Neuroscience 337 (2016) 191–199

LONG NONCODING RNA MEG3 ACTIVATION OF p53 MEDIATES ISCHEMIC NEURONAL DEATH IN STROKE

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Abstract—Maternally expressed gene 3 (MEG3) is suggested to function as a long non-coding RNA (lncRNA) and to play roles in various human cancers. However, the functional properties of MEG3 in ischemic stroke remain unknown. Here, we report that expression of MEG3 is upregulated following ischemia in adult mice. Moreover, cerebral ischemia recruits p53 into the MEG3 complex in ischemic tissues. MEG3 directly binds with the p53 DNA binding domain (DBD) consisting of amino acids 271–282 (p53-DBD$^{271–282}$), which stimulates p53-mediated transactivation and mediates ischemic neuronal death. Administration of the membrane-permeable peptide inhibitor Tat-p53-DBD$^{271–282}$ uncouples p53 from MEG3 *in vivo* and protects against cerebral ischemic insults *in vitro* and *in vivo*. Our data demonstrate that MEG3 functions as a cell death promoter in ischemia and physically and functionally interacts with p53 to mediate ischemic damage. Intervention in the MEG3–p53 interaction presents a new target for the therapeutic treatment of ischemic insults.

Key words: maternally expressed gene 3, p53, ischemia, Tat-p53-DBD$^{271–282}$, neuronal death.

INTRODUCTION

Stroke is a cerebrovascular disease that presents a serious hazard to human health and safety. Stroke has a high incidence rate, a high disability rate and a high mortality rate (Hachinski and World Stroke, 2015). Compared to other tissues and organs of the body, the brain is the most prone to ischemic injury. Approximately 80% of stroke patients experience ischemia and, different from the instantaneous ischemic injury observed in other tissues, a transient cerebral ischemia (lasting approximately 10 min) can produce profound neuronal damage (Lai et al., 2011). Immediately following ischemia, oxygen and glucose are depleted, hindering the energy-dependent processes necessary for cell and tissue survival and leading to a series of adverse reactions including ion homeostasis, the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and mitochondrial injury (Carelli and Chan, 2014; George and Steinberg, 2015). Affected cells subsequently die as a result of necrosis (swelling, rupture and cell lysis), apoptosis (chromatin condensation and DNA fragmentation) or both (Pei et al., 2014). According to previous research, secondary neuronal death following ischemia is the primary cause of death and disability in stroke patients. However, the complex cell death pathway involved remains unclear. To clarify the signal transduction mechanism involved in ischemic cell death is a key scientific problem involved in the treatment of stroke. A growing body of evidence indicates that non-coding RNAs (ncRNAs) may be involved in ischemia pathogenesis and is providing new insights into the biology of this disease (Dharap et al., 2012; Zhang et al., 2016).

Up to 90% of the genes in the human genome are transcribed into RNA, but only 2.94% of the genome represents protein-encoding genes, while the remaining are transcribed into non protein-encoding ncRNAs (Consortium, 2012). Noncoding transcripts are further divided into various classes of ncRNA that range from 17 to >9000 nucleotides in length (Vemuganti, 2013). Small ncRNAs such as microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-associated RNAs (piRNAs) are those less than 200 nucleotides in length, while those greater than 200 bases in length are usually referred to as long noncoding RNAs (lncRNAs) (Nagano and Fraser, 2011). Although the current literature on miRNAs is more extensive than on other small ncRNAs, there is an increasing body of research on lncRNAs. Initially, lncRNAs were considered to be transcriptional noise lacking biological functions. However, recent evidence has shown that several lncRNAs are involved in chromatin modification, transcriptional regulation, post-transcriptional regulation, X chromatin inactivation, genomic imprinting, splicing, transcription interference, post cell cycle regulation and epigenetic regulation (Mercer

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Abbreviations: CNS, central nervous system; ECA, external carotid artery; FJ, Fluoro-Jade C; ICA, internal carotid artery; lncRNA, long non-coding RNA; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MEG3, maternally expressed gene 3; miRNAs, microRNAs; MRI, magnetic resonance imaging; OGD, oxygen glucose deprivation; PDL, poly-D-lysine; PI, propidium iodide; PIC, preinitiation complex; piRNAs, piwi-associated RNAs; qRT-PCR, quantitative real-time PCR; RNS, reactive nitrogen species; ROS, reactive oxygen species; siRNAs, small interfering RNAs; TTC, 2,3,5-triphenyltetrazolium chloride.

http://dx.doi.org/10.1016/j.neuroscience.2016.09.017
0306-4522/

et al., 2009). Several studies have also suggested that altering the expression and function of ncRNAs such as BACE1-AS, 17A, TUG1, NEAT1 and maternally expressed gene 3 (MEG3) modulates the pathophysiology of central nervous system (CNS) disorders (Massone et al., 2011; Johnson, 2012; Ng et al., 2013; Zhang et al., 2016).

