Dynamic changes in DNA demethylation in the tree shrew (Tupaia belangeri chinensis) brain during postnatal development and aging

Shu Wei1,2,#, Hai-Rong Hua3,#, Qian-Quan Chen1, Ying Zhang1, Fei Chen1,4, Shu-Qing Li3, Fan Li3,*, Jia-Li Li1,5,*
1 Key Laboratory of Animal Models and Human Disease Mechanisms of the Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming Yunnan 650223, China
2 Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming Yunnan 650223, China
3 Department of Pathology and Pathophysiology, School of Basic Medical Science, Kunming Medical University, Kunming Yunnan 650500, China
4 School of Life Science, University of Science and Technology of China, Hefei Anhui 230027, China
5 Kunming Primate Research Center of the Chinese Academy of Sciences, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming Yunnan 650223, China

ABSTRACT

Brain development and aging are associated with alterations in multiple epigenetic systems, including DNA methylation and demethylation patterns. Here, we observed that the levels of the 5-hydroxymethylcytosine (5hmC) ten-eleven translocation (TET) enzyme-mediated active DNA demethylation products were dynamically changed and involved in postnatal brain development and aging in tree shrews (Tupaia belangeri chinensis). The levels of 5hmC in multiple anatomic structures showed a gradual increase throughout postnatal development, whereas a significant decrease in 5hmC was found in several brain regions in aged tree shrews, including in the prefrontal cortex and hippocampus, but not the cerebellum. Active changes in Tet mRNA levels indicated that TET2 and TET3 predominantly contributed to the changes in 5hmC levels. Our findings provide new insight into the dynamic changes in 5hmC levels in tree shrew brains during postnatal development and aging processes.

Keywords: Tree shrew; DNA demethylation; 5-hydroxymethylcytosine; Brain development and aging

INTRODUCTION

Epigenetic systems emphasize the heritable changes in gene expression that do not involve coding sequence modifications, e.g., DNA methylation, histone modification and chromatin remodeling, and non-coding RNA regulation. These types of changes are proposed to be responsible for controlling the expression and function of genes and have emerged as important mediators of development and aging (Abel & Zukin, 2008; Agis-Balboa et al., 2013; Alagiakrishnan et al., 2012; Bakulski et al., 2012; Barbash & Soreq, 2012; Bihaqi et al., 2012; Coppieters & Dragunow, 2011; Deaton & Bird, 2011). Complex disorders, such as cardiovascular disease, cancer, diabetes, and neuropsychiatric and neurodegenerative diseases (Kinney & Pradhan, 2013; Konsoula & Barile, 2012; Kudo et al., 2012; Kwok, 2010; Maekawa & Watanabe, 2007; Marques et al., 2011; Urdinguio et al., 2009), have multifactorial origins, depending not only on genetic but also on environmental factors. The most extensively studied neurological disorder regarding epigenetic changes is Rett syndrome. Patients with Rett syndrome exhibit neurodevelopmental defects associated with MeCP2 mutations, which encode methyl CpG binding protein 2 and bind to methylated DNA (Ballester et al., 2000; Hansen et al., 2010). Other mental retardation disorders are also linked to the disruption of genes involved in epigenetic mechanisms, e.g., alpha thalassemia/mental retardation X-

Received: 03 January 2017; Accepted: 06 March 2017
Foundation items: This work was supported by the Hundred-Talent Program of Chinese Academy of Sciences (Y406541141; 1100050210) to J.L., the National Natural Science Foundation of China (81471313; 91649119) to J.L., the National Science and Technology Infrastructure Program (2014BAI01B01-04) to S.L., and the National Natural Science Foundation of China (31260242 to F.L).
*Corresponding authors, E-mail: leefan623@sina.com; lijiali@mail.kiz.ac.cn
#Authors contributed equally to this work
DOI: 10.24272/j.issn.2095-8137.2017.013
linked syndrome, Rubinstein-Taybi syndrome, and Coffin-Lowry syndrome (Urdinguio et al., 2009). Aberrant DNA methylation followed by abnormal gene expression was first reported as an epigenetic hallmark in cancer (Haffner et al., 2011; Malzkorn et al., 2011). The cytosine base in situ to produce 5-methylcytosine (5mC) is catalyzed by DNA methyltransferases (Lian et al., 2012). It is estimated that 5mC accounts for 2%–8% of total cytosines in human genomic DNA and affects a broad range of biological functions, including gene expression, genome integrity maintenance, parental imprinting, X-chromosome inactivation, development regulation, aging, and cancer (Guo et al., 2011; Lian et al., 2012; Szwagierczak et al., 2010). Ten-eleven translocation (TET) proteins, which are the enzymes necessary for demethylating 5mC, display enzymatic activity for the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) (Dahl et al., 2011; Kudo et al., 2012). This oxidized form of 5mC is known as the “sixth base”. Its presence adds a layer of complexity to the epigenetic regulation of DNA methylation (Ito et al., 2010; Tahiliani et al., 2009) and it is found in a variety of mammalian cells, particularly in self-renewing and pluripotent stem cells and neuronal cells (Guo et al., 2011; Szwagierczak et al., 2010). Although the biological role of 5hmC is not fully understood at present, it has gained attention due to its cancer biomarker potential (Davis & Vaisvila, 2011). It is assumed that, like 5mC, 5hmC also plays an important role in switching genes on and off.

