脳特異的miR-29ノックダウンマウス

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Generation and some characterization of BAC transgenic mouse that neurally expresses antisense RNA against the potential longevity determinant miR-29

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Synopsis

miR-29, a gene family of microRNA, is highly expressed across the entire adulthood until life termination in the mouse central nervous system. Its expression is almost unique to astroglia throughout the adult brain. Given a leading part that microRNAs play in a broad spectrum of biological events, it is convincing to hypothesize that miR-29 present in the central nervous system is causally involved in the setting of the maximum duration of mammalian life. To address this notion, establishment of an experimental regime is needed that allows for induction of stable loss-of-function phenotypes for miR-29 specifically in the adult mouse brain. BAC (bacterial artificial chromosome)-mediated transgenesis is a powerful technology that facilitates spatial and temporal modulation of gene expression *in vivo*. Here we set out a BAC transgenic mouse that displays brain-specific conditional knockdown of miR-29 by overexpressing antagonistic transcripts against this microRNA. We first selected *Ntrk2*, a neurotrophin receptor gene, as the BAC driver gene providing the promoter and enhancer activities necessary for brain-specific expression of the transgene. The mouse *Ntrk2* gene is essentially specific to the brain and predominantly expressed in astroglia. We identified two mouse BAC clones harboring a distinct *Ntrk2* genomic segment by web searches, and unified them via their overlapping sequence to represent a genomic region large enough to cover appropriate regulatory elements for optimal transgene expression. We next modified the fusion BAC clone as the vector for delivery and expression of the transgene in the brain by recombining a DNA cassette encoding antisense RNAs against the miR-29 just downstream of the *Ntrk2* translation start codon in order for transcription of the miR-29 antagonist to be driven by the *Ntrk2* promoter. Finally, the resulting BAC targeting construct was injected into fertilized oocytes, and six independent transgenic founder lines that transferred the transgene to their offsprings were obtained. Out of these, five founder lines showed successful expression of the anti-miR-29 transcripts. Analysis of one representative founder line revealed a substantial decrease in the endogenous miR-29 level only in the brain compared with non-transgenic littermates.

Keywords: miR-29, microRNA, lifespan, aging, brain

1 Introduction

MicroRNAs (miRNAs) are a major category among non-coding RNAs that normally function as negative regulators of expression of their target mRNA at the posttranscriptional level[1]. It has also recently been thought that they may positively regulate the target expression in some

cases[2]. Generally, the identified miRNA targets show only slight to moderate repression, implying the action of miRNA as a 'dimmer switch' fine-tuning gene regulatory networks, either directly or indirectly. Although the function of only a small percentage of known miRNAs is clarified, yet bioinformatics predictions have estimated that the vast majority of mammalian gene transcripts are susceptible to miRNA-mediated regulation[3]. miRNAs were initially associated with developmental regulation, but a rapidly growing body of literature has described that these small transcripts contribute to a wide variety of key biological processes such as cell growth, embryonic development, tissue differentiation, and metabolism and homeostasis[4, 5]. Compared with protein-protein interactions in which a single protein interacts with a limited number of proteins, each miRNA has the potential ability to repress the translation of hundreds of transcripts that carry common target sequences; *vice versa*, a target gene message may possess several target sequences, vulnerable to control by multiple miRNAs. It is also becoming clear that most miRNAs target multiple genes that have related functions, and thereby sensitively exert strong effects on a particular regulatory pathway and even programmatically shift a signaling activation towards another physiological facet depending on the cellular context, such as the example of committing cells to apoptotic death by shut-down of pro-survival pathways. Consequently, aberrant expression of miRNA could in turn be responsible for unwanted organismal dysfunction leading to many body disorders, diseases and aging[6-8].

