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Altered Long Non-coding RNA Transcriptomic Profiles in Brain Microvascular Endothelium after Cerebral Ischemia

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Abstract

The brain endothelium is an important therapeutic target for the inhibition of cerebrovascular dysfunction in ischemic stroke. Previously, we documented the important regulatory roles of microRNAs in the cerebral vasculature, in particular the cerebral vascular endothelium. However, the functional significance and molecular mechanisms of other classes of non-coding RNAs in the regulation of cerebrovascular endothelial pathophysiology after stroke are completely unknown.

Using RNA sequencing (RNA-seq) technology, we profiled long non-coding RNA (lncRNA) expressional signatures in primary brain microvascular endothelial cells (BMECs) after oxygen-glucose deprivation (OGD), an *in vitro* mimic of ischemic stroke conditions. After 16h of OGD exposure, the expression levels for 362 of the 10,677 lncRNAs analyzed changed significantly, including a total of 147 lncRNAs increased and 70 lncRNAs decreased by more than 2-fold. Among them, the most highly upregulated lncRNAs include Snhg12, Malat1, and lnc-OGD 1006, whereas the most highly downregulated lncRNAs include 281008D09Rik, Peg13, and lnc-OGD

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3916. Alteration of the most highly upregulated/downregulated ODG-responsive lncRNAs was further confirmed in cultured BMECs after OGD as well as isolated cerebral microvessels in mice following transient middle cerebral artery occlusion (MCAO) and 24h reperfusion by the quantitative real-time PCR approach. Moreover, promoter analysis of altered ODG-responsive endothelial lncRNA genes by bioinformatics showed substantial transcription factor binding sites on lncRNAs, implying potential transcriptional regulation of those lncRNAs. These findings are the first to identify OGD-responsive brain endothelial lncRNAs, which suggest potential pathological roles for these lncRNAs in mediating endothelial responses to ischemic stimuli. Endothelial-selective lncRNAs may function as a class of novel master regulators in cerebrovascular endothelial pathologies after ischemic stroke.

Keywords

RNA sequencing; long non-coding RNAs; transcriptome; brain microvascular endothelial cells; oxygen-glucose deprivation; ischemic stroke

INTRODUCTION

Stroke is one of the severest health issues in the United States (Moskowitz et al., 2010). Currently, thrombolytic therapy is not enough for the acute intervention of ischemic stroke and only small percentage of patients can receive it because of its narrow therapeutic time window (Schellinger et al., 2004; Stapf and Mohr, 2002). Thus, development of effective therapies is urgently required. During the past two decades, extensive studies showing the effectiveness of neuroprotectants in animal stroke models, but not in stroke clinical trials, imply that solely focusing on neuroprotection is not sufficient (Ginsberg, 2009; Iadecola and Anrather, 2011). Thus, greater attention has been paid to the local environment of the surviving neuron, such as the cerebral microvasculature, neurovascular units, and vascular neural network (Barone, 2009; del Zoppo, 2006; Ginsberg, 2009; Lok et al., 2007; Yin et al., 2010a; Yin et al., 2011; Yuan, 2009; Zhang et al., 2012). As a major structural and functional element of the brain microvasculature, the vascular endothelium plays a dominant role in maintaining its normal physiological functions. 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, contributing to ischemic brain injury (del Zoppo and Hallenbeck, 2000; Ishikawa et al., 2004; Sandoval and Witt, 2008). Thus, it is important to identify the insightful mechanisms by which cerebrovascular disorders can be effectively inhibited or reduced through protection of the brain endothelium under ischemic stroke conditions (Fagan et al., 2004; Fisher, 2008; Rodriguez-Yanez et al., 2006). Understanding the critical mediators in regulating cerebrovascular endothelial dysfunction may eventually lead us to discover novel targets for the treatment of stroke.

Recently, accumulating studies have shown that non-coding RNAs (ncRNAs) are functional RNA molecules that are generally not translated into proteins but can actively regulate the expression and function of protein-coding genes by different mechanisms. 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 (Derrien et al., 2012; Qureshi and Mehler, 2012). In addition to well-known ncRNAs such as rRNAs and tRNAs, ncRNAs can be broadly classified as small (<200 nt) and long (>200 nt) ncRNAs (lncRNAs) (Bartel, 2004; Kim, 2005; Qureshi and Mehler, 2012; Schonrock et al., 2012; Taft et al., 2010; Vemuganti, 2013; Yin et al., 2014). NcRNAs have become a focus in biomedical research in the last half decade and are now regarded as important and essential mediators in major biological/physiological processes impacting development, differentiation, and metabolism, as well as pathologies in a variety of human diseases (Qureshi and Mehler, 2012; Schonrock et al., 2012; Suarez and Sessa, 2009; Taft et al., 2010; Vemuganti, 2013; Wienholds and Plasterk, 2005; Yin et al., 2014). So far in the stroke field, the role of a specific class of small ncRNAs, miRNAs, was mainly investigated in the pathogenesis of stroke (Eacker et al., 2013; Liu et al., 2013; Ouyang et al., 2013; Rink and Khanna, 2010; Saugstad, 2010; Tan et al., 2011; Vemuganti, 2010; Yin et al., 2013b, 2014). Notably, our research team has focused on studying the functional significance of stroke-associated endothelial ncRNAs with a special focus on miRNAs in cerebral endothelial pathophysiology after stroke (Yin et al., 2010a; Yin et al., 2013a; Yin et al., 2013b, 2014; Yin et al., 2012).