MEG3 was the first lncRNA to be found to function as a tumor suppressor (Zhang et al., 2003; Benetatos et al., 2011). MEG3 has recently been shown to be related to the pathogenesis of many nervous system diseases. MEG3 is expressed in the nucleus accumbens of normal human brain tissue, and overexpressed in heroin abusers (Michelhaugh et al., 2011). In contrast, MEG3 expression is downregulated in the caudate nucleus of patients with neurodegenerative diseases such as Huntington's disease (Johnson, 2012). MEG3 is also associated with the proliferation of glioma, and the ectopic overexpression of MEG3 inhibits cell proliferation and induces apoptosis (Zhang et al., 2010). From a functional perspective, MEG3 inhibits DNA synthesis and cell colony formation in meningioma cells and stimulates p53 mediated transcriptional activation (Zhang et al., 2010). However, very little is known about MEG3 expression levels in ischemia and the role of MEG3 in the development of ischemia.

We found that MEG3 expression was significantly upregulated after ischemia both *in vitro* and *in vivo*. We further confirmed that the overexpression of MEG3 *in vitro* increased neuronal death and that this effect could be suppressed through the use of siRNA targeting MEG3. Moreover, MEG3 stimulates p53 mediated transcriptional activation in N2a cells. As a transcription factor, p53 recruits diverse transcriptional coregulators such as histone-modifying enzymes, subunits of the mediator complex, chromatin remodeling factors, and components of general transcription machinery and preinitiation complex (PIC) by binding to its response element (Beckerman and Prives, 2010). On the other hand, one important way of lncRNAs playing their functions is to interact with transcriptional regulatory proteins (Wang and Chang, 2011). In hepatoma cells, MEG3 interacts with p53 DNA binding domain (p53-DBD) to activate p53-mediated transcriptional activity and regulates partial p53 target genes (Zhu et al., 2015). In this study, we further determined a more exact binding region consisting of amino acids 271–282 of p53-DBD (p53-DBD$^{271–282}$) at which MEG3 interacts with p53 in neurons. Interfering with the MEG3–p53 interaction through the use of Tat-p53-DBD$^{271–282}$ was shown to decrease ischemic neuronal death and protect against cerebral ischemic insults *in vivo*. These findings indicate that lncRNA MEG3 plays an important role in ischemic neuronal death and could be a new target for the treatment of ischemia.

EXPERIMENTAL PROCEDURES

Animals

The care of, and experiments with, animals were performed in accordance with institutional guidelines and the Animal Care and Use Committee (Wuhan University, Wuhan, China). Adult (90 ± 2 days of age) male C57BL/6 mice were used.

Focal cerebral ischemia model

Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO). Cerebral blood flow was monitored using a BLF21C Laser Doppler Flow-meter with an LL-M1043 fiber flow-probe (Transonic System Inc). Briefly, MCAO was induced by advancing a length of 10–13 mm 6-0 surgical nylon monofilament into the lumen of the internal carotid artery (ICA) from the left external carotid artery (ECA) until it blocked the origin of the middle cerebral artery (MCA). When the cerebral blood flow has been reduced by more than 60%, the MCA was occluded. The surgical nylon monofilament was left in place for 60 min and then withdrawn. The sham-operated animals were treated identically except that their left ECAs were exposed without inserting the surgical nylon monofilament into the ICA and the MCA was not occluded after the neck incision was made.

Primary cortical neurons and N2a cells

Primary cortical neurons were isolated from E18 mice. Cells were dissociated, purified using trypsin (Invitrogen, Shanghai, China) and plated at a density of 600–800 cells/mm^2 on coverslips coated with 30 μg/ml poly-D-lysine (PDL). The cells were placed in fresh DMEM/F12 medium (Invitrogen, Shanghai, China) plus 8–10% fetal bovine serum and the medium was replaced with serum-free Neurobasal medium plus 4% B27 6 h later. The cells were then fed every 3 days with fresh Neurobasal medium containing 4% B27. N2a cells were cultured in DMEM medium (Invitrogen, Shanghai, China) plus 8–10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Shanghai, China) and fed every 3 days.

Oxygen-glucose deprivation

After 12 days (DIV12), primary cortical neurons were challenged with oxygen-glucose deprivation (OGD) by exchanging the medium for a glucose-free bicarbonate buffer (pH 7.4) containing 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 2 mM $CaCl_2$ and 0.8 mM $MgCl_2$. The culture plate was then transferred to a special chamber containing 5% CO_2/10% H_2/85% N_2 and maintained at 32 °C for 1 h. Beginning up to 1 h after OGD, the cultures were maintained for 12 h, 24 h and 48 h in an incubator with 5%CO_2/10% H_2/85% O_2 at 37 °C, respectively. The cultures were then used for RNA extraction or propidium iodide (PI) staining.

RNA extraction and quantitative real time PCR analysis

Total RNA was isolated from ischemic tissues using TRIzol reagent (Invitrogen, Shanghai, China) 24 h, 48 h or 72 h after the MCAO operation, and from cultured neurons 12 h, 24 h or 48 h after OGD. Reverse transcription was performed using MMLV reverse