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Profiling neuron-autonomous lncRNA changes upon ischemia/reperfusion injury

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ABSTRACT

Extensive changes of neuronal transcriptome occur post ischemic stroke and during the following reperfusion. Although numerous studies focused on transcriptome changes of mRNAs associated with ischemic stroke, little is known about whether and how long non-coding RNAs (lncRNAs), which play critical roles in cellular homeostasis, are involved in this process. In this study, we performed high throughput screening to analyze expression changes of lncRNAs in primarily cultured hippocampal neurons under an oxygen-glucose deprivation/reperfusion (OGD/R) condition at 0 h, 6 h, 12 h, and 18 h, respectively. Knock down of one validated lncRNAs (Tnxa-ps1) promoted neuronal survival by inhibiting apoptosis. Coding non-coding co-expression network analysis revealed that the expression of Tnxa-ps1 was highly correlated with changes of a particular group of genes, many of which are associated with neural protection. Finally, we showed that down-regulation of Tnxa-ps1 reversed the expression changes of four mRNAs post OGD/R, revealing a regulatory effect between Tnxa-ps1 and selected genes. Together, our data revealed possible participation of lncRNAs in the pathophysiology of OGD/R and thereby provided new insights into the studies of potential therapeutic targets for ischemic stroke.

1. Introduction

One of the leading causes for motor and mental disabilities is ischemic stroke [1,2]. Due to limited neuron and axon regrowth in the adult CNS, functional deficits caused by ischemic stroke are largely permanent. A prevailing therapeutic intervention, if time permitted, is to re-perfuse immediately, which tries to restore the insufficient blood flow caused by the stroke. However, in many cases, reperfusion often causes a secondary injury and exacerbates the initial damages caused by ischemia [3,4]. Therefore, to explore the underlying mechanisms of the injury induced brain damages, an efficient way is to perform thorough and comprehensive analysis of the repertoire of transcriptome changes post ischemic stroke/reperfusion.

Within the transcriptome, long non-coding RNAs (lncRNAs), although typically transcribed by RNA polymerase II, are different from mRNAs due to their lacking of the open reading frame [5–7]. Mechanistically, lncRNAs can regulate gene expression in both *cis* or *trans* way. For example, they can recruit proteins or molecular complexes to specific DNA loci, provide platform to install nuclear or cytoplasmic complexes, and sometimes bind other RNAs to trigger posttranscriptional regulation [5–7]. Such high flexibility renders lncRNAs the abilities to orchestrate multiple gene expression changes in a real-time manner to respond sudden events. Indeed, studies showed that knockdown of FosDT, a lncRNA, reduced the infarct volume and promoted motor functional recovery in an ischemic stroke model [8].

To mimic brain ischemia and reperfusion, we sought out to investigate lncRNA changes in primarily cultured hippocampal neurons conditioned by oxygen-glucose deprivation/reperfusion (OGD/R) [9]. We first validated the expressional changes of selected key lncRNAs at various time points post OGD/R. Among them, we further showed that knock down of a persistently up-regulated lncRNA, Tnxa-ps1, significantly alleviated neuronal apoptosis and thereby promoted cell viability after OGD/R. Using bioinformatics analysis, we showed that elevation of Tnxa-ps1 is closely connected

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with mRNA expression changes of a group of transcripts. Silencing Tnxa-ps1 reversed expression changes of specific genes post OGD, confirming its regulatory roles post OGD/R. Our results demonstrated the involvement of lncRNAs in ischemic stroke pathology and therefore provided new directions for drug screening to treat ischemic stroke and reperfusion caused brain damages.

2. Materials and methods

2.1. Cell culture, transfection and OGD/R treatment

All experiments were approved by the Ethics Committee of the Affiliated Hospital of Nantong University. The rat hippocampal cells were isolated as described previously [9]. In brief, E18-E19 Sprague-Dawley (SD) rat (experimental animal center of Nantong University) hippocampal tissues were quickly harvested, mechanically and enzymatically dissociated on a cold stage. Hippocampal cells were then plated onto poly-lysine-coated plates at the density of 1×10^6 per ml in DMEM supplemented with 10% FBS (Invitrogen, GrandIsland, NY) and incubated for 4 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cultures were incubated for 7–8 days with half of the medium being changed every 3 days.

To transfect, control siRNA (5′-ACGUGACACGUUCGGAGAAdTdT-3′, 200 nM), siRNA-1 (5′-GCAUUAAACUUGACUGACA dTdT-3′, 200 nM) & siRNA-2 (5′-GGAGGACAGAUUCAGGAAA dTdT-3′, 200 nM) against Tnxa-ps1 were mixed with Lipofectamine® RNAiMAX Reagent (1:1 ratio, Thermo Fisher Scientific, 13778030) in opti-MEM and added directly into the DMEM culture medium. Culture medium was changed at 16 h after transfection. 48 h after transfection, cultures were treated for OGD-R injury (see below).

Before inducing OGD-R injury, cultured hippocampal neurons were rinsed twice with PBS and maintained in glucose-free DMEM. Cells were then placed into a hypoxic incubator (Don Whitley Scientific, England) with 1% O_2, 5% CO_2, and 94% N_2 for 45 min at 37 °C to mimic OGD injury [9], (named OGD 45 min/R 0 h). Control cells (OGD 0 min/R 0 h) were harvested after incubating with DMEM with glucose in a humidified incubator with 5% CO_2 at 37 °C for the same period of time. After 45 min hypoxia challenge, some hippocampal cells were transferred to Primary Neuron Basal Medium with 2% B27 supplement and returned back into normoxic conditions for 6 h (OGD 45 min/R 6 h), 12 h (OGD 45 min/R 12 h) or 18 h (OGD 45 min/R 18 h) before harvest to mimic reperfusion caused injury.

2.2. RNA extraction and RNA-Seq analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and assessed by the Agilent 2200 Bioanalyzer for RNA quality. Samples were processed using the Illumina mRNA-Seq Sample Preparation Kit (containing 1 Box, part # 1004824 and 1 Bag, part # 1004825). Briefly, polyA containing mRNA was enriched by oligo-dT magnetic beads from 5 μg total RNA and fragmented into small pieces (using divalent cations at 94 °C) for cDNA synthesis. The cDNA fragments then underwent an end repair process, the addition of a single 'A' base, and the ligation of the adapters. The products were then purified and PCR amplified to create final cDNA libraries. RNA-seq libraries were 100 bp, paired-end sequenced on an Illumina HiSeq 2000. Sequencing reads after removing polymers, primer adaptors, and ribosomal RNAs were aligned to rat genome with SOAPaligner/SOAP2. The alignment data is utilized to calculate distribution of reads on reference genes and perform coverage analysis. The expression level for each gene was measured by the reads per kilo-base per million (RPKM) after quality controls. Genes were considered significantly differentially expressed if they exhibited at least a two-fold difference in expression with a false discovery rate (FDR) less than 0.001.

2.3. Quantitative real-time reverse transcription polymerase chain reaction

Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). qPCR was performed on an ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, USA) by using SYBR Green Real-Time PCR Master Mix (Toyobo, Japan). GAPDH was used for normalization. All quantitative PCR reactions were performed in biological triplicates. Primer sequences were listed in Supplemental Table 1.

2.4. Cell viability and TUNEL assay

The cell viability of hippocampal neuron cells was evaluated using thereduction 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as described previously [9]. In brief, the 96-well culture plates were seeded in hippocampal neurons with a density of 1×10^6 cells/ml for 7 days. After pretreatment with control, siRNA-1 & 2 against Tnxa-ps1 for 48 h, all groups were exposed to OGD + R18h except the normal control (OGD only). Each well was then added with 10 μl of MTT solution (5 mg/ml), incubated for 4 h, and followed by adding the 20% SDS solution (100 μl) for 20 h. Spectrophotometry was used to measure the absorbance at 570 nm by Tecan M200 Microelisa reader (Tecan company, Austria). For TUNEL assay, cultured cells were washed (1xPBS), fixed (2% paraformaldehyde, 1xPBS), permeated (0.1% Triton X-100), and incubated with TUNEL reaction mixture (in situ cell death detection kit, Roche). Results were analyzed by fluorescence microscope.

2.5. Coding non-coding co-expression network analysis

To perform the coding non-coding co-expression network analysis, we first identified mRNAs that showed consistent changes upon multiple time points post OGD/R ($n = 811$). We then calculated the Pearson correlation and choose the significant correlation pairs (>0.999) with which to construct the network [9].

2.6. Statistical analysis

For all figures, error bars figures represent mean ± SEM, the number (n) of samples employed is indicated in legends. Student's *t*-test, One-way ANOVA with Bonferroni correction for multiple comparisons (all were shown in figure legends) were performed to determine the significance difference between different groups. For all statistics, **, $p < 0.01$, *, $p < 0.05$, n.s., no statistical significance.

3. Results

3.1. Changes of lncRNAs in hippocampal neurons with oxygen-glucose deprivation and reperfusion (OGD/R)

To examine neuronal lncRNA responses to stroke and reperfusion, we isolated total RNAs and performed RNA-Seq from primary cultured hippocampal neurons with no treatment (control), with oxygen-glucose deprivation (OGD only), or with oxygen-glucose deprivation followed by reperfusion of various time periods (OGD + R 6 h, OGD + R 12 h, or OGD + R 18 h, respectively). To our surprise, in contrast to about a thousand differentially expressed transcripts identified in hippocampal neurons immediately after OGD [9], only two lncRNAs (Vof16 and RT1-T24-2, Fig. 1A), which were down regulated, showed different expression promptly after stroke. At 6, 12 and 18 h post reperfusion, 9, 8, 9 and 3, 4, 2 lncRNAs were up-regulated and down-regulated, respectively (Fig. 1A). We

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