It is important to illuminate the differences in 5hmC expression in different tissues and cells (Almeida et al., 2012a, b). Of note, 5hmC is particularly abundant in the brain (Globisch et al., 2010; Kriaucionis & Heintz, 2009; Li & Liu, 2011), and accounts for approximately 40% of modified cytosine, suggesting a potential role in neuronal plasticity (Szulwach et al., 2011). Low levels of 5hmC are reportedly associated with brain tumor differentiation and anaplasia (Kraus et al., 2012; Lian et al., 2012). In the mouse cortex and cerebellum, 5hmC is enriched within genes and appears to promote gene transcription (Jin et al., 2011; Song et al., 2011). As an intermediate state of complete DNA demethylation, 5hmC affects functional demethylation by blocking transcriptional repressor binding (Guo et al., 2011; He et al., 2011; Ito et al., 2011; Zhang et al., 2012). Synchronous neuronal activity promotes active DNA demethylation of plasticity-related genes in the mouse brain through TET-mediated formation of 5hmC (Guo et al., 2011). However, it is not clear whether such demethylation solely accounts for the enrichment of 5hmC in the brain. Recent research has reported that DNA methylation dynamics influence brain function and are altered in neurological disorders (Chia et al., 2011). Genome-wide mapping of 5hmC in the mouse hippocampus and cerebellum at different developmental stages indicate an increase with age and gene expression related enrichment in genes implicated in neurodegeneration (Szulwach et al., 2011). In addition, our lab recently revealed that selective changes in 5hmC-mediated epigenetic regulation promote Purkinje cell neurodegeneration in Ataxia-Telangiectasia disease and regulate DNA damage response (Jiang et al., 2015, 2017).

The tree shrew (Tupaia belangeri chinensis) belongs to Order Scandentia and is similar to primates in many biological features (Fan et al., 2013; Rockland & Lund, 1982; Xu et al., 2012). Moreover, its high brain-to-body mass ratio makes it a promising non-human primate animal model in brain and biomedical research (Cao et al., 2003; Li et al., 2017). In the present study, we sought to define dynamic changes in 5hmC levels in the tree shrew brain during postnatal brain development and aging, as well as its potential epigenetic regulation. During postnatal development, the levels of 5hmC showed a gradual increase in multiple anatomic structures of the tree shrew brain. In contrast, a significant loss of 5hmC was found in several selective regions of the aged tree shrew brains. Thus, dynamic changes in 5hmC levels suggest its potential epigenetic regulation in the process of brain development and aging.

MATERIALS AND METHODS

Animal use and care

Wild-type tree shrews were obtained from the Kunming Primate Research Center, Kunming Institute of Zoology, Chinese Academy of Sciences. All experimental procedures and animal care and handling were performed per the protocols approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences. For 5hmC immunohistochemistry, P10-, 3-month, 1-, 2-, 4-, and 5-year-old age-matched groups of animals (three males and three females) were used; for dot-blot assay, 3-month, 1-, 2-, and 4-year-old age-matched groups of animals (three males and three females) were used; for Tet mRNA quantitative real-time PCR, P10-, 3-month, 1-, 2-, and 4-year-old age-matched groups of animals (three males and three females) were used. The tree shrew brain cryostat section preparation

The tree shrews were anesthetized with ketamine (0.2 mg/g, i.m.) and perfused with phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) after complete anesthesia. After the blood was rinsed off from the body of animal, the perfusion buffer was changed to 4% paraformaldehyde (PFA) fixative solution for 20 min. Immediately, the whole brain was dissected using scissors and fixed with 4% PFA overnight at 4 °C. The fixed brain tissue was placed in 10, 20, and 30% sucrose solution sequentially for dehydration until it sank, and was then completely immersed with embedding agent optimal cutting temperature compound (OCT, Ted Pella Inc., USA). Finally, 15 μm cryostat brain sections were prepared at -25 °C and stored at -80 °C.

Immunohistochemistry

For 3,3′-diaminobenzidine (DAB)/bright field staining, all frozen brain sections were pretreated with 0.3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity, then rinsed in Tris-buffered saline (TBS), and treated with 0.1 mol/L citrate buffer in a microwave at sufficient power to keep the solution at 100 °C for 20 min. Sections were cooled in the same buffer at room temperature (RT) for 30 min and rinsed in TBS. Slides were incubated in 10% goat serum in PBS blocking
solution for 1 h at RT, after which anti-5hmC antibody (Abcam, ab106918, 1:200 dilution) was applied to the sections, followed by incubation at 4 °C overnight. The sections were washed three times in TBS before applying the secondary antibody (Vector Laboratories) for 1 h at RT. Afterwards, sections were rinsed three times in TBS. Rinsed sections were then incubated in Vectastain ABC Elite reagent (Vector Laboratories) for 1 h and developed using diaminobenzidine, according to the manufacturer's protocols. The sections were counterstained with hematoxylin, and finally mounted in Permount under a glass cover slip after dehydration. Control sections were subjected to identical staining procedures, except for the omission of the primary antibody.

Dot-blot analysis
Genomic DNA was isolated from brain tissue using a QIAamp DNA Mini Kit (Qiagen). Dot-blot assays were performed as described previously (Jiang et al., 2015). Briefly, 10 μg of sample DNA was diluted in Tris-EDTA (10 mmol/L, pH 8.0) buffer to 90 μL, with 2 μL of 5 mol/L NaOH buffer then added. After denaturing at 98 °C for 10 min, samples were immediately chilled on ice and neutralized with 10 μL of 6 mol/L (NH₄)₂Ac to a final DNA concentration at 100 ng/μL. The indicated amount of mixed DNA solution was spotted on a N+-Nylon membrane (Amersham Biosciences) and then crosslinked by UV-x-linker at 0.5 Joule/cm². Blots were incubated in blocking buffer (5% non-fat milk and 1% BSA in PBS-T) for 1 h at RT and then in 5hmC (Active Motif 39769) solution (1:10 000 in blocking buffer) overnight at 4 °C. Corresponding secondary Ab-HRP (1:5 000 in 5% non-fat milk) was used and blots were detected with the ECL system following the manufacturer's instructions. Methylene blue (0.02% in Na₂Ac, pH 5.5) staining was also performed to measure total DNA loading.

RNA isolation and real-time quantitative PCR (RT-qPCR)
Total RNA was isolated from tree shrew brain tissue using Trizol reagent (Invitrogen) and cDNA was generated with a Fast Quant RT Kit (Vazyme). Real-time qPCR reactions were performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using SYBR Green reagent (Invitrogen). Expression levels of target genes were analyzed using the comparative cycle threshold (Ct) method, where Ct is the cycle threshold number normalized to that of β-actin. The primers used in this study were: for Tet1, Tet1-F 5'-CGAATCATC CTACACGCTCT-3'/Tet1-R 5'-GGACAGTTATTACCTCTTA-3'; for Tet2, Tet2-F 5'-ACCCCTCTATTACATACCC-3'/Tet2-R 5'-CACCCTCATAAAACACCTGAC-3'; and for Tet3, Tet3-F 5'-CAAGGCTGAGACCACCTGACT-3'/Tet3-R 5'-CTTCTCCCTACTA TTTGTTCCGAC-3'. β-actin was applied as the standard for normalization by using β-actin-F 5'-TGCGTGACATCAAGGA GAAG-3'/β-actin-R: 5'-ACCTGACCATTAGGCAACCT-3'.