Non-coding transcripts >200 nucleotides up to ~100kb are defined as lncRNAs (Mattick, 2009; Mercer et al., 2009; Ponting et al., 2009; Vemuganti, 2013; Yin et al., 2014). Recently, whole transcriptome sequencing (e.g. RNA-seq) of human and other genomes has revealed significant numbers of lncRNAs which are long transcripts similar to mRNAs but lack protein coding (translation) potential (Derrien et al., 2012). LncRNAs can control gene expression by various mechanisms that include recruitment of histone modification complexes, modulating transcription factor activity and splicing machinery, increasing mRNA stability, and acting as transcription enhancers. LncRNAs function in various biological processes including gene transcription, cell differentiation, imprinting, immune responses, and stem cell pluripotency, and have cell-specific expression patterns that respond to external stimuli (Mattick, 2009; Mercer et al., 2009; Ponting et al., 2009). Involvement of lncRNA in human diseases, including stroke, have begun to appear and are expected to escalate. For example, recent microarray profiling studies identifying stroke-responsive alterations of lncRNAs (Dharap et al., 2012; Dharap et al., 2013; Vemuganti, 2013) underscore the importance of lncRNAs in the pathogenesis of ischemic stroke. However, the essential role and molecular mechanisms of lncRNA in ischemic stroke still remain poorly understood. In particular, nothing is known to date about the functional significance of lncRNAs in cerebral vascular endothelial biology and pathophysiology in ischemic stroke.

In this article, we utilized RNA-seq technology to transcriptome profiling lncRNAs in brain microvascular endothelial cells (BMECs) after exposure to Oxygen-Glucose Deprivation (OGD), an *in vitro* mimic of stroke conditions. For the first time, we have identified substantial OGD-responsive lncRNAs in BMECs, and further confirmed the altered pathological lncRNA expression in the cerebral vascular endothelium in a mouse ischemic stroke model. Moreover, we also found a number of conserved transcription factor binding sites in the promoter region of these OGD-responsive endothelial lncRNAs. Therefore, our

studies could uncover lncRNAs associated with brain endothelial dysfunction in ischemic stroke, potentially advancing the field by linking lncRNA biology to stroke-induced cerebral vasculature pathologies.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO), while cell culture supplies were purchased from Invitrogen Corporation (Carlsbad, CA) unless specified.

BMEC cultures and oxygen-glucose deprivation (OGD)

Mouse BMECs were prepared as previously described (Yin et al., 2002b). Briefly, mouse cerebral cortex from adult male C57BL/6J mice (male, body weight 25-30g, 3-4 months-old) (Jackson Laboratory, Bar Harbor, ME) was homogenized, filtered and sequentially digested with collagenase B, then collagenase/dispase (Roche Molecular Biochemicals, Indianapolis, IN), followed by centrifugation in 40% Percoll solution. The second band containing microvessels was collected and plated onto collagen-coated dishes. Mouse BMECs (1-5 passages, > 95% purity based on expression of factor VIII and exhibiting bradykinin receptor function) were grown to 85-95% confluency before use (Yin et al., 2002b).

To mimic ischemic-like conditions *in vitro*, cell cultures were exposed to OGD for a fixed time (Yin et al., 2002a). Briefly, mouse BMEC cultures were transferred into a temperature controlled (37±1°C) anaerobic chamber (Forma Scientific, Marietta, OH) containing a gas mixture composed of 5% CO_2 and 95% N_2. The culture medium was replaced with deoxygenated glucose-free Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, CA) and cells were maintained in the hypoxic chamber for 16 h. Control mouse BMECs were not exposed to OGD.

RNA sequencing and Bioinformatics analysis

RNA was extracted from cultured BMECs by using a miRNeasy Mini Kit (Qiagen, Valencia, CA). Eluted RNA was prepared for sequencing using Illumina protocols, and then sequenced on the HiSeq 2000 (Illumina) to generate 50 bp paired-end reads. We obtained about 41.4~59.1M reads for each sample (four independent biological replicates per group). The reads were aligned to the mouse genome (from UCSC Genome Browser) using Bowtie2 (2.1.0) (Langmead et al., 2009) with Tophat (2.0.8b) (Trapnell et al., 2009). We used Cufflinks to first assemble transcripts from all datasets using the NCBI Reference Sequence (RefSeq) mouse gene annotation as a reference guide. All mouse XenoRefSeq annotations that overlapped with the unannotated transcripts were identified. Nonoverlapping transcripts were assessed for protein-coding potential using PhyloCSF (Lin et al., 2011). Using a threshold previously used to identify non-coding RNAs, all multi-exonic transcripts with a PhyloCSF score >20 were called novel protein-coding transcripts and <20 as lncRNAs (Leung et al., 2013; Lin et al., 2011; Pauli et al., 2012). FPKM values were calculated for expression levels of each transcript. To assess the biological reproducibility between the replicates for each condition, Pearson's Correlation value was also determined for each pair

of replicates. Cuffdiff (Trapnell et al., 2010) was used to identify differentially expressed transcripts in two conditions with the reference annotation containing mouse RefSeq genes and lncRNAs with an FPKM >0.5.

Hierarchical cluster analysis was performed on normalized array values with Cluster 3.0 with Euclidean distance and single linkage for >10,000 genes and average linkage for smaller gene sets. Heatmaps were generated with Java TreeView. Gene ontology (GO) analysis was completed using GO Elite with default settings.

To assess the significance of the proximity of co-regulated lncRNAs and protein-coding genes, we carried out 100,000 simulations of proximity measurements on randomly placed co-regulated lncRNAs and protein-coding genes in the mouse genome (Leung et al., 2013; Lin et al., 2011; Pauli et al., 2012). We analyzed 4,301 coding genes and 362 lncRNAs to be co-regulated and then counted the number of simulated OGD-responsive lncRNAs within 500kb of a simulated OGD-responsive gene.

Promoter analysis of transcription factor binding sites

As transcription factors are crucial in the process of transcription of non-coding RNAs, we checked and analyzed the promoter region of OGD-responsive lncRNA genes for the identification of conserved transcription factor binding sites. For each lncRNA, a 2-kb promoter sequence upstream to the transcription start site was analyzed for conserved TF binding sites by using the Genomatix Software. All TF matrices with a Z-score of >2 (representing P>0.05) were considered statistically significant (Dharap et al., 2011).