Our previous work exhaustively analyzed the expression of small-sized RNAs from young and extremely old mouse brains, and identified three miRNAs belonging to the miR-29 family, miR-29a/b/c, that remarkably up-regulated in the aged brain, showing up to ~ 70-fold higher levels than those in the younger animal[9]. miR-29 expression in the mouse brain, below detectable levels during embryonic and perinatal development, is increasingly enhanced from the pre-pubertal to early adult stage[10-12], and plateaus thereafter[9, 13]. In addition, miR-29 is expressed across the entire brain region[14-16], but is mostly localized to astroglia[17, 18]. We recently conducted an in-depth scanning for transcriptional and translational alterations after overexpression of miR-29 in cultured mouse astrocytes of prenatal origin to explore the biological implication of this miRNA in earlier ages[19]. This gain-of-function study detected a large set of putative miR-29 targets and their downstream signal responders, and computational annotations of these co-regulated genes predicted functional roles of miR-29 in the developing brain that support differentiation-linked cellular metabolic and signaling activities while counteracting abnormal cell proliferation and undesirable cell death. The result of the annotations is incompatible with the pro-aging/apoptotic nature characteristic of miR-29 in adult tissues as reviewed in our article[19], which is reminiscent of the term 'antagonistic pleiotropy'[20]. However, administration of exogenous miRNA into cells that do not normally express it may lead an artifactual identification. Instead, specific inhibition of endogenous miRNAs would be a preferable approach for pinpoint detection of physiological targets. miRNAs bind to the 3'-untranslated region (UTR) of target mRNAs through base-pair interaction, followed by cleavage or translational attenuation of the targets. The specific base pairing between miRNAs and their cognate mRNAs is the molecular basis for the gene knockdown action of miRNA. Therefore, a logical approach of silencing miRNAs is to use an antisense oligonucleotide to the miRNA of interest[21-24]. Such miRNA antagonists, anti-miRNAs, block the interactions between miRNAs and the binding partners by competitive inhibition, resulting in the de-repression function of the targets as a phenotypic readout. As such, development of vector systems allowing efficient delivery and tissue-specific expression of anti-miRNA is essential for better understanding of functional roles of miRNA *in vivo*.

Remarkable advances of DNA cloning

techniques exploiting episomal replicons, in particular, F-factor-backed bacterial artificial chromosome (BAC)[25, 26] have made large genomic fragments extending to several hundred kilobases (kb) of human, mouse, and other organismal origins readily available through web-administered public genomic databases and repositories. BAC clones, on average 100-300 kb in size, have been the templates for most genome sequencing projects, and as a consequence are mapped to the entire human and rodent genomes[27, 28]. Different strategies have been developed to engineer BACs for animal transgenesis[29-33], which all rely on *in vivo* homologous recombination, recombineering[34, 35], between a cloned or synthesized DNA fragment and the corresponding genomic site within a BAC-holding gene named driver gene, generating insertion of a heterologous DNA sequence into a specified locus, deletion of a defined endogenous sequence, or site-directed sequence alteration without the need for classical in vitro cloning techniques. After experiencing the modification on the targeted locus, BACs are purified and delivered into animal fertilized eggs as in the conventional transgenic method using a small-sized cDNA transgene with minimal promoter. Given their large size, BACs encode most, if not all, *cis*-regulatory elements of a gene of interest, thus offering a powerful transgenic technology combining advantages of both conventional transgenic and standard knock-in/out gene targeting approaches[36]: in essence, BAC-mediated transgenesis features creation of animals showing spatiotemporally faithful expression of transgenes under the control of tissue- and/or stage-specific regulatory domains present in a genomic configuration and minimal interference by the chromatin region neighboring the transgene insertion site (position effect), while it also serves as a versatile, efficient, and relatively easy-to-handle alternative to stem cell technologies in which an allele is modified in mouse stem cells before those are re-implanted into blastocytes. Thus, BAC transgenic technology has being the best choice for recombinogenic techniques in the study of functional genomics to uncover the biological significance of a large variety of animal genes including those associated with the central nervous system (CNS)[37-39]. This sophisticated engineering tool to assess gene function at the organismal level is also applicable to assay *in vivo* functionality of miRNA. A recent study reported that BAC-encoded miRNAs designed to target the *Gpc5* gene in an apodocyte-specific manner effectively silenced expression of this potential mediator of nephrosis, and thereby alleviated nephrotic symptoms[40]. The same BAC-based methodology would have success in loss-of-function studies of miRNA by specifically expressing its antisense antagonist. In this article, we describe a BAC transgenic mouse in which the miR-29 family members are antagonized through the BAC engineering exclusively in the brain under the control of an astroglia-specific promoter activity.