Statistics
All data were presented as means±SE of a minimum of three replicates. For most analyses, we evaluated statistical differences using the Student's t-test. For all analyses, P<0.05 indicated statistical significance.

RESULTS
Spatiotemporal-biased increase of 5hmC in tree shrew brains during postnatal development
To address if postnatal brain development could be attributed to active DNA demethylation, we examined the 5hmC levels in the prefrontal cortex (PFC), parietal cortex (PC), occipital cortex (OC), hippocampus (HP), and cerebellum (CB) of P10, 3-month, and 1-year-old tree shrew brains using immunohistochemistry. Compared with P10 brains, the intensity of 5hmC in the PFC, PC, OC, and HP neurons showed substantial increases in the 3-month and 1-year-old tree shrew brains, and peaked at 2-years old (Figure 1A-B). However, despite of high abundance of 5hmC in CB neurons in 1-year-old animals, high populations of CB neurons from P10 and 3-month-old animals showed low intensities of 5hmC (Figure 1A-B). The high abundance of 5hmC in adult brains (1- and 2-year-old tree shrews), but not developing brains (P10 and 3-month-old tree shrews), suggests that dynamic changes in 5hmC levels are involved in postnatal brain development. We further analyzed 5hmC-specific immunostaining data and revealed a significant increase in 5hmC intensity from P10 to 1-year, with a 2.31±0.12-fold increase in the PFC (P<0.05), 2.26±0.13-fold increase in the PC (P<0.01), 2.1±0.16-fold increase in the OC (P<0.001), 2.25±0.18-fold increase in the HP (P<0.01), and a 4.5±0.15-fold increase in the CB (P<0.001) (Figure 1B).

Age-related decrease of 5hmC in tree shrew brains during aging
Although 5hmC-mediated epigenetic regulation plays an important role in brain development, whether it is involved in brain aging remains unclear. To explore if changes in 5hmC levels diminished its dynamics during brain aging, we examined the abundance of 5hmC in the PFC, PC, OC, HP, and CB in 2-, 4- and 5-year-old tree shrew brains. In contrast to the abundance of 5hmC in 2-year-old brains, the intensities of 5hmC were substantially decreased in the PFC, PC, OC, and HP neurons of 4- and 5-year-old tree shrews (Figure 1A-B). In both the cerebral cortices and HP, we found selective loss of 5hmC in most neurons in 4- and 5-year-old tree shrew brains. Nevertheless, decreased 5hmC levels were not significantly observed in CB neurons (Figure 1A-B). The decreased abundance of 5hmC in aged brains (4- and 5-years old), but not adult brains (2-years old), suggests that changes in 5hmC levels are involved in brain aging. We analyzed 5hmC-specific immunostaining and revealed a significant decrease in 5hmC intensities from 2-year-old to 4- and 5-year-old brains, with a 1.75±0.16-fold decrease in the PFC (P<0.05), 1.36±0.14-fold decrease in the PC (P<0.05), 1.6±0.13-fold decrease in the OC (P<0.01), and 1.5±0.17-fold decrease in the HP (P<0.05), but a 0.35±0.08-fold increase in the CB (P>0.05) (Figure 1B).

To verify dynamic changes in 5hmC levels in tree shrew brains during postnatal brain development and aging, we determined the global changes in 5hmC levels through dot-blot assay. Genomic DNA from the PFC, HP, OC, PC, and CB of 3-month, 1-, 2-, and 4-year-old tree shrew brains was extracted.
Figure 1  Dynamic changes in 5hmC levels in tree shrew brains during postnatal development and aging
A: 15 µm cryostat sections of P10-, 3-month-, 1-, 2-, 4-, and 5-year-old wild-type tree shrew brains were immunostained with 5hmC (brown). Representative images were taken of the prefrontal cortex (PFC), parietal cortex (PC), occipital cortex (OC), hippocampus (HP), and cerebellum (CB). Signals from 100–300 neurons from each anatomic structure were averaged and normalized to the highest intensity across the observation. Scale bar, 200 µm. B: Normalized intensity of 5hmC staining illustrated in panel A. *: P<0.05. Error bars represent SE (unpaired t-test, n=3 repetitions of the experiment).

and sonicated for examination. As expected, the levels of 5hmC increased from the P10 to the 2-year-old brains in five regions during postnatal brain development (Figure 2A,B). In contrast, the levels of 5hmC in the aged brains showed a slight decrease in the PC, OC, and CB, but not in the PFC or HP (Figure 2A,B). This indicates that selective reduction in 5hmC levels might play a distinct role in different brain compartments during aging.

Figure 2  Decreases in 5hmC levels in the prefrontal cortex (PFC) and hippocampus (HP) during aging confirmed by dot-blot assay
A: Dot-blot analysis of changes in 5hmC levels in 3-month, 1-, 2- and 4-year-old wild-type tree shrew brains. Sample loadings were 3 µL, 1.5 µL, and 0.75 µL per-sample. Images were taken of the prefrontal cortex (PFC), parietal cortex (PC), occipital cortex (OC), hippocampus (HP), and cerebellum (CB). B: Quantitative statistics of 5hmC illustrated in panel A. *: P<0.05. Error bars represent SE (unpaired t-test, n=3 repetitions of the experiment).
Differential patterns of Tet mRNA expression in tree shrew brains during development and aging

The TET family enzymes, including TET1, TET2, and TET3, are responsible for oxidizing 5mC into 5hmC. To test if changes in 5hmC levels were directly associated with levels of Tet mRNA expression in tree shrew brains during postnatal development and aging, we examined their expression patterns. Total RNA from the PFC, HP, OC, PC, and CB of P10-, 3-month, 1-, 2-, and 4-year-old tree shrew brains were extracted, followed by RT-qPCR. In the PFC, Tet3 mRNA expression increased from P10- to 2-years old (2.15±0.11-fold, P<0.05), and then decreased at 4-years old. This is consistent with the patterns of changes in 5hmC levels described above. However, Tet1 and Tet2 mRNA expressions exhibited little change during postnatal development in the PFC (Figure 3A). The mRNA levels of Tet3 showed an exclusive increase in the HP (~2-fold from P10- to 2-years old) (Figure 3B). In contrast, mRNA levels of Tet1 and Tet2 in the HP significantly decreased during postnatal brain development (from P10- to 2-years old) (Figure 3B). Interestingly, the mRNA levels of all three Tet genes in the PC, and Tet2/Tet3 in the OC showed significant increases during postnatal development (Figure 3C,D). Lastly, while mRNA levels of Tet1 and Tet2 were upregulated in the CB during postnatal development and aging, the level of Tet3 mRNA remained quite stable (Figure 3E).

Figure 3 Expression patterns of Tet genes in tree shrew brains during postnatal development and aging

A-E: Relative mRNA levels of Tet1, Tet2, and Tet3 in the prefrontal cortex (PFC), parietal cortex (PC), occipital cortex (OC), hippocampus (HP), and cerebellum (CB) were determined by RT-qPCR. β-actin was used as a quantitative and qualitative control. All expression data were normalized to the expression of Tet1 at P10. *: P<0.05. Error bars represent SE (unpaired t-test, n=3 repetitions of the experiment).

DISCUSSION

DNA methylation/demethylation-mediated epigenetic regulation is involved in the processes of brain development and aging (Hutnick et al., 2009; Wilson et al., 1987). Abnormal alterations in this regulatory system leads to changes in brain function (Jiang et al., 2015; Szulwach et al., 2011); however, the underlying mechanisms remain uncertain in many biological processes and disease states. Loss of DNA methylation can occur through passive or active demethylation (Tahiliani et al., 2009). In active processes, the 5mC methyl group is removed by an enzymatic process. It is proposed that this mechanism requires the action of TET dioxygenases, which hydroxylate 5mC to 5hmC so that it can be deaminated and subsequently repaired by the base excision repair system (Chaudhry & Omaruddin, 2012; Kriaucionis & Heintz, 2009). As a novel epigenetic hallmark, the levels of 5hmC in genomic DNA vary significantly depending on cell type (Guo et al., 2011; Kriaucionis & Heintz, 2009).

In the present study, we observed and assessed dynamic changes in 5hmC levels in the tree shrew brain during postnatal development and aging. We found that the levels of 5hmC in the tree shrew brains increased in multiple anatomic structures during postnatal development, whereas a significant decrease in 5hmC abundance in aged brain neurons was found in the cerebral cortices and hippocampus, but not in the cerebellum. Previous research has reported that 5hmC levels increase in
the neurons of the mouse hippocampus and cerebellum during postnatal brain development (Szulwach et al., 2011), similar to the change patterns of the tree shrew brain observed in this study. These findings suggest that the dynamic change in 5hmC levels is an epigenetic hallmark and potential key factor in regulating the processes of brain development and aging. In addition, dynamic changes in 5hmC levels in different neural cell types are different, and its mediated epigenetic regulation during brain development could be different to its role in brain aging.

Brain function is linked to two significant factors: (1) processing of normal, usually abundant, proteins, e.g., neurotransmitter receptors and ion channels; and (2) maturation and aging. Age is not disease-specific, but is a prerequisite in brain development and aging. Our data indicate that understanding abnormal alteration in 5hmC during brain aging will be a useful epigenetic basis to explore the consequences of aging. We revealed that the dynamic pattern of changes in 5hmC levels in tree shrew brains predicts a possible link between DNA demethylation and brain development and aging. This novel perspective significantly broadens the search for new explanations of the complex biology of brain development and aging in tree shrews. The present study will help deepen our understanding of whether abnormal changes in 5hmC levels are epigenetic hallmarks of age-related neurodevelopmental and neurodegenerative disorders, and how changes in 5hmC levels in specific types of neurons produce a broad array of physiological and neurological symptoms.

We presented original data and research on the tree shrew brain, but recognize that our approach entails significant challenges. In the future, we will expand our observations in deciphering the mechanisms of the dynamic changes in 5hmC levels and provide new information about how the 5hmC-mediated regulatory system functions in brain development and aging.

ACKNOWLEDGEMENTS

We are grateful to De-Wei Jiang for critical reading of the manuscript.

REFERENCES

Abel T, Zukin RS. 2008. Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. Current Opinion in Pharmacology, 8(1): 57-64.

Agis-Balboa RC, Pavelka Z, Kerimoglu C, Fischer A. 2013. Loss of HDAC5 impairs memory function: implications for Alzheimer's disease. Journal of Alzheimer's Disease, 33(1): 35-44.

Alagiarishkhan K, Gill SS, Fagarasaru A. 2012. Genetics and epigenetics of Alzheimer's disease. Postgraduate Medical Journal, 88(1043): 522-529.

Almeida RD, Loose M, Sottile V, Matsa E, Denning C, Young L, Johnson AD, Gering M, Ruzov A. 2012a. 5-hydroxymethyl-cytosine enrichment of non-committed cells is not a universal feature of vertebrate development. Epigenetics, 7(4): 383-389.

Almeida RD, Sottile V, Loose M, De Sousa PA, Johnson AD, Ruzov A. 2012b. Semi-quantitative immunohistochemical detection of 5-hydroxymethyl-cytosine reveals conservation of its tissue distribution between amphibians and mammals. Epigenetics, 7(2): 137-140.

Bakulski KM, Rozek LS, Dolinoy DC, Paulson HL, Hu H. 2012. Alzheimer's disease and environmental exposure to lead: the epidemiologic evidence and potential role of epigenetics. Current Alzheimer Research, 9(5): 563-573.

Ballestar E, Yusufzai TM, Wolfe AP. 2000. Effects of Rett syndrome mutations of the methyl-CpG binding domain of the transcriptional repressor MeCP2 on selectivity for association with methylated DNA. Biochemistry, 39(24): 7100-7106.

Barbash S, Soreq H. 2012. Threshold-independent meta-analysis of Alzheimer's disease transcriptomes shows progressive changes in hippocampal functions, epigenetics and microRNA regulation. Current Alzheimer Research, 9(4): 425-435.

Bilhaqi SW, Schumacher A, Maloney B, Lahiri DK, Zawia NH. 2012. Do epigenetic pathways initiate late onset Alzheimer disease (LOAD): towards a new paradigm. Current Alzheimer Research, 9(5): 574-588.

Cao J, Yang EB, Su JJ, Li Y, Chow P. 2003. The tree shrews: adjuncts and alternatives to primates as models for biomedical research. Journal of Medical Primatology, 32(3): 123-130.

Chaudhry MA, Omaruddin RA. 2012. Differential DNA methylation alterations in radiation-sensitive and -resistant cells. DNA and Cell Biology, 31(6): 908-916.

Chia N, Wang L, Lu XY, Senut MC, Brenner CA, Ruden DM. 2011. Hypothesis: environmental regulation of 5-hydroxymethylcytosine by oxidative stress. Epigenetics, 6(7): 853-856.

Coppieters N, Dragunow M. 2011. Epigenetics in Alzheimer's disease: a focus on DNA modifications. Current Pharmaceutical Design, 17(31): 3398-3412.

Dahl C, Grenbaek K, Guldberg P. 2011. Advances in DNA methylation: 5-hydroxymethylcytosine revisited. Clinica Chimica Acta, 412(11-12): 831-836.

Davis T, Vaisvila R. 2011. High sensitivity 5-hydroxymethylcytosine detection in Balb/C brain tissue. Journal of Visualized Experiments, (48): 2661.

Deaton AM, Bird A. 2011. CpG islands and the regulation of transcription. Genes & Development, 25(10): 1010-1022.

Fan Y, Huang ZY, Cao CC, Chen CS, Chen YX, Fan DD, He J, Hou HL, Hu L, Hu XT, Jiang XT, Lai R, Lang YS, Liang B, Liao SG, Mu D, Ma YY, Niu YY, Sun XQ, Xia JQ, Xiao J, Xiong ZQ, Xu L, Yang L, Zhang Y, Zhao W, Zhao XD, Zheng YT, Zhou JM, Zhu YB, Zhang GJ, Wang J, Yao YG. 2013. Genome of the Chinese tree shrew. Nature Communications, 4: 1426.

Globisch D, Münzel M, Müller M, Michalakis S, Wagner M, Koch S, Brückl T, Biel M, Carell T. 2010. Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One, 5(12): e15367.

Guo JU, Su YJ, Zhong C, Ming GL, Song HJ. 2011. Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain. Cell, 145(3): 423-434.

Haffner MC, Chaux A, Meeker AK, Esopi DM, Gerber J, Pelliakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, De Marzo AM, Yegnasubramanian S. 2011. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget, 2(6): 627-637.

Hansen JC, Ghosh RP, Woodcock CL. 2010. Binding of the Rett syndrome protein, MeCP2, to methylated and unmethylated DNA and chromatin. IUBMB Life, 62(10): 732-738.
He YF, Li BZ, Li Z, Liu P, Wang Y, Tang QY, Ding JP, Jia YY, Chen ZC, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL. 2011. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science*, 333(6047): 1303-1307.

Hutnick LK, Golshani P, Namihira M, Xue ZG, Matynia A, Yang XW, Silva AJ, Schweizer FE, Fan GP. 2009. DNA hypomethylation restricted to the murine forebrain induces cortical degeneration and impairs postnatal neuronal maturation. *Human Molecular Genetics*, 18(15): 2875-2888.

Ito S, D’Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. 2010. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*, 466(7310): 1129-1133.

Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. 2011. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*, 333(6047): 1300-1303.

Jiang DW, Zhang Y, Hart RP, Chen JM, Herrup K, Li JL. 2015. Alteration in 5-hydroxymethylcytosine-mediated epigenetic regulation leads to Purkinje neuronal maturation. *Brain*, 138(12): 3520-3536.

Jiang DW, Wei S, Chen F, Zhang Y, Li JL. 2017. TET3-mediated DNA oxidation promotes ATR-dependent DNA damage response. *EMBO Reports*, doi: 10.15252/embr.201643179.

Jin SG, Wu XW, Li AX, Pfeifer GP. 2011. Genomic mapping of 5-hydroxymethylcytosine in the human brain. *Nucleic Acids Research*, 39(12): 5015-5024.

Kinney SRM, Pradhan S. 2013. Ten eleavlone translocation enzymes and 5-hydroxymethylcytosine in mammalian development and cancer. In: Karpf AR. Epigenetic Alterations in Oncogenesis: Advances in Experimental Medicine and Biology. New York: Springer, 754: 57-79.

Konsoula Z, Barile FA. 2012. Epigenetic histone acetylation and deacetylation mechanisms in experimental models of neurodegenerative disorders. *Journal of Pharmacological and Toxicological Methods*, 66(3): 215-220.

Kraus TFJ, Globisch D, Wagner M, Eigenbrod S, Widmann D, Münzel M, Müller M, Paffeneder T, Hackner B, Feiden W, Schüller U, Carell T, Kretzschmar HA. 2012. Low values of 5-hydroxymethylcytosine (5hmC), the "sixth base," are associated with anaplasia in human brain tumors. *International Journal of Cancer*, 131(12): 1577-1590.

Kriaucionis S, Heintz N. 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science*, 324(5929): 929-930.

Kudo Y, Tateishi K, Yamamoto K, Yamamoto S, Asaoka Y, Iijichi H, Nagae G, Yoshida H, Aburatani H, Koike K. 2012. Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation. *Cancer Science*, 103(4): 670-676.

Kwok BJ. 2010. Role of epigenetics in Alzheimer's and Parkinson's disease. *Epigenomics*, 2(5): 671-682.

Li CH, Yan LZ, Ban WZ, Tu Q, Wu Y, Wang L, Bi R, Ji S, Ma YH, Nie WH, Lv LB, Yao YG, Zhao XD, Zheng P. 2017. Long-term propagation of tree shrew spermatogonial stem cells in culture and successful generation of transgenic offspring. *Cell Research*, 27(2): 241-252.

Li WW, Liu M. 2011. Distribution of 5-hydroxymethylcytosine in different human tissues. *Journal of Nucleic Acids*, 2011: 870726.

Lian CG, Xu YF, Ceol C, Wu FZ, Larson A, Dresser K, Xu WQ, Tan L, Hu YG, Zhan Q, Lee CW, Hu D, Lian BQ, Kleffel S, Yang Y, Neiswender J, Khorasani AJ, Fang R, Lezcano C, Duncan LM, Scolyer RA, Thompson JF, Kakavand H, Houvras Y, Zon LI, Mihm MC Jr, Kaiser UB, Schatton T, Woda BA, Murphy GF, Shi YG. 2012. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell*, 150(6): 1135-1146.

Maekawa M, Watanabe Y. 2007. Epigenetics: relations to disease and laboratory findings. *Current Medicinal Chemistry*, 14(25): 2642-2653.

Maizel B, Wolter M, Riemenschneider MJ, Reifenberger G. 2011. Unraveling the glioma epigenome—from molecular mechanisms to novel biomarkers and therapeutic targets. *Brain Pathology*, 21(6): 619-632.

Marques SCF, Oliveira CR, Pereira CM, Outeiro TF. 2011. Epigenetics in neurodegeneration: a new layer of complexity. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 35(2): 348-355.

Rockland KS, Lund JS. 1982. Widespread periodic intrinsic connections in the tree shrew visual cortex. *Science*, 215(4539): 1532-1534.

Song CX, Szulwach KE, Fu Y, Dai Q, Yi QG, Li XK, Li YJ, Chen CH, Zhang W, Jian X, Wang J, Zhang L, Looney TJ, Zhang BC, Godley LA, Hicks LM, Lahn BT, Jin P, He C. 2011. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nature Biotechnology*, 29(1): 68-72.

Szulwach KE, Li XK, Li YJ, Song CX, Wu H, Dai Q, Iriver H, Upadhyay AK, Gearing M, Levey AI, Vasanthakumar A, Godley LA, Chang Q, Cheng XD, He C, Jin P. 2011. 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. *Nature Neuroscience*, 14(12): 1607-1616.

Szwagierczak A, Bultmann S, Schmidt CS, Spada F, Leonhardt H. 2010. Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. *Nucleic Acids Research*, 38(19): e181.

Tahiliani M, Koh KP, Shen YH, Pastar WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*, 324(5929): 930-935.

Urdinguio RG, Sanchez-Mut JV, Esteller M. 2009. Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. *The Lancet Neurology*, 8(11): 1056-1072.

Wilson VL, Smith RA, Mor S, Cutler RG. 1987. Genomic 5-methyldeoxycytidine decreases with age. *The Journal of Biological Chemistry*, 262(21): 9948-9951.

Xu L, Chen SY, Nie WH, Jiang XL, Yao YG. 2012. Evaluating the phylogenetic position of Chinese tree shrew (*Tupaia belangeri chiniensis*) based on complete mitochondrial genome: implication for using tree shrew as an alternative experimental animal to primates in biomedical research. *Journal of Genetics and Genomics*, 39(3): 131-137.

Zhang L, Lu XY, Liu JY, Liang HH, Dai Q, Xu GL, Luo C, Jiang HL, He C. 2012. Thymine DNA glycosylase specifically recognizes 5-carboxylcytosine-modified DNA. *Nature Chemical Biology*, 8(4): 328-330.