Mouse model of transient focal cerebral ischemia

Male C57BL/6J mice (male, 8- to 10-weeks-old) were purchased from Jackson Laboratory. Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO) using a nylon monofilament suture as described previously (Yin et al., 2010a; Yin et al., 2011; Yin et al., 2010b; Yin et al., 2013a). Briefly, mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg). After a midline skin incision, the left common carotid artery was exposed and then its branches were electrocoagulated. A 2-cm length of 6-0 rounded tip nylon suture was gently advanced from the external carotid artery up to the internal carotid artery until regional cerebral blood flow (rCBF) was reduced to less than 16% of baseline. After 60 minutes of proximal MCA occlusion, blood flow was restored by removing the suture. Changes in cerebral blood flow (CBF) at the surface of the cortex were recorded using a laser-Doppler flowmetry monitor (BPM2 System, Vasamedic, St. Paul, MN). Sham control animals were subjected to similar operations to expose the carotid arteries without occlusion of the middle cerebral artery. After 60 minutes of MCAO, the mice were allowed to recover for 24 hours. Arterial blood gases, mean arterial pressure, and heart rate were also monitored in selected animals 30 min before, during, and 30 min after MCAO. The rectal temperature was controlled at 37.0 ± 0.5°C during surgery with a feedback-regulated heating pad (Harvard, Holliston, MA). After the ischemic insult, mice were kept in an air-ventilated incubator at 24.0 ± 0.5°C. The animals were sacrificed at 24 h of reperfusion, and the brains were quickly removed for biochemical assays as well as

infarct determination. All procedures using laboratory animals were approved by the University of Michigan Animal Care and Use Committee.

Cerebral microvessel isolation

Cerebral microvessel isolation employed previously described methods with modifications (Pardridge et al., 1985; Zlokovic et al., 1993; (Yin et al., 2010a; Yin et al., 2006b). Briefly, mice were sacrificed by decapitation under anesthesia. The brains were immediately removed from the skull and immersed in ice-cold buffer A (103 mM NaCl, 4.7mM KC1, 2.5 mM $CaC1_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM Hepes, pH 7.4). The brain was homogenized in a 5-fold volume excess of buffer B (103 mM NaCl, 4.7mM KC1, 2.5 mM $CaC1_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM Hepes, 25 mM HCO_3, 10 mM glucose, 1mM sodium pyruvate, and 1 g/100 ml dextran pH 7.4) with a Teflon homogenizer. The homogenate was suspended in an equal volume of 25% BSA and was centrifuged at 5,800 × g at 4°C for 30 min. The pellet was resuspended in 10 ml of buffer B and passed over an 85-μm nylon mesh (Tetko, Depu, NY). This filtrate was then passed over a 3 × 4-cm glass bead column (0.45-mm glass beads) with a 44-μm nylon mesh at the bottom, and then washed with 400 ml of buffer B. Microvessels adhered to the glass beads while contaminants passed unimpeded. Microvessels were recovered by repeated gentle agitation of the glass beads in buffer B. The supernatant with microvessels was decanted and spun at 500 × g for 5 min, and the final pellet was stored at −80°C until various biochemical assays were performed.

Quantitative real-time PCR

Total RNA was isolated from BMEC cultures or isolated cerebral microvessels by using a miRNeasy Mini Kit (Qiagen, Valencia, CA) or Trizol (Invitrogen). A quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out with a Bio-Rad thermocycler and an SYBR green kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. Specific lncRNA primers used for the reaction are listed in Supplemental Table 1. The relative lncRNA expression was normalized by 18S RNA levels. The PCR experiments were repeated 3 times, each using separate sets of cultures (Yin et al., 2006a; Yin et al., 2006b).

Immunofluorescence staining

Mouse BMECs grown on coverslips were then fixed with 4% paraformaldehyde for 30 min and washed 3 times with 0.1 M PBS (pH 7.4). The cells were incubated with a primary rat anti-CD31 antibody (1:50; BD Bioscience), mouse anti-Claudin 5 (1:50; Invitrogen), or mouse anti-Occludin antibody (1:50; Invitrogen) overnight at 4°C. On the following day, the cells were incubated with fluorescein-conjugated anti-rat or anti-mouse IgG (1:100; Vector Labs, Burlingame, CA) for 1 h. BMECs were counterstained with 1 μg/ml of DAPI (Molecular Probes; Eugene, OR) to visualize nuclear morphology. Slides were washed, wet mounted and examined with an Olympus fluorescence microscope (Yin et al., 2006a; Yin et al., 2002b).

Quantitative and Statistical analysis

Quantitative data are expressed as mean ± SD or SEM based on at least three independent experiments of triplicate samples. Differences among groups were statistically analyzed by oneway analysis of variance followed by Bonferroni's post-hoc test. Comparison between two experimental groups was based on a two-tailed t-test. A p-value less than 0.05 was considered significant.

RESULTS

RNA-seq identification of transcriptome profiling in mouse BMECs after OGD

To determine the transcriptomic effects of OGD, an *in vitro* mimic of ischemic stroke conditions, on the cerebral vascular endothelium, we treated mouse BMECs (Fig. 1) with OGD for 16 hours and then performed RNA sequencing (Fig. 2) of RNA isolated from non-OGD control and OGD-treated cells. RNA sequencing reads were generated and mapped to the mouse genome reference. A total of 79.68% of filtered reads could be aligned to the mouse reference genome. Four independent biological replicates were performed for each treatment and the transcript expression level (FPKM) was calculated as described in the Methods.

We discovered 55,832 transcripts using this approach, and further divided them into protein-coding or non-coding by assessing their protein-coding potential. We classified a total of 10,677 transcripts with limited protein-coding potential as lncRNAs and 43,705 as protein-coding transcripts (Table 1). Approximately 5,241 lncRNA transcripts that did not overlap with the known mouse genome were classified as novel (non-annotated), and named as lnc-OGD in numerical order (lnc-OGD 1-5,241) compared to 5,436 annotated lncRNAs (Table 1). Further bioinformatics analysis using Cuffdiff discovered 4,663 significantly differentially expressed transcripts (FDR<0.05), including 362 annotated or unannotated lncRNAs and 4,301 coding genes (Table 1). Hierarchical clustering showed systematic variations in the expression of lncRNAs and protein-coding RNAs in mouse BMECs after ischemic stimuli (Fig. 3). Among the 362 significantly changed lncRNAs analyzed, a total of 147 lncRNAs increased and 70 lncRNAs decreased by more than 2-fold in mouse BMECs after 16h of OGD exposure, and were classified as OGD-responsive endothelial lncRNAs (Table 1). The most highly-upregulated annotated lncRNAs are Snhg12, Malat1, and Srsf3, whereas the most highly upregulated non-annotated lncRNAs are lnc-OGD 1645, lnc-OGD 2327, and lnc-OGD 1006 (Table 2). Accordingly, the most highly downregulated annotated lncRNAs include 281008D09Rik, Peg13, and Pisd-ps1, whereas the most highly downregulated non-annotated lncRNAs include lnc-OGD 3916, lnc-OGD 2838, and lnc-OGD 301 (Table 3).

