Small RNAs in mouse brain specifically expressed at the end-life stage

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Synopsis

The organismal life span is supposed to be mechanistically linked to the system of sexual reproduction specified by a yet uncharacterized species-specific life program. On the basis of previous studies, we predicted that small non-coding RNA serves as the primary signal that directs the termination of the maximum life span inherent in each species. Here, we prepared small-sized transcripts from the whole brain of newborn mice and extremely aged ones, and compared the transcriptome of the two age groups by deep sequencing. Annotation of their transcriptional differences identified a few kinds of microRNA markedly up-regulated in the older mouse brain. In particular, the miR-29 family was noteworthy for its high expression level. A functional attribution to this microRNA in the molecular mechanism for the determination of the murine life span was proposed.

Keywords: miR-29, microRNA, small RNA, life span, aging, brain

1 Introduction

From the viewpoint of evolution, organisms are considered to acquire their life span simultaneously with the system of sexual reproduction, when organismal death coincides with the completion of reproduction[1]. We suppose that this close coupling with reproduction is the original mode of the termination of the organismal life span, which is now still observed in semelparous species such as Pacific salmon[2, 3] as exceptional cases. Organismal mortality is the essential precondition to limit population size and accelerate the turnover of generations, thereby allowing future generations to readily adapt to changing environments. Organismal aging is certainly subjected to the influence of genetics, but this is unlikely to be of programmable nature. It is instead convincing that genetically programmed is the maximum duration of life characteristic of each species[4]. Therefore, it can be argued for that the sequence of events leading to organismal death is designed by natural selection just like the innate schedule in ontogenesis[5, 6]. We suppose that organismal post-reproductive phase was acquired as a result of the evolution of life span and its duration has also been under the influence of natural selection, because in higher animals post-reproductive survival of parental generations is vital for their children to successfully grow until self-reliant. Although it has been proven that it is easy to delay aging by genetic or environmental interventions in many model organisms under nonselective circumstances[7], laboratory animals that successfully underwent the engineering manipulations will not be able to outlive the species-specific upper limit of life established by the force of selection. Even though such an 'engineered' longevity outlasts the evolutionarily optimized one, physiological mortality per se should not be abolished.

Most species of the unicellular ciliated protozoans such as *Paramecium* and *Tetrahymena* have an intrinsic maximum life span[8]. They are characterized by nuclear dimorphism, in which two types of nuclei, macronucleus and

micronucleus, carry a distinct life-maintenance function in the same cell: the former serves as the somatic nucleus providing all transcription during vegetative growth, while the latter regarded as germline is transcriptionally silent until sexual conjugation[9]. Upon conjugation, the micronucleus produces a zygotic nucleus, which divides and differentiates to form the new macro- and micronucleus of the progeny cell. At this time, the parental old macronucleus is destroyed while presenting a fragmentation of DNA, a feature reminiscent of apoptosis[10, 11]. This degradation of old macronucleus is considered as the termination of the parental life. A series of studies on developmentally programmed genome rearrangements in ciliates suggests that a sort of small RNA referred to as scan RNA epigenetically triggers the degradation of old macronuclear DNA[12, 13]. We suppose that this event caused by scan RNA is the molecular basis underlying the termination of life span of ciliates[14]. Scan RNA, the processing product of micronuclear transcripts generated during conjugation, is originally identified as the primary agent responsible for elimination of transposon-like DNA elements on the formation of new macronucleus. A parallel phenomenon has been reported for fruit fly and mouse, in which a germline-specific small RNA called Piwi-interacting RNA (piRNA) plays a central role in suppression of transposable elements through an RNA silencing mechanism at the initial step of germline development[15, 16]. These findings led us to analogize that the key role of scan RNA at the end of ciliates' life is also phylogenetically conserved in a manner linked to reproduction. If this is the case, the causal determinant of life span of higher organisms could be found in the population of small RNA.

Thus, we planned to perform an exhaustive comparison in the expression profile of small RNAs of brain origin between mice at the initial life stage and those at the point of death in hope of detecting ones showing highly elevated expression at the end-life, promising candidates for the master regulator of organismal mortality. The reason for focusing on the brain in this study is that in higher animals this organ is the principal controller of all the body functions including natural death.

2 Materials and Methods

RNA samples

Total RNA of mouse brain was purchased from TAKARA BIO INC., where the following procedures were conducted for the RNA preparation: Normal wild-type female C57BL/6CrSlc mice were maintained on a CE-2 commercial diet (CLEA Japan) under specific pathogen free conditions in a room with controlled temperature (23 ± 2℃) and photoperiod (12:12 hr). Euthanasia was carried out by cervical dislocation to avoid confounding factors associated with the isoflurane sacrificing procedure, which may cause changes in gene expression. Whole brains were excised from mice of 4 days, 24 months, 30 months and 34 months in age, with 3 individuals per age cohort. Extraction of total RNA from the brain tissues and the subsequent isolation of a small RNA fraction of 18-50 nucleotides were carried out by using an RNAiso (TAKARA BIO) and a Small RNA Gel Extraction Kit (TAKARA BIO), respectively, according to the manufacture's instruction.

Sequencing and annotation of small RNA

Small RNA libraries were constructed from the above small RNA fraction using a Small RNA Cloning Kit (TAKARA BIO), and then subjected to high-throughput pyrosequencing on a Genome Sequencer FLX System (Roche) which was done through an experimental service by TAKARA BIO. All reads were mapped to the mm9 assembly of the mouse genome and to sequences with known function. The genome assembly and some functional annotation are available from the genome browser at the UCSC (http://genome.ucsc.edu). To identify small RNAs corresponding to microRNA (miRNA), piRNA, rRNA, tRNA,

small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small cytoplasmic RNA (scRNA), miscellaneous RNA (miscRNA) and mRNA based on sequence similarity, we downloaded the sequences of these known RNAs from the following databases: miRNA, miR Base (ftp:// ftp.ac.uk/mirbase/sequences/13.0/mature.fa.gz, ftp://ftp.ac.uk/mirbase/sequences/13.0/hairpin. fa.gz); piRNA, NCBI Entrez Nucleotide Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide); mRNA, NCBI Reference Sequences (ftp://ftp.ncbi.nih.gov); other RNAs, Ensembl Genome Browser (ftp://ftp.ensembl.org/pub/release55). Then, BLASTN search was performed using our RNA sequences as queries and the downloaded sequences as a database. Since the sequences in the databases could not cover all of our RNA species, we aligned the query sequences with the database sequences using a 90% match criterion including gaps. If a small RNA had more than one annotation, we used the following order of priority: miRNA, piRNA, rRNA, tRNA, snRNA, snoRNA, scRNA, miscRNA and mRNA. Any un-annotated reads were classified as "match genome" or "no-match genome" when they were mapped to the mouse genome with a perfect or imperfect match, respectively.

Northern blotting

Northern blot was produced by running 20 μg of total RNA into a 15% denaturing polyacrylamide gel and electro-transferring the RNA sample onto a nylon membrane. Hybridization with probe and detection of signal was performed using a Detector AP Chemiluminescent Blotting Kit (KPL). Prehybridization, hybridization and post-hybridization washing were done at 42℃. 5' biotinylated oligo DNA probes, which are perfectly complementary to mature miRNAs, were as follows (5' to 3'): miR-29b, AACACTGATTTCAAATGGTGCTA; miR-219, AGAATTGCGTTTGGACAATCA; miR-338, CAACAAAATCACTGATGCTGGA

3 Results

Profiling of small RNAs in extremely young and old mouse brains

The aim of this study is to deeply compare the expression level of small non-coding RNAs between the initial and the final life stages of mouse brain, and identify ones predominantly found in the latter stage. We extracted small-sized RNAs from the whole brain of 4-day-old neonates and 34-month-old adults, and sequenced extensive pools of the RNA in each age group. A total of 97725 and 121450 sequence reads were obtained for analysis from the newborn and the extremely aged mice, respectively, after removing sequence artifacts generated in the library construction step. Of these, 33172 and 51447 reads were unique sequences in the respective libraries, indicating that a huge diversity of small RNA sequences exists in mouse brains.

These sequences were annotated and categorized as one of 11 broad classes based on their alignment to sequences deposited in the above-mentioned databases (Table 1). No clear difference was observed in the composition of these RNA classes between the two libraries. For both libraries, miRNAs contributed substantially (23-25%) to the brain small RNAs. piRNAs and mRNAs comprised 12% and 18-26%, respectively, of these libraries. Roughly 5-9% corresponded to presumed breakdown products of abundant non-coding RNAs such as rRNA and tRNA. The remaining 30-37% excluding minor nuclear small RNAs were un-annotated clones, a substantial fraction of which is likely to represent functionally unknown small RNAs. All the sequence data of the neonate small RNAs and the aged adult ones has been deposited in DDBJ under accession numbers ANAAA0000001-ANAAA0033164 and ANAAB0000001-ANAAB0051442, respectively. A full detail of the annotation results is available at http://www.nara.kindai.ac.jp/bio/tanabe/AnnotationData.

Table 1. Small RNA repertoire of 4-day-old and 34-month-old mouse brains

RNA class	Read counts (%)	
	4-day-old brain	34-month-old brain
miRNA	24571 (25.1)	28236 (23.2)
piRNA	11367 (11.6)	14066 (11.6)
rRNA	2937 (3.0)	3780 (3.1)
tRNA	1280 (1.3)	6136 (5.0)
snRNA	887 (0.9)	1001 (0.8)
snoRNA	429 (0.4)	416 (0.3)
scRNA	0 (0.0)	0 (0.0)
miscRNA	598 (0.6)	695 (0.6)
mRNA	25921 (26.5)	21660 (17.8)
Match genome	3055 (3.1)	4306 (3.5)
No-match genome	26680 (27.3)	41154 (33.9)
Total	97725 (100)	121450 (100)

Identification of end-life-specific small RNAs

We normalized the raw data showing the number of reads of each RNA species including isoforms to the total reads of the RNA class to which the RNA species belongs. A thorough comparison in the normalized value between the two libraries detected three miRNA species, miR-29 family (miR-29a/b/c paralogs), miR-219 and miR-338, derived from the older brain that markedly outnumbered the newborn controls (Supplementary Table 1). Here we defined the marked read difference as more than a tenfold difference in read counts, and excluded RNA species less than 100 in the sum of reads in the two libraries from further investigation since their significant differences are not clear due to very low read frequency. The fold difference (aged vs. neonate) of miR-29 family, miR-219 and miR-338 were 66.0 11.1 and 10.5, respectively.

Expression alterations of end-life-specific miRNAs during normal brain aging

We obtained total RNA from the brain of mice aged 4 days, 24 months, 30 months and 34 months, and analyzed the expression of the above three kinds of miRNAs with advancing age by Northern blotting (Fig. 1). Every miRNA showed a much stronger expression in the extremely old brain compared with the newborn level (lane 1 vs. lane 4), validating the results of the deep-sequencing analysis. However, all the miRNAs were found to have reached the highest expression level at 24 months old, and thereafter remain unchanged or at a somewhat decreased abundance (lanes 2 to 4).

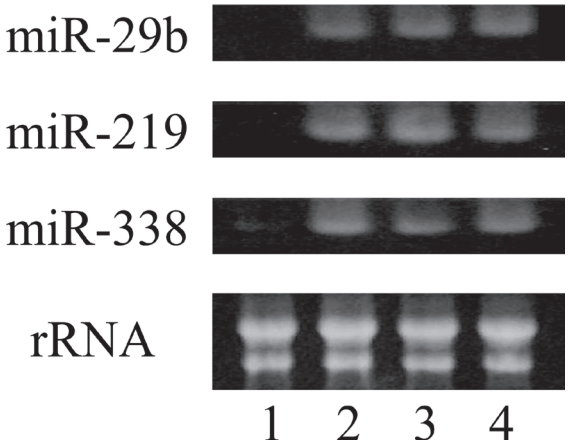

Fig. 1. Northern analysis of miRNA prevalence in aging mouse brains.
Each lane contained 20 μg of total RNA isolated from whole brain at different ages: lane 1, 4 days old; lane 2, 24 months old; 3, 30 months old; 4, 34 months old. After electrophoretic separation, these RNAs were transferred onto nylon membranes to prepare a triplicate blot. Each blot was hybridized with a biotinylated oligonucleotide probe for miR-29b, miR-219 or miR-338 (from top to bottom). Chemiluminescent signals from the labeled probes were photographed with an ECL camera. We ran the same RNA samples on a 1% denaturing agarose gel and detected 28S and 18S rRNAs by ethidium-bromide staining of gels to verify equal loading (the lowest panel). Since similar miRNA sequences are difficult to distinguish under lower hybridization stringency, the signal from miR-29b probe is supposed to represent the total abundance of the miR-29 family. Also, since these blots were exposed for different lengths of time, the signal intensity of each blot does not reflect the relative expression level of miRNA in the brain tissues.

4 Discussion

It has been established that miRNAs play leading parts in post-transcriptional regulation of cellular gene expression involved in a wide range of biological phenomenon[17, 18]. Although numerous miRNAs were shown to be expressed in a spatially and/or temporally restricted manner in mammalian brain and be associated with brain morphogenesis, neuronal fate and

synaptic plasticity, very limited knowledge has been clarified about the functional roles of miRNAs in the physiological events in the central nervous system[19]. In this study, surveying expression of small RNAs in mouse brains, three kinds of miRNAs, miR-29 family, miR-219 and miR-338, were found to be remarkably abundant in very old adults compared to their newborn counterparts. Unexpectedly, a relatively small set of small RNAs accounted for most of the difference in the expression profile between the two age groups.

Of particular interest is the miR-29 family: its net expression change over life span deserves special attention. Previous studies indicated that the miR-29 family is ubiquitously distributed across the entire brain in adult mice[20-22], and is enriched in astrocytes in comparison to neurons[23]. Expression of miR-29 is well conserved in lower animals as well as among mammals, implying its high biological significance. In Drosophila melanogaster, its miR-29 homolog, miR-285, is detected almost exclusively in the adult head during ontogenic development[24, 25]. Although expression of miR-83, the Caenorhabditis elegans homolog of miR-29, progresses consistently at a low level overall in the body throughout the life span of this nematode[26, 27], its localization in nervous system is unclear. Miska et al. reported using microarray technology that the abundance of miR-29 in mouse brains remained at a basal level through early development but rapidly rose when mice grew to young (4-month-old) adults[28], which is consistent with our result. Another microarray analysis dealing with miR-29 expression in adult mouse brains presented an inconsistent expression change during adulthood with an activation peak of around 24 months in age[29], again in agreement with our expression kinetics data, although such a subtle change in expression was not detected by Northern analysis.

These results together raise the possibility that miR-29 works intensively at the mid-life stage in mouse brains as an initiator of the late-life program, being followed by highly dynamic and temporally regulated waves of gene expression in later life. In the context of our presumption mentioned in the Introduction, here we would like to propose that organismal mortality is the final step of programmed sequential events in ontogenesis and adulthood, in which miR-29 acts as a master switch that turns on or off an unidentified signaling network directing the brain-wide neurological disorder and cell death. Although future functional investigations of this miRNA family are needed to make this hypothesis conclusive, given the crucial role of miRNAs as causal regulators of development in other vertebrates and invertebrates, our notion is fully plausible and should shed light on the mechanistic aspect of the genetic program designed for species-specific life potential.

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寿命終末期のマウス脳内で特異的に発現している小分子 RNA

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要　約

生物種固有の生物寿命は老化の結果としての偶発的な死とは無関係な先天的要因により規定されており，その決定機構には有性生殖のシステムが何らかの様式で関与していると考えられる。原生動物をモデル生物として行われてきた一連の研究から，寿命終了と連動して開始される有性生殖の際に発現する小分子非コード RNA が寿命決定のマスター因子であるとの知見が得られた。この現象が大きな生物学的意義を有し生物進化の過程で保存される性格のものであれば，同様な寿命決定機構が高等動物でも作動していることになる。そのような仮定のもとに，寿命決定因子候補としての小分子 RNA の同定を目的として新生マウスと老齢マウスの全脳から短鎖 RNA をそれぞれ調製し，それらの塩基配列を網羅的に決定した。各転写産物のアノテーションの結果，老齢マウス脳の方では miR-29 などの数種類の microRNA が顕著に発現していることがわかった。