2 Materials and Methods

Engineering of BAC clones

The BAC transgene for this study was prepared using the standard lambda-Red recombination protocol[41] with minor modifications. The Red recombination machinery is composed of three lambda-phage proteins, exo, beta and gam[42]. Exo, an exonuclease, processes linear double-stranded DNA and provides 3'-single-stranded overhangs, and then beta binds the 3'-overhangs and catalyzes strand annealing/exchange reactions starting from single-stranded DNA extremities to form recombinant DNA. To stabilize the DNA substrate in host cells, the recombination event is assisted by gam which prevents the host endogenous RecBCD nuclease from degrading the substrate. The basic principle of this method requires a pair of DNA fragments longer than 42 bp, designated homology arm, homologous to the target site being attached on the termini of the substrate DNA in order for recombination to take place.

Assembling of two BAC clones into a single clone was performed fundamentally as previously described[43]. This method consists of two recombineering steps mediated by the aforementioned Red recombination system. In the first step, a large DNA segment of one BAC insert was subcloned into a retrieving vector. In the second step, the subcloned DNA was recombined with the other BAC insert in a site-specific manner via an overlapping area between the two BAC inserts, yielding a chimeric BAC clone that holds a larger regulatory element dictating a more faithful expression of the BAC driver gene.

A targeting DNA construct to express miR-29 antisense RNA, anti-miR-29, was designed in such a way that a homology arm comprising 50 residues of nucleotides immediately ahead of the translation initiator codon ATG present in the second exon (exon 2) of *Ntrk2* was followed by three tandemly arranged anti-miR-29 DNA sequences directed individually against miR-29a, -29b, and -29c[44], the polyadenylate addition signal of the herpes simplex virus thymidine kinase gene, and lastly another 50-bp homology arm corresponding to the sequence just behind the *Ntrk2* start codon (from 5'- to 3'-termini: Supplementary Fig. 1). These anti-miR-29 modules were placed between 5'- and 3'- flanks of the mouse miR155 precursor that served as a platform to aid proper processing of the co-cistronically synthesized anti-miR-29 transcripts by nuclear Drosha ribonuclease.

The modified BAC DNA was linealized and purified for the following microinjection experiment according to a published procedure[45]. The very large DNA fragment was separated by pulsed-field gel electrophoresis on a 1% agarose gel after PI-*Sce*I digestion, and dialyzed by ultrafiltration against a microinjection buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 100 mM NaCl, 30 μM spermine, 70 μM spermidine).

miRNA-specific quatitative RT-PCR (miR-qRT-PCR)

Total RNA was extracted from mouse tissues with Trizol reagent (Invitrogen). Small-sized RNA was enriched from total RNA by incubation with PEG 8000[46]. RNA quality and concentration was measured using an Agilent 2100 Bioanalyzer and a NanoDrop ND-1000 spectrophotometer, respectively.

Mature miRNAs were quantified with TaqMan MicroRNA Assays and TaqMan premade probes (Assay ID: miR-29a=5'-UAGCACCAUCUGAAA UCGGUU-3', 000412; miR-29b=5'-UAGCACCAUU UGAAAUCAGUGUU-3', 000413; miR-29c=5'-UAG CACCAUUUGAAAUCGGU-3', 000415) as recommended by the manufacturer (Applied Biosystems). One hundred nanograms of total RNA was reverse transcribed to cDNA with miRNA-specific stem-looped RT-primers, and PCR-amplified in triplicate using a 7500 Real-Time PCR System (Applied Biosystems). The PCR conditions were as follows: 95℃ for 10 min followed by 60 cycles of 95℃ for 15 sec and 60℃ for 1 min. Data were normalized by the 2-ΔΔCT method using snoRNA202 as internal reference.

Northern blotting

Total RNAs (10 μg per sample) were electrophoresed on a 1% agarose-2% formaldehyde MOPS gel, transferