairment of endothelial function increase cerebrovascular permeability and BBB leakage, leading to primary and secondary ischemic brain injury (del Zoppo and Hallenbeck, 2000; Ishikawa et al., 2004; Sandoval and Witt, 2008). Therefore, the brain endothelium becomes an important therapeutic target for the inhibition of cerebrovascular dysfunction in ischemic stroke (Fagan et al., 2004; Rodriguez-Yanez et al., 2006; Fisher, 2008). However, the molecular mechanisms of cerebral endothelial injury and dysfunction after cerebral ischemia remain largely unexplored.

Non-coding RNAs (ncRNAs) are generally defined as untranslated regulatory RNA molecules. In humans, DNA sequences responsible for non-protein coding regions comprise at least 98% of the total genome. Similar to the gene/protein regulators, recent evidence suggests that ncRNAs also have functional importance, and one ncRNA may be able to mediate one or more genes to affect multiple cellular pathways in a biological process. In addition to well-known ncRNAs such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), the remaining ncRNAs can be broadly classified as small (<200 nt) and long (>200 nt) ncRNAs (lncRNAs) (Bartel, 2004; Kim, 2005; Taft et al., 2010; Qureshi and Mehler, 2012; Schonrock et al., 2012; Vemuganti, 2013). Small RNAs include microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and promoter-associated small RNAs. LncRNAs are further subdivided into long intergenic ncRNAs, long intronic ncRNAs, telomeric ncRNAs, pseudogene transcripts, enhancer RNAs and promoter-associated long RNAs. Small and long ncRNAs differ in their origin, processing, and mechanisms of action (Bartel, 2004; Kim, 2005; Taft et al., 2010; Lee et al., 2011; Qureshi and Mehler, 2012; Rajasethupathy et al., 2012; Schonrock et al., 2012; Vemuganti, 2013).

Currently, the exact roles and molecular mechanisms of many classes of ncRNAs have not been clearly explored. So far in the stroke field, the role of a specific class of small ncRNAs, miRNAs, was mainly investigated (Rink and Khanna, 2010; Saugstad, 2010; Vemuganti, 2010; Tan et al., 2011; Eacker et al., 2013; Liu et al., 2013; Ouyang et al., 2013; Yin et al., 2013a). Moreover, recent breakthrough microarray profiling studies identifying stroke-responsible alteration of piRNAs (Dharap et al., 2011) and lncRNAs (Dharap et al., 2012; Dharap et al., 2013; Vemuganti, 2013) underscore the importance of other ncRNAs in the pathogenesis of ischemic stroke. In this review, we will discuss the functional significance of stroke-associated endothelial ncRNAs with a special focus on miRNAs in cerebral endothelial pathophysiology after stroke.

2. Cerebrovascular endothelial pathophysiology after ischemic stroke

In ischemic stroke, both the cerebral vasculature and neuronal tissues are affected and damaged. Cerebral vascular damage contributes to a severe degree of ischemic brain injury.

2.1. BBB breakdown and dysfunction

The BBB is a brain-specific physical barrier between blood and the central nervous system that strictly controls molecule/nutrient exchange between the blood and brain compartments, thereby playing an important role in brain homeostasis and maintaining a healthy microenvironment for complex neuronal activities (Weiss et al., 2009). One of the dominant components of the BBB is brain capillary endothelial cells and its adjacent junction complexes (tight junctions and adherens junctions), which form the structural and functional basis for the BBB (Hawkins and Davis, 2005; Sandoval and Witt, 2008; Weiss et al., 2009). Accumulating experimental and clinical evidence has documented that ischemic stroke induces rapid and dual phases of alterations in the endothelial permeability barrier, an early phase occurring minutes after reperfusion followed by a second phase several hours after ischemia (Kuroiwa et al., 1985; Belayev et al., 1996; Aronowski et al., 1997). Several major mechanisms involving changes in production of reactive oxidants and activation of matrix metalloproteinases and inflammatory cytokines have been shown to disrupt the BBB permeability properties, leading to degradation of endothelial junctions, loss of vascular integrity, and BBB dysfunction. BBB damage may further aggravate neurological outcomes in ischemic stroke by facilitating edema formation and hemorrhagic transformation (Dirnagl et al., 1999).

2.2. Cerebrovascular endothelial injury and death

During cerebral ischemia, the pathologic processes resulting in vascular endothelial injury and death can be classified as three temporal stages: acute (hours), subacute (hours to days), and chronic (days to months) (Fagan et al., 2004; Rodriguez-Yanez et al., 2006; Fisher, 2008). Mechanistic regulators have been identified for three different stages. Acutely, superoxide is the predominant mediator, followed by inflammatory mediators/proteases subacutely, and proapoptotic genes/proteins in the chronic phase. Cerebral vascular endothelial injury due to reperfusion is largely regulated by reactive oxygen species, resulting in endothelial cell membrane damage and subsequent cell death. Inflammation also contributes to cerebral vascular endothelial injury, and the expression of inflammatory mediators in endothelial cells rapidly increases following cerebral ischemia, leading to enhanced leukocyte-endothelial interactions mediated by selectins and adhesion molecules. Ischemic endothelial cell death undergoes both necrosis and apoptosis, with the latter most prominent in the ischemic penumbral regions. In rodent experimental stroke models, activation of both caspase-dependent and caspase-independent apoptotic pathways have been shown to be responsible for endothelial cell apoptosis. In response to various ischemic stimuli, many pro-apoptotic factors such as BH3-only proteins, have been identified in cerebral endothelium. In contrast, several key antiapoptotic proteins including Bcl-2, Bcl-xl, and the inhibitor of apoptosis proteins are also upregulated, suggesting that a balance of pro- and anti-apoptotic proteins may be critical for the survival of brain endothelium under ischemic conditions. Hopefully, therapeutic options will be focused on the development of

anti-oxidant, anti-inflammatory, and anti-apoptotic interventions to avoid or attenuate ischemic vascular endothelial injury and provide vascular endothelial protection in patients with ischemic stroke (Fagan et al., 2004; Rodriguez-Yanez et al., 2006; Fisher, 2008).

2.3. Cerebrovascular endothelial activation and inflammation

Inflammatory mediators generated/released from various neural cells are involved in molecular cascades determining stroke outcome (Huang et al., 2006). During cerebral ischemia, cerebral endothelial cells are stimulated by inflammatory mediators in ischemic brains, and undergo proinflammatory activation. Activated cerebral endothelium have the capacity to produce and/or secrete many inflammatory mediators, most important of which are the selectins (P-selectin, E-selectin), immunoglobulin gene superfamily (VCAM, ICAM-1), and integrins, thus becoming a source of inflammation themselves in the cerebral vasculature (del Zoppo and Hallenbeck, 2000; Ruetzler et al., 2001; Huang et al., 2006). As a result, these proinflammatory mediators in vascular endothelium attract and actively mediate the transmigration process of peripheral circulating leukocytes into ischemic brain tissue (Okada et al., 1994; Haring et al., 1996). This leukocyte-endothelial cell interaction includes several steps: leukocyte rolling, adhesion, and transendothelial migration. The recruited leukocytes further contribute to and amplify reperfusion injury through the release of proteases, reactive oxygen species, and other inflammatory mediators. Circulatory leukocytes can also plug cerebral capillaries and induce capillary no-reflow, leading to further amplification of the inflammatory response. Thus, cerebral endothelial cells at the BBB interface play a principal regulatory role in vascular and brain inflammatory events during stroke via facilitation of the trans-endothelial migration of leukocytes (Wang and Lo, 2003; Ishikawa et al., 2004; Huang et al., 2006).

2.4. Vasogenic brain edema

In general, two major types of brain edema (cytotoxic and vasogenic) have been reported in the pathogenesis of stroke (Klatzo, 1967). The cytotoxic type is an intracellular edema, and represents a water shift from extracellular to intracellular compartments. Depending on the severity of ischemia, cytotoxic edema may occur within minutes or hours after the stroke onset. Several mechanisms have been identified for the cytotoxic brain edema, including ischemia-induced failure of the Na^+/K^+ pump that leads to subsequent loss of membrane ionic gradients and dysfunction of neuronal membrane potential, finally resulting in the accumulation of intracellular Na^+, a net influx of water, and cellular swelling. Obviously, cytotoxic edema is independent of structural and functional BBB alterations. It is worth noting that the volume of cytotoxic edema may reflect the degree of ischemic cellular damage, brain infarction size, and stroke severity but cytotoxic edema is not an important cause of brain swelling (Ayata and Ropper, 2002; Kahle et al., 2009).

In contrast, vasogenic edema is a type of extracellular brain swelling, mainly due to impaired BBB integrity, and subsequent water movement from intravascular to extravascular compartments. Compared to cytotoxic edema, vasogenic edema takes place a few days after stroke onset (Gotoh et al., 1985; Menzies et al., 1993; Slivka et al., 1995). The formation and procession of vasogenic edema are closely correlated with the severity of brain ischemia and BBB breakdown. It has become clear that disruption of cerebral vascular

endothelium and endothelial tight junctions greatly contribute to impairment of BBB integrity, resulting in a shift of intravascular serum proteins and ions into the extravascular space, with water passively following the osmotic gradient. Vasogenic edema is a dominant cause of ischemic brain swelling, and closely associated with delayed neuronal death in several animal models of stroke (Ayata and Ropper, 2002; Kahle et al., 2009).

2.5. Cerebrovascular endothelial proliferation and angiogenic remodeling

Angiogenesis generates new blood vessels from existing vascular endothelial cells to deliver nutrients and oxygen to various tissues and organs (Folkman, 1995; Carmeliet, 2005). In addition to functioning as a normal physiological process in tissue growth and development, angiogenesis also occurs as a natural defense and beneficial response against ischemic human diseases including ischemic heart or cerebrovascular disease, peripheral arterial disease, and wound healing. Extensive studies have shown that post-ischemic angiogenesis plays a crucial role in the recovery of blood flow in affected brain tissue (Hayashi et al., 2006; Arai et al., 2009; Beck and Plate, 2009; Zhang and Chopp, 2009) and promotes stroke recovery.

In humans, active angiogenesis takes place at 3–4 days and may persist for at least 3 months in brains following an ischemic insult (Krupinski et al., 1994). Analysis of post-mortem brain tissue obtained from patients with various survival times after stroke revealed increased microvessel density in the penumbral areas in comparison with the contralateral normal hemisphere. Of importance, stroke patients with greater cerebral blood vessel density appear to make better progress and survive longer than patients with lower vascular density (Krupinski et al., 1993, 1994), suggesting active angiogenesis may be beneficial for neurological functional recovery. In rodent models of experimental focal cerebral ischemia, cerebral vascular endothelial cells start to proliferate in the peri-infarcted region as early as 12–24h after the onset of stroke (Beck et al., 2000; Marti et al., 2000; Hayashi et al., 2003). This ischemia/hypoxia-induced vascular remodeling leads to increased microvessel density surrounding the infarcted brain area three days following ischemic injury. Neovascularization can occur actively for a longer time in the ischemic region, as evidenced by the findings of Hayashi *et al.* showing that vessel proliferation continued for more than 21 days following experimental cerebral ischemia (Hayashi et al., 2003). Accumulating endogenous pro-angiogenic molecules have also been identified, including growth factors, matrix metalloproteinases, cytokines, and integrins. Growth factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-beta (TGF-beta), and epidermal growth factor (EGF). Thus, promoting post-ischemic angiogenesis may become a useful therapeutic strategy for the treatment of acute ischemic stroke in the clinical setting.

3. Non-coding RNAs in vascular and cerebrovascular EC biology

3.1. Overview of non-coding RNAs

Recent genome-wide studies by deep sequencing technology have revealed that eukaryotic genomes are extensively transcribed to produce many thousands of regulatory non-protein-coding RNAs (ncRNAs). It is worth noting that only approximately 1.5% of DNA sequences

in the human genome are responsible for protein coding whereas at least 98% of the genome does not contain protein-coding DNA sequences but transcribed into various ncRNAs. NcRNAs are diverse in molecular structure, biogenesis, cellular distribution and biological function, and can be broadly classified according to their size (Table 1) (Taft et al., 2010; Qureshi and Mehler, 2012; Schonrock et al., 2012; Vemuganti, 2013). It is gradually becoming clear that ncRNAs are not junk DNA but actively functional regulatory elements in the epigenetic control of gene function at different levels, including but not limiting to chromatin-modifying, transcriptional and post-transcriptional mechanisms (Bartel, 2004; Kim, 2005; Mercer et al., 2009; Ponting et al., 2009; Taft et al., 2010).

NcRNAs have become a focus in biomedical research in the last half decade and are now regarded as important and essential mediators in multiple major biological/physiological processes impacting development, differentiation, and metabolism, as well as pathologies in a variety of human diseases (Wienholds and Plasterk, 2005; Suarez and Sessa, 2009; Taft et al., 2010; Qureshi and Mehler, 2012; Schonrock et al., 2012; Vemuganti, 2013). In particular, miRNAs have been mainly investigated as disease biomarkers and therapeutic targets (Tan et al., 2009) and miRNA-based therapeutics (Broderick and Zamore, 2011; van Rooij and Olson, 2012; van Rooij et al., 2012) are under development and progressing very rapidly.

3.2. Non-coding RNAs in vascular endothelial cells and roles in vascular biology and pathologies

The vascular endothelium functions as a primary barrier between blood flow and the rest of the body, and also plays an essential role in maintaining blood vessel homeostasis by dynamically responding to alterations in the cellular microenvironment. Vascular endothelial cells are enriched with various classes of ncRNA, and it is now recognized that these non-translated RNA molecules are involved in controlling endothelial biology and physiological function. For example, recent studies have identified a specific role for endothelial miRNAs as key regulators of vascular function, including senescence, angiogenic, vascular repair and inflammatory signaling cascades (See multiple reviews (Suarez and Sessa, 2009; Wang and Olson, 2009; Yin et al., 2013a)). Additionally, long ncRNAs in endothelial cells have also been reported in the regulation of vascular biology. In embryonic zebrafish, the *tie-1AS* lncRNA directly binds *tie-1* mRNA and inhibits *tie-1* transcript levels, resulting in specific defects in vascular endothelial tight junction and adherens junction proteins *in vivo* and *in vitro*. Moreover, *tie-1AS* lncRNA significantly inhibits VEGF-induced endothelial tube formation in HUVEC cultures via down-regulation of the Tie-1 protein and disruption of endothelial cell junctions and stability of the endothelial network. Also, the aberrant ratio of *tie-1* versus *tie-1AS* lncRNA is observed in human vascular anomaly samples. This is the first report on the identification of a lncRNA that plays a functional regulatory role in vascular development (Li et al., 2010).

On the other hand, elucidation of the functional importance of endothelial ncRNAs in vascular disease is a burgeoning field, particularly since previous studies primarily focused on the involvement of vascular miRNAs. For instance, much work has shown that endothelial miRNAs play key roles in the pathogenesis of multiple human diseases

including atherosclerosis, coronary artery disease, thrombosis, renal artery stenosis, abdominal aortic aneurysms, stroke, macular degeneration, diabetes, and cancer, among others (Quintavalle et al., 2011; Staszel et al., 2011; Lorenzen et al., 2012; Madrigal-Matute et al., 2013; Yin et al., 2013a). In addition, a recent study (Yuan et al., 2012) reported that lncRNA associated with microvascular invasion in hepatocellular carcinoma (HCC) (lncRNA MVIH) was significantly upregulated in HCC, which was associated with frequent microvascular invasion, a higher tumor node metastasis stage, and decreased survival rate. Moreover, lncRNA MVIH can inhibit the secretion of phosphoglycerate kinase 1, thus promoting tumor growth and intrahepatic metastasis by activating angiogenesis in mouse models. Deregulation of lncRNA MVIH could be utilized as a potential target for new adjuvant therapies against active angiogenesis (Yuan et al., 2012).

3.3. Non-coding RNAs in brain vascular EC biology and pathologies

Compared to ncRNA research in general vascular biology, there has been little focus on exploring the roles of these untranslated RNA molecules in the cerebral vasculature, in particular brain vascular endothelial cells. In a recent study (Mishra and Singh, 2013) examining the mechanisms of perturbation of the BBB during HIV-1 infection, it was reported that the HIV-1 Tat protein is implicated in HIV neuropathogenesis by exerting an adverse effect on BBB integrity and permeability. This study shows that HIV-1 Tat C significantly increased miR-101 expression in human brain microvascular endothelial cells (BMVECs), which led to translational inhibition of VE-cadherin. Consistent with this finding, *in vivo* gain- or loss-of-miR-101 function effectively suppresses or enhances the expression of VE-cadherin, respectively. Moreover, they also found that the expression level of claudin-5 is also governed by the expression of VE-cadherin. These findings suggest a novel mechanism for the regulation of BBB permeability by miR-101 via posttranscriptional regulation of VE-cadherin in human BMVECs exposed to the HIV-1 Tat C protein (Mishra and Singh, 2013).

In another study (Reijerkerk et al., 2013) investigating whether miRNAs play a role in BBB function, a set of BBB-associated miRNAs were identified in cultured human brain endothelial cells under normal and inflammatory conditions. The data demonstrated that inflammatory stimuli significantly changed the expression of 107 miRNAs. Among them, most (98 of 107) of the differentially expressed miRNAs were significantly down-regulated by TNFα and IFNγ treatment, which was also shown to impair endothelial barrier function. Most importantly, levels of BBB-associated miRNAs were also diminished in freshly isolated brain capillaries from postmortem patients with multiple sclerosis (MS). In particular, miR-125a-5p is further identified as a key regulator of brain endothelial tightness and leukocyte efflux, thus significantly improving or enhancing brain endothelial cell barrier function. These findings reveal a novel miRNA regulatory mechanism of brain endothelial cell barrier function. Since many neurological disorders are related to pathological alterations in the microvasculature of the brain, it is anticipated that therapeutic application of miRNAs, such as a miR-125a-5p mimic, may potentially repair a dysregulated BBB to re-establish normal functioning of the brain vasculature, in particular MS.

Consistent with this finding, a very recent study also demonstrated that miR-155 was significantly induced at the neurovascular unit in brains of patients with MS and in spinal cords of mice with experimental autoimmune encephalomyelitis (EAE). Genetic mutation of miR-155 in mice prevented BBB leakage/breakdown in EAE and lipopolysaccharide-induced acute systemic inflammation. Mechanistically, miR-155 was found to regulate human brain endothelial permeability by directly targeting cell-cell complex molecules such as annexin-2 and claudin-1, as well as focal adhesion components such as DOCK-1 and syntenin-1. Thus, miR-155 is proposed to function as a negative BBB regulator that may become a potential therapeutic target for CNS neuroinflammatory diseases associated with BBB breakdown(Lopez-Ramirez et al., 2014).

Except for endothelial miRNAs, there have been only a few reports to show the functional importance of other classes of ncRNAs in cerebrovascular biology and function at both physiological and disease conditions. For instance, the Meg3 gene encodes a long noncoding RNA and is expressed in many normal tissues including brain vasculature. Genetic inactivation of Meg3 in mice leads to a significant increase in the expression of pro-angiogenic genes, in particular the VEGF signaling pathway (VEGF and VEGFR1), and promotes new microvessel formation in the brain cortex. These findings strongly suggest that Meg3 functions as a novel lncRNA tumor suppressor (Gordon et al., 2010; Zhou et al., 2012).

4. Stroke-associated cerebrovascular endothelial non-coding RNAs

Although miRNAs are mainly investigated in the pathogenesis of stroke, less attention has been paid to the expression profile and functional significance of miRNAs in the cerebral vasculature, in particular the cerebral vascular endothelium.

Recent studies from our laboratory have demonstrated that miR-15a is significantly increased in cerebral vascular endothelial cell cultures (CECs) after 4–16 hour oxygen glucose deprivation (ODG) exposure as well as in cerebral vasculature 1 day after ischemic stroke. Intriguingly, *in vitro* gain- or loss-of-miR-15a function using a miR-15a mimic or inhibitor can effectively reduce or increase OGD-induced CEC death, caspase-3 activation, and Golgi fragmentation by post-transcriptional enhancement or repression of its direct downstream target bcl-2, respectively (Yin et al., 2010). Furthermore, we have further identified that miR-15a is a direct target of PPARδ and PPARγ, and transcriptional inhibition of miR-15a activity by PPARδ or PPARγ appears to contribute to PPARδ and PPARγ-mediated cerebrovascular protection *in vitro* and *in vivo* after cerebral ischemic stimuli (Yin et al., 2010; Yin et al., 2013b). Taken together, these findings indicate that miR-15a is a master regulator in cerebral endothelial injury and subsequent cerebrovascular dysfunction after ischemic stroke, and pharmacological inhibition of miR-15a may be a potentially suitable therapeutic option for stroke-induced cerebral vascular damage.

Endothelial miR-15a is also a critical regulatory molecule in the regulation of angiogenesis in some ischemic pathological conditions (Yin et al., 2012). We have recently shown a novel finding that EC-selective miR-15a transgenic overexpression in mice leads to reduced blood vessel formation and local blood flow perfusion in the ischemic hindlimbs at 1–3 weeks

after hindlimb ischemia. Consistent with the miR-15a anti-angiogenic effects *in vivo*, lentivirus-mediated gain- or loss-of-miR-15a function in ECs significantly reduces or increases tube formation, cell migration, and cell differentiation, respectively. Mechanistically, by FGF2 and VEGF 3'UTR luciferase reporter assays, real-time PCR, and immunoassays, we further identified that miR-15a directly targets FGF2 and VEGF in ECs to facilitate its anti-angiogenic effects (Yin et al., 2012). Thus, our data suggest that endothelial miR-15a can significantly suppress cell-autonomous angiogenesis through direct inhibition of endogenous FGF2 and VEGF activities. Moreover, we further investigated the role of miR-15a in the regulation of postbrain ischemic angiogenesis (unpublished data). We have shown that expression of miR-15a is significantly increased in the cerebral vasculature at the penumbral area 7 days after mouse middle cerebral artery occlusion (MCAO). Accordingly, endothelial cell (EC)-selective miR-15a transgenic overexpression leads to reduced cerebral capillary density, increased brain infarction, and neurological deficits in mice 7 days post-MCA occlusion. These findings may suggest miR- 15a can suppress post-ischemic cerebral angiogenesis. Elucidating the molecular mechanisms of miR-15a/16-1 cluster-mediated angiogenesis will provide new insights into the understanding of miR function in post-ischemic neurovascular remodeling and neurological recovery and open a new area with potential promise for further development of neurorestorative therapies after ischemic stroke.

Concurrently, another group led by Dr. Zhang investigated miRNA expression profiles in cerebral endothelial cells after stroke in the rat (Teng et al., 2012). In this study, primary brain microvascular endothelial cells were isolated from microvessels of adult non-ischemic rats and from rats subjected to 7 days of MCAO. By using miRNA microarray screening techniques and real-RT-PCR analysis, they found that nine miRNAs (miR-100, -101a, -204, -210, m-224, -322, - 322*, -331, and -450a) were significantly upregulated in BMECs after stroke, whereas seven miRNAs (miR-139-5p, -193*, -203, -225, -335, -494, and -708) were downregulated. Obviously, ischemic stroke alters miRNA expression profiles in cerebral endothelial cells, implying their regulation of cerebral angiogenesis through multiple signaling pathways.

In addition, other groups have also recently found that miR-210, a hypoxia-induced miRNA, is significantly up-regulated in adult rat ischemic brain cortexes in which the expression of Notch1 signaling was also increased. Overexpression of miR-210 in HUVEC cultures activated the Notch1 signaling pathway and subsequently induced endothelial cells to migrate and form capillary-like structures, suggesting that miR-210 mediates post-ischemic neovascularization (Lou et al., 2012). Consistent with this finding, miR-210 has been also shown to regulate angiogenesis in normal brain tissue (Zeng et al., 2013).

On the other hand, emerging evidence shows that circulating miRNAs may be involved in the regulation of post-ischemic angiogenesis after stroke. In young stroke patients, circulating miRNAs in blood that are associated with vascular endothelial function and angiogenesis (hsalet- 7f, miR-130a, -150, -17, -19a, -19b, -20a, -222, and -378) and vascular remodeling (miR-21, -126, and -150) have been found to be differentially regulated under ischemic conditions. Similarly, miRNAs that are expressed in hypoxic conditions (miR-23, -24, -26, -103, -107, and - 181) and cardiac ischemia/reperfusion (miR-15, -16, -21, -23a,

-29, -30a, -150, and -195) have also been detected in blood samples from stroke patients (Tan et al., 2009). Moreover, miR-320, which is a negative angiogenic mediator in myocardial microvascular endothelial cells of type 2 diabetic rats, is significantly reduced in all stroke patients, especially those with good outcome (Tan et al., 2009).

In addition to miRNAs, the research group led by Dr. Vemuganti has recently published a series of pioneering studies, demonstrating that cerebral ischemia rapidly changes the expression profiles of other classes of ncRNAs, such as lncRNAs (Dharap et al., 2012; Dharap et al., 2013; Vemuganti, 2013) and piRNAs (Dharap et al., 2011), and imply that manipulation of these ncRNAs may regulate post-ischemic neuronal death and remodeling. To our knowledge, little is known regarding the expression and function of cerebral vasculature and/or endothelium lncRNA and piRNA molecules responsible for stroke.

5. Future prospects for endothelial ncRNA studies in cerebrovascular diseases

Up to now, accumulating evidence has shown that dysregulated endothelial ncRNAs, in particular endothelial miRNAs, contribute to the cerebrovascular pathophysiology of stroke. However, we are still at the very early stages in fully understanding the functional importance of endothelial ncRNAs in the stroke research field. Although continuing investigation on endothelial miRNAs (endomiRs) (Santoro and Nicoli, 2013) are necessary, future studies will be needed to explore the particular functions of other classes of ncRNA members (e.g. piRNAs, lncRNAs, and snoRNAs) by using *in vitro* brain vascular endothelial cell cultures and experimental stroke animal models. It is worth noting that cell-specific genetic manipulation in mouse models is a valuable approach for defining the specific role of target genes in cerebrovascular diseases. In order to clarify the role of ncRNAs in stroke-induced cerebrovascular pathophysiology *in vivo*, a model that can distinguish between vascular endothelial-dependent and -independent signaling is required. Thus, utilization of endothelial cell-specific ncRNA transgenic and conditional knockout animals will be necessary to further clarify the role and underlying mechanisms of individual ncRNAs in the cerebrovascular pathogenesis of stroke (Yin et al., 2012). For the translational aspects of stroke research, data from human stroke patients or from population-based stroke epidemiology studies will better address the functional importance of endothelium-specific ncRNAs in stroke and other neurological disorders (Tan et al., 2009).

Specific attention must also be paid to the careful identification of ncRNA downstream target genes, and related signaling pathways/networks to fully understand the acting mechanisms of stroke-associated endothelial ncRNAs (Yin et al., 2012). In addition, we also need to understand how these endothelial ncRNAs are regulated in the pathophysiology of stroke, which will provide new insights into the development of pharmaceutical agents that affect ncRNA activity in the cerebral vasculature and intervene against stroke-related cerebrovascular disorders.

On the other hand, due to the complex nature of brain structure and function, as evidenced by the neurovascular unit and vascular neural network (Lo et al., 2003; Zhang et al., 2012), further investigation is also needed to explore the interaction or cross-link of ncRNAs

between the cerebrovascular endothelium and other neural cells (pericytes, astrocytes, neurons, oligodendrocytes, microglia, etc.) as an integrative ncRNA regulatory network in ischemic brain injury. It is also worth noting that the precise mechanisms of these interacting networks in regulating neural cell survival as well as post-ischemic remodeling are still elusive.

6. Conclusion

As illustrated in this review, we have focused on recent advancements in the regulation of multiple vascular endothelium-dependent cerebrovascular pathologies such as BBB disruption, vascular inflammation, edema, and angiogenesis after ischemic stroke. In particular, we have summarized the essential role of endothelial ncRNA molecules in controlling cerebral vascular endothelial responses to brain ischemic stimuli. NcRNAs have been broadly implicated in vascular development, maintenance, remodeling, and disease. NcRNA research in cerebrovascular biology and cerebrovascular diseases such as stroke is quite young but fastgrowing, and the functional importance and molecular regulatory mechanisms of ncRNAs in the cerebral vasculature, especially in the endothelium following ischemic stroke, are largely unknown. The elucidation of stroke-responsible endothelial ncRNA-related mechanisms may be important for our understanding in-depth the pathogenesis of ischemic cerebrovascular injury/remodeling and brain damage. It is speculated that more insights into the molecular mechanisms of normal brain vascular function and neurological disease-related pathophysiology mediated by endothelial ncRNA regulatory molecules will allow us to identify novel targets for a promising translational future of ncRNA-based diagnostics and therapeutics in ischemic stroke.

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Table 1

Overview of various ncRNAs

Class	Full Name	Size	Function
Small ncRNAs			
siRNAs	small/short interfering RNAs	20–21nt	mRNA degradation
miRNAs	microRNAs	18–24nt	post-transcriptional silencing of proteincoding genes and ncRNAs
piRNAs	piwi-interacting RNAs	26–31nt	silencing of mRNAs and transposons
rasiRNAs	repeat-associated siRNAs	24–29nt	transposon silencing
crasiRNAs	centromere repeat associated short interacting RNAs	34–42nt	heterochromatin recruitment
tel-sRNAs	telomere small RNAs	23–28nt	telomere maintenance
snoRNAs	small nucleolar RNAs	60–300nt	rRNA methylation, pseudouridylation, and pre-mRNA processing
snRNAs	small nuclear RNAs	<200nt	pre-mRNA splicing
PASRs	promoter-associated short RNAs	20–200nt	transcriptional regulation of gene expression
tiRNAs	transcription initiation RNAs	18nt	transcriptional regulation of gene expression
TSSa-RNAs	transcription start site-associated RNAs	20–90nt	transcriptional regulation of gene expression
PROMPTs	promoter upstream transcripts	18nt	transcriptional regulation of gene expression
spliRNAs	splice junction-associated RNAs	17–18nt	epigenetic regulation of gene expression
TASRs	termini-associated short RNAs	20–200nt	pervasive transcription
CRISP	clustered regularly interspaced short palindromic repeats	24–48nt	prokaryotic immune control
tasiRNAs	trans-acting siRNAs	20–22nt	gene silencing in plants
vRNAs	vault RNAs	88–98nt	multidrug resistance, apoptosis resistance and innate immunity
mito-sncRNAs	mitochondrial small non-coding RNAs	<200nt	regulation of mitochondrial gene expression
MSY-RNAs	MSY2-associated RNAs	26–30nt	unknown
moRNAs	microRNA-offset RNAs	~20 nt	unknown
tRNA-derived RNAs	tRNA-derived RNAs	19–40nt	Inhibition of mRNA translation
sdRNAs	sno-derived RNAs	<50nt	miRNA-like regulators of translation
xiRNAs	X-inactivation RNAs	<50nt	X-chromosome inactivation
Y RNAs	Y RNAs	~100nt	DNA replication
Long ncRNAs			
LincRNAs	long intergenic non-coding RNAs	>200nt	epigenetic regulation and chromatin remodeling; transcriptional control
eRNAs	enhancer RNAs	>200nt	promoting mRNA synthesis
uaRNAs	3′ untranslated region (UTR)-derived RNAs	>200nt	regulation of mRNA stability, translation, and localization
NATs	natural antisense transcripts	>200nt	chromatin modifications; regulation of transcription, mRNA stability, splicing, and translation
TERRA	telomeric repeat-containing RNAs	>200nt	maintenance of telomere integrity

Class	Full Name	Size	Function
T-UCR/E	transcribed ultraconserved region/element	>200 nt	splicing and epigenetic modifications, transcriptional coactivator
PALRs	promoter-associated long RNAs	>200 nt	transcriptional regulation of gene expression
SUT	stable unannotated transcript	>200 nt	transposon silencing
CUT	cryptic unstable transcript	>200 nt	gene regulation and silencing
mito-lncRNAs	mitochondrial long non-coding RNA	>200 nt	regulation of mitochondrial gene expression
Pseudogenes	pseudogenes	>200 nt	modulators of gene expression

Table 2

NcRNAs in brain vascular EC biology and pathologies

NcRNAs	Function	Targets	References
miR-101	mediates the HIV-1 Tat C-induced impairment of endothelial barrier integrity and subsequent BBB dysfunction in human brain microvascular endothelial cells.	VE-cadherin	Mishra *et al.*, 2013
miR-125a	significantly diminishes in isolated multiple sclerosis patient capillaries and functions as a key regulator of brain endothelial tightness and immune cell efflux.	VE-cadherin, ZO-1	Reijerkerk *et al.*, 2013
miR-155 Meg3	Negatively regulates BBB breakdown in CNS neuroinflammatory disorders such as MS and EAE.inhibits angiogenesis in mouse brains.	Annexin-2, Claudin-1, DOCK-1, Syntenin-1 VEGF signaling pathway	Lopez-Ramirez MA, 2014Gordon *et al.*, 2010 Zhou *et al.*, 2012

Table 3

Stroke-Associated Cerebrovascular Endothelial Cell ncRNAs

NcRNAs	Functions	Targets	Cell types	References
miR-15a	Increases in ischemic brain vasculature. Gain- or loss-of-miR-15a function reduces or increases EC viability, tube formation, migration, and differentiation, respectively. EC-miR-15a transgenic overexpression leads to reduced blood vessel formation and local blood flow perfusion after hindlimb ischemia.	Bcl-2 FGF2, VEGF	CECs HUVECs	Yin et al., 2010b Yin *et al.*, 2012
miR-100 *et al.*, miR-139 *et al.*,	Nine miRNAs (miR-100, -101a, -204, - 210, m-224, -322, -322*, -331, -450a) are significantly upregulated in BMECs after stroke, whereas seven miRNAs (miR- 139-5p, -193*, -203, -225, -335, -494, -708) downregulated.	Unknown	BMECs	Teng *et al.*,2012
miR-210	Upregulates in rat ischemic brain cortexes. Overexpression increases Notch1 expression and induces ECs to migrate and form capillary-like structures on matrigel. Induces angiogenesis and neurogenesis in normal brain tissues.	Notch1 VEGF	HUVECs CECs Neurons Astrocytes	Lou *et al.*, 2012 Zeng *et al.*, 2013
miR-320	Downregulates in all young stroke patients with good outcome.	Unknown	Blood	Tan *et al.*, 2009