Interestingly, we also found that a total of 992 protein-coding RNAs increased and 748 decreased by more than 2-fold in mouse BMECs after the same ischemic exposure (Table 1). Within the listed differentially expressed genes (Supplementary Table 2-3), GO analysis results were determined and KEGG pathway analysis (Supplementary Fig.1) revealed substantial enrichment-related pathways, including cell degradation, cell adhesion, cell cycle, p53, MAPK, VEGF, and other significant signaling pathways in this profile, many of

which are associated closely with cell death, abnormal cell metabolism, ER stress, oxidative stress, inflammation, or cerebral endothelial pathologies under ischemic stroke.

It has been reported that lncRNAs may prefer to co-regulate with neighboring genes and exert enhancer-like activities (Cabili et al., 2011; Leung et al., 2013; Orom et al., 2010a; Orom et al., 2010b). In order to determine the significance of the proximity of co-regulated OGD-responsive endothelial lncRNAs and protein-coding genes, we carried out 100,000 simulations of proximity measurement on randomly placed co-regulated lncRNAs and protein-coding genes in the mouse genome. In particular, we focused on 4,301 significantly changed genes and the most highly altered lncRNAs (Table 2-3) to be co-regulated. We counted the number of simulated OGD-responsive lncRNAs that were within 500kb of a simulated OGD-responsive gene. We found that most highly upregulated or downregulated lncRNAs are proximal (< 500kb) to differentially expressed OGD-responsive genes (Supplementary Table 4-5). Further functional association or regulation between OGD-responsive lncRNAs and their neighboring/adjacent protein-coding genes needs to be further verified in the future.

QPCR verification of RNA-seq identified OGD-responsive brain endothelial lncRNAs

In order to validate the expression profiles generated from RNA-seq, we performed quantitative real-time PCR to identify the correlation between the two datasets. As shown in Figure 4, the most highly upregulated or downregulated brain endothelial lncRNAs found in RNA-seq profiling experiments were further verified and showed consistent alteration tendencies in our qPCR study. These findings are the first to identify OGD-responsive brain endothelial lncRNAs, which suggest potential functional roles for these lncRNAs in mediating vascular endothelial responses to ischemic stimuli.

The expression of OGD-responsive endothelial lncRNAs in cerebral microvasculature in mice after transient focal cerebral ischemia

Extending our *in vitro* observations into *in vivo* studies, we further examined if OGD-responsive endothelial lncRNAs (upregulated and downregulated) have similar pathological expression patterns in mouse cerebral vasculature after ischemic stroke. To quantitatively determine expression of OGD-responsible lncRNAs in the cerebral vasculature, whole microvessel isolates from mouse brains after MCAO (n=6) were prepared by vessel-enriched isolation as described in our previous publications (Yin et al., 2010a; Yin et al., 2006b). Total RNA was isolated and subjected to quantitative PCR for the detection of lncRNA levels. As demonstrated in Figure 5, microvessels were isolated from mouse brains and qPCR analysis was characterized for high expression of brain EC markers ZO-1, claudin-5, CD31, and vWF with much lower levels of the astrocyte marker, GFAP and pericyte marker, platelet-derived growth factor receptor-β, indicating a relative high EC abundance (Fig. 5A). Of note, three most highly upregulated (Snhg12, Malat1, lnc-OGD1006) or downregulated (281008D09Rik, Peg13, and lnc-OGD3916) OGD-responsive endothelial lncRNA transcriptswere also significantly increased or reduced in isolated mouse cerebral microvessels in response to ischemic stroke (Fig. 5B), respectively. These data suggest that the expression of OGD-responsive endothelial lncRNAs will also be significantly altered in the cerebral vascular endothelium after *in vivo* ischemic insults,

suggesting that these OGD-responsive lncRNA transcripts are associated with cerebrovascular pathogenesis after ischemic stroke, and warrant further investigation.

Identification of transcription factors in OGD-responsive brain endothelial lncRNAs

The function of non-coding RNAs appears to be under the control of many transcription factors (TFs) (Bethke et al., 2009; Schanen and Li, 2010; Yin et al., 2010a). We analyzed a total of 128 transcription factors with FPKM>1 in all conditions of our RNA-seq data. To explore the possibility that TFs regulate OGD-responsive lncRNA expression in a transcriptional manner, we employed Genomatix software to screen for TF binding sites in the promoter region of 362 OGD-responsive endothelial lncRNAs (Table 4). Our bioinformatics analysis has identified that Sp1 and Krüppel-like transcription factors (KLFs) are the two highest overrepresented TFs in the promoter region of OGD-responsive lncRNA genes, whereas LOC100861682 and 2610019E17Rik have the highest number of TFs bound in their promoters (Table 4). These first-step results suggest that TFs may specifically affect the activity of OGD-responsive endothelial lncRNAs under ischemic stimuli.

DISCUSSION

The major finding of our study is the identification of OGD-responsive brain endothelial lncRNAs by a state-of-the-art RNA-seq profiling approach. For the first time, our data have shown that brain vascular endothelial cells respond to ischemic stimuli by dramatically altering hundreds of lncRNA transcriptomic profiles, suggesting potential and uncovered functional roles for these differentially expressed lncRNAs in the brain vascular pathogenesis of ischemic stroke.

During the past few years, our understanding of the complex aspects of vascular endothelial biology dramatically increased by the discovery of ncRNAs (Bartel, 2004; Kim, 2005; Qureshi and Mehler, 2012; Schonrock et al., 2012; Taft et al., 2010; Vemuganti, 2013; Yin et al., 2014). In that context, ncRNAs such as miRNAs have been shown to play crucial roles in the control of endothelial cell biology, including cerebral endothelial biology (Yin et al., 2013b, 2014). Except for endothelial miRNAs, only a few reports have demonstrated the participation of specific lncRNAs in the regulation of endothelial biology and function during physiological and disease conditions (Gordon et al., 2010; Li et al., 2010; Yuan et al., 2012). A recent study has shown that genetic inactivation of Meg3 in mice leads to a significant increase in the expression of pro-angiogenic genes that promote new microvessel formation in the brain cortex (Gordon et al., 2010; Li et al., 2010; Yuan et al., 2012). Up to now, the expression and function of lncRNAs in cerebral vascular endothelial biology and stroke-induced cerebral vascular pathologies are totally unknown (Yin et al., 2014).

In this study, we employed RNA-seq approaches to provide definitive evidence that cerebral vascular endothelial cells express high levels of lncRNA transcripts. In particular, we identified a total of 362 annotated and unannotated OGD-responsive endothelial lncRNAs in cultured BMECs, including 147 OGD-upregulated and 70 OGD-downregulated lncRNA transcripts. These most highly altered expression profiles of cerebral endothelial lncRNAs were further confirmed by our quantitative PCR analysis in cultured BMECs after *in vitro* stimuli. Importantly, the expression levels of the most highly upregulated lncRNAs

(Snhg12, Malat1, and lnc-OGD 1006) as well as the most highly downregulated lncRNAs (281008D09Rik, Peg13, and lnc-OGD 3916) have also been found to increase or decrease respectively in cerebral microvasculature after *in vivo* ischemic stimuli (mouse ischemic stroke model). These findings strongly suggest that OGD-responsive brain endothelial lncRNAs may have functional roles in mediating endothelial responses to ischemic stimuli, and may serve as endogenous modulators to regulate endothelial cell biology and also pathophysiology after cerebral ischemia. Further studies are needed in the future to show the significance of individual lncRNA molecules in the regulation of post-stroke cerebrovascular pathophysiology and neurological outcomes. Also, the molecular/transcriptional regulation of selective lncRNAs in cerebral vascular endothelium was warranted for further investigation. These results will provide novel insights into a better understanding of lncRNA-based molecular regulatory mechanisms in the control of impaired cerebral vascular endothelial structure and function after cerebral ischemia.

LncRNAs are known to recruit chromatin-modifying proteins such as Polycomb Repressive Complex 2 (PRC2) to specific sites of genome and affect gene expression through regulating chromatin states (Rinn and Chang, 2012). Human lncRNA HOTAIR is the first such RNAs recognized: HOTAIR physically associates with PRC2, and modulates PRC2 and H3K27me3 localization of hundreds of sites throughout the genome (Rinn et al., 2007; Tsai et al., 2010). Also, a number of studies indicated that many TFs are able to transactivate or transrepress the transcription of lncRNAs and thus affect their functions (Bethke et al., 2009; Huang et al., 2015; Schanen and Li, 2010; Yin et al., 2010a). For example lincRNA-p21 expression can be activated by p53 through physical interaction with p53, lincRNA-p21 then serves as a transcriptional repressor in the p53 pathway and plays a role in triggering apoptosis (Huarte et al., 2010). In another study, lncRNA TUG1 expression was found induced by nuclear transcription factor SP1 and TUG1 further repressed Krüppel-like factor 2 transcription in the epigenetic level (Xu et al., 2015).

Consistent with the previous studies, our bioinformatics analysis has shown that there are a substantial number of TF binding sites in the promoter regions of the 362 OGD-responsive lncRNAs, including Krüppel-like transcription factors (KLFs) and Sp1 as the two highest overrepresented TFs in the promoter regions of most OGD-responsive lncRNA genes. These data imply the possibility that TFs may regulate OGD-responsive lncRNA expression in a transcriptional manner. In order to further validate TF transcriptional regulation of OGD-responsive lncRNAs, a series of further experiments, including luciferase transcription activity assay, chromatin immunoprecipitation (ChIP) analysis, qPCR, and Western blotting, will need to be performed in the future as described in our previous publications (Yin et al., 2010a; Yin et al., 2013a).

In summary, this study is the first to profile lncRNA regulatory transcripts in cerebral vascular endothelium under ischemic conditions by using high-throughput deep sequencing (RNA-seq) technology. The functional importance and molecular regulatory mechanisms of lncRNAs in the cerebral vasculature, especially in the endothelium following ischemic stroke, are totally unknown. Identification of new lncRNA transcripts will provide a novel window of opportunity to study RNA-directed epigenetic regulators in cerebral endothelial biology and their essential role in stroke-induced vascular endothelium-dependent

cerebrovascular pathologies, as well as likely reveal novel targets for a promising translational future of lncRNA-based diagnostics and therapeutics in ischemic stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

➢ A first study to profile ischemic-responsive brain endothelial long non-coding RNAs.

➢ Total 217 lncRNAs were classified as ischemic-responsive endothelial lncRNAs.

➢ The most highly altered lncRNAs were confirmed in brain microvessels after stroke.

➢ Identified key transcription factors that regulate ischemic-responsive lncRNAs.

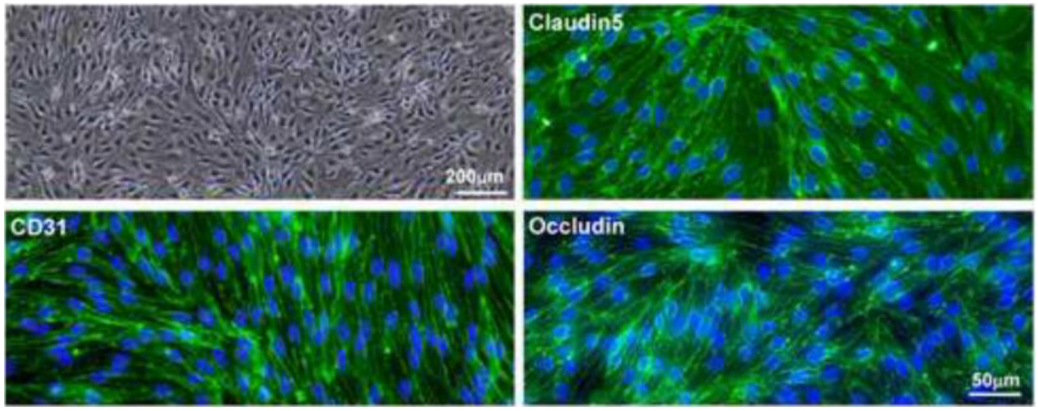

Figure 1.

Brain microvascular endothelial cell cultures. Phase-contrast light microscopy shows primary mouse BMEC cultures. Immunofluorescent stain using antibody against brain vascular endothelial cell marker, CD31 and BBB markers, Claudin 5 and Occludin reveals typical circumcellular signals of CD31, Claudin 5, and Occludin. Scale bars are indicated.

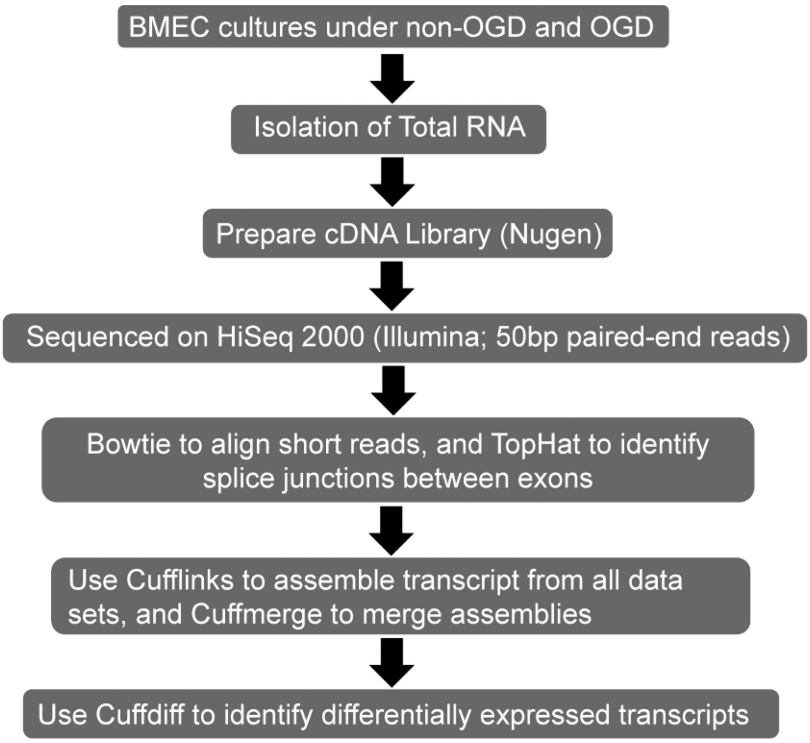

Figure 2.
Schematic representation of RNA-seq experiments and bioinformatics analysis.

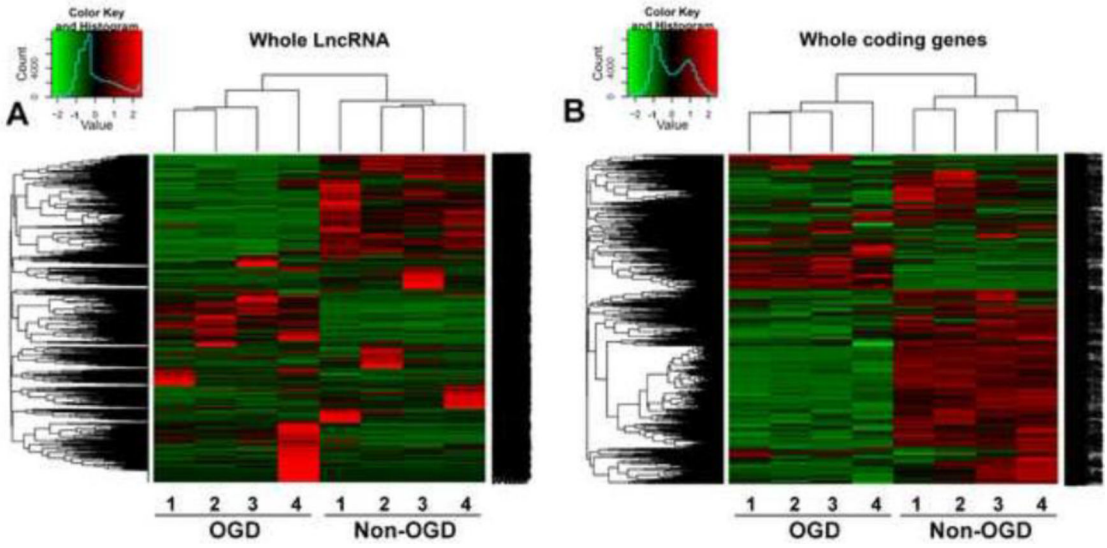

Figure 3.

Altered lncRNA and mRNA profiles in mouse BMECs after OGD. Heatmap and hierarchical clustering analysis of 10,677 lncRNAs (A) and 43,705 protein-coding RNAs (B) that were differentially expressed ($P<0.045$ and $FDR<0.05$) in mouse BMECs 16 hours after OGD exposure. Four independent biological replicates were analyzed for non-OGD and OGD conditions. The clustering tree for lncRNAs and protein-coding RNAs is shown at the top. The expression values are represented in shades of red and green, indicating expression above and below the median value across all samples (log scale 2, from −2.00 to +2.00), respectively.

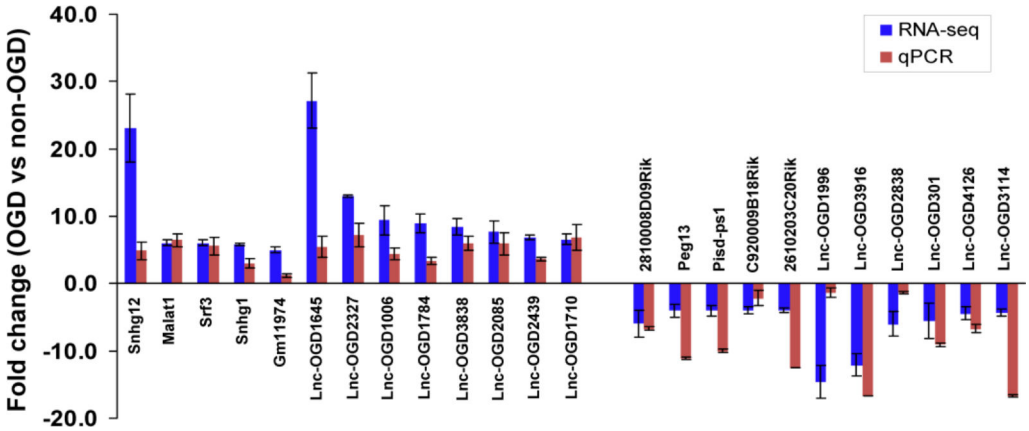

Figure 4.

PCR verification of expression profiles of lncRNA in mouse BMECs after OGD. The relative expression levels of most highly changed lncRNAs as indicated were examined using qRT-PCR in mouse BMECs that were treated with OGD for 16 hours. Data from RNA-seq were well-matched to quantitative PCR results. Data are expressed as mean ± SEM from 3 separate experiments.

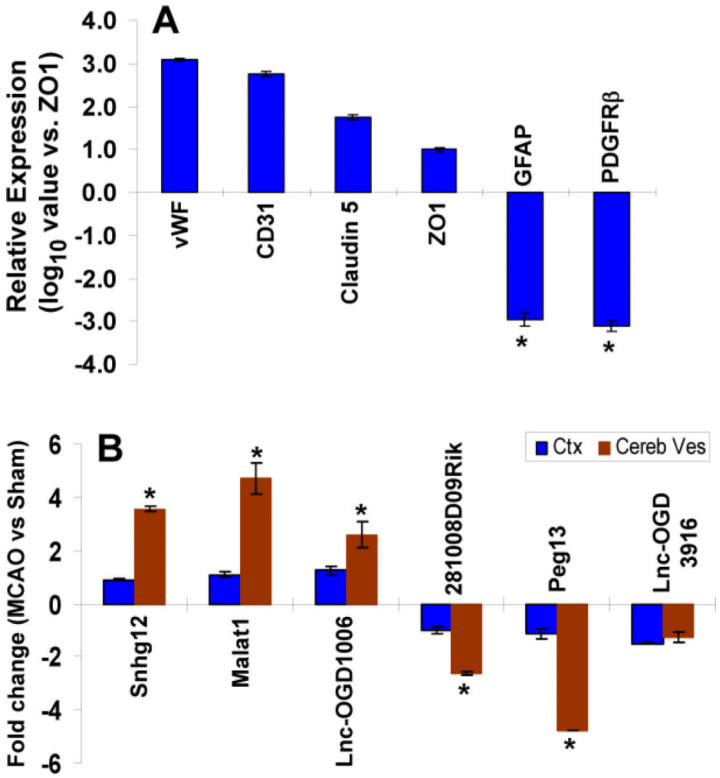

Figure 5.

Altered lncRNA expression profiles in cerebral microvasculature and brain in mice after focal cerebral ischemia. (A) Brain microvessels were isolated from mouse cerebral cortex and the expression of multiple neurovascular cell markers was analyzed by qPCR. Characterized high expression of brain EC markers, ZO-1, claudin-5, CD31, and vWF with much lower levels of the astrocyte marker, GFAP and pericyte marker, platelet-derived growth factor receptor-β, indicates a relative high EC abundance. (B) QPCR data demonstrated that three most highly upregulated and downregulated OGD-responsive lncRNAs as indicated are also significantly increased and reduced respectively in cerebral microvessels (Cereb Ves) but not in brain parenchyma (cerebral cortex, Ctx) in mice after 1h MCAO followed by 24h reperfusion. Data are expressed as mean + SEM. *$p<0.05$ vs. Sham or ZO-1 group.

Table 1

Overview of RNA-seq results In mouse BMECs after OGD

Transcriptome	Classification	Number	Access ID
ncRNAs	Total	12,127	
LncRNAs	Total	10,677	
	Annotated	5,436	NR_ or XR_
	Non-annotated	5,241	Lnc-OGD
	Significant change	362	
	>2 fold increase	147	
	<2 fold decrease	70	
mRNAs	Total	43,705	NM_ or XM_
	Significant change	4,301	
	>2 fold increase	992	
	<2 fold decrease	748	

Total 55.832 transcriptome isoform expression in mouse primary BMEC culture

Table 2

Most highly upregulated lncRNAs in BMECs after OGD

LncRNAs	Ref. ID	Transcript length	Fold change	Chromosome	Start site	End site	p value
Lnc-OGD1645	-	268	27.13	5	113592196	113592464	5.00E-05
Snhg12	NR_029468.1	606	23.06	4	132308677	132311C33	5.00E-05
Gm19660	XR_104882.2	636	21.40	4	119108744	119139596	5.00E-06
Lnc-OGD2327	-	237	12.93	7	126644602	126644839	0.0022
LOC100861877	XR_140599.1	554	12.05	3	65527482	65560603	5.00E-05
Lnc-OGD1006	-	381	9.39	3	149326946	149327327	5.00E-05
Lnc-OGD1784	-	367	9.00	6	28410358	26410725	5.00E-06
Lnc-OGD3838	-	665	8.43	13	5860536	5851201	5.00E-05
Lnc-OGD2085	-	445	7.66	7	25076565	25077010	5.00E-05
Lnc-OGD2439	-	259	6.87	8	54441066	54441325	5.00E-05
Gm3601	XR_105655.1	219	6.63	14	49971362	49989708	5.00E-05
Lnc-OGD1710	-	305	6.55	5	140403591	140403B95	5.00E-05
Lnc-OGD3723	-	254	6.19	12	74803907	74804506	0.00825
Malat1	NR_002847.2	6424	6.05	19	5795687	5802950	5.00E-05
Srsf3	NR_036613.1	3041	6.05	17	29032659	29043735	5.00E-05
LOC100861991	XR_141282.1	535	5.88	15	80255183	80265023	5.00E-05
Snhg1	NR_002896.3	476	5.86	19	8723486	8726445	5.00E-05
Lnc-OGD5236	-	1873	5.68	Y	90731450	90736460	5.00E-05
Lnc-OGD3641	-	265	5.58	13	9834264	9834529	5.00E-05
Gm13187	XR_104650.1	866	5.46	2	4152862	4614043	0.0041
Lnc-OGD3413	-	1160	5.34	11	67455361	67689220	0.00035
Gm13351	XR_140493.1	1957	5.31	2	18055236	18392830	5.00E-05
Lnc-OGD456	-	476	5.25	2	98664878	98665371	5.00E-05
Lnc-OGD2596	-	1689	5.24	9	13414327	13416072	0.00555
Gm11974	NR_045893.1	592	5.01	11	6525590	6528805	5.00E-05
1810026B05Rik	NR_037569.1	709	5.00	7	73426454	73558615	0.0017
LOC100861894	XR_140466.1	494	4.96	1	143999337	144016631	5.00E-05
Gm20367	XR_105161.1	315	4.67	7	126623245	126635195	5.00E-05
2410006H16Rik	NR_030738.1	461	4.39	11	62602876	62604806	5.00E-05
Gm16702	NR_045795.1	2384	4.35	17	8372395	8422728	5.00E-05
Lnc-OGD3606	-	477	4.38	11	109011641	109012118	5.00E-06
4930470H14Rik	NR_045764.1	1734	4.12	17	4044657	40B2995	5.00E-05
Lnc-OGD4481	-	204	4.10	16	17222307	17222511	5.00E-05
Lnc-OGD2556	-	1703	4.09	8	125418062	125569817	5.00E-05
4930405P13Rik	NR_045955.1	811	4.03	11	88905927	88955442	0.01145

Table 3

Most highly downregulated IncRNAs in BMECs after OGD

LncRNAs	Ref. ID	Transcript length	Fold change	Chromosome	Start site	End site	p value
LHC-OGD1996	-	408	0.07	6	128843185	128843593	5.00E-05
Lnc-OGD3916	-	988	0.08	13	34875479	34906067	0.0011
Lnc-OGD2838	-	280	0.17	13	3198305	3198585	5.00E-05
2810008D09Rik	NR_027059.1	600	0.17	11	117076719	117079086	5.00E-05
LHC-OGD301	-	660	0.18	2	13011326	13014685	5.00E-05
Gm19767	XR_105707.2	1138	0.21	15	99029890	99038110	5.00E-05
LOC100882008	XR_140736.1	399	0.22	5	110135761	110176504	5.00E-05
Lnc-OGD4126	-	1184	0.23	14	54431485	54443711	0.0012
Lnc-OGD3114	-	1938	0.23	10	61428297	61430235	5.00E-05
Lnc-OGD124	-	273	0.24	1	93918070	93918343	0.00605
Peg 13	NR_002864.1	4721	0.25	15	72589619	73061204	5.00E-05
Pisd-ps1	NR_003517.1	606	0.25	11	3124020	3194157	5.00E-05
C920009B18Rik	NR_015465.1	336	0.25	10	22173444	22374139	0.001
Gm10374	XR_106218.1	584	0.25	11	84870922	84871631	5.00E-05
2610203C20Rik	NR_015483.2	2578	0.25	9	41474936	41615185	5.00E-05
Lnc-OGD4922	-	1866	0.28	18	60593012	60649002	0.00125
LOC100861838	XR_140809.1	540	0.33	7	35772345	35838074	0.0089
2700023E23Rik	NR_015531.1	798	0.30	5	74093064	74094336	0.0117
LOC100861735	XR_140828.1	611	0.31	7	55794147	55833964	5.00E-05
LOC100861846	XR_140548.1	364	0.31	2	72476218	72499861	5.00E-05
LOC100861633	XR_140781.1	593	0.32	6	127116352	127212419	5.00E-05
LOC100861797	XR_140919.1	722	0.32	8	106150398	106198704	0.00075
Lnc-OGD5097	-	1675	0.33	19	56601953	56603628	5.00E-05
2700038G22Rik	NR_045040.1	1370	0.33	5	23850512	23855109	5.00E-05
LHC-OGD2057	-	941	0.34	7	16348401	16049342	5.00E-05
Lnc-OGD3950	-	1881	0.34	13	57999967	58001848	5.00E-05
Gm19841	XR_104928.1	713	0.35	5	35697179	35729383	0.00265
Lnc-OGD1718	-	582	0.35	5	142866909	142867491	5.00E-05
LOC100862093	XR_140579.1	478	0.35	3	108591237	108722299	0.00035
LOC100862295	XR_141255.1	541	0.35	15	10486033	10493909	5.00E-05
Gm14966	XR_106193.1	2631	0.35	19	6341160	6378158	0.00445

Table 4

Top transcription factors in the promoter of OGD-responsible lncRNAs

Name of TFs	# of lncRNA promoters bound	Name of lncRNAs	# of TF bound in the promoter
SP1	141	LOC100861682	137
KLF4	137	2610019E17Rik	127
MAZ	123	Msx1as	119
PATZ1	119	D330041H03Rik	111
TFAP2C	112	Lnc-OGD4751	103
MZF1	107	LOC100862031	102
TFDP1	107	Lnc-OGD3606	101
YY1	86	LOC100861830	93
ELF2	85	Prr3	89
TFAP2B	83	LOC100861838	89
SPI1	79	Malat1	76
SPIB	79	Srsf7	76
GTF2I	78	LOC100861979	74
PAX5	78	LOC100862175	74
SPZ1	76	LOC100862295	74
TCF4	75	Gm10374	72
KLF12	74	1110038B12Rik	70
MTF1	71	Lnc-OGD3898	70
MAFB	70	Lnc-OGD648	68
MYOD1	70	Gm17690	67
HIC1	69	Lnc-OGD3838	65
GABPA	68	LOC100862023	65
HOXB6	66	Gm19767	65
EGR3	65	LOC100862184	64
GATA1	65	Gm20184	63
FEV	64	Lnc-OGD2327	62
RREB1	63	Lnc-OGD2789	62
GFI1	62	Snhg5	61
NHLH1	62	Gm5853	61
NKX3-2	61	A030001D20Rik	61
ZNF148	60	Lnc-OGD4044	60
ARNT	56	LOC100861862	60
NFE2L1	56	LOC100861829	60
BPTF	55	LOC100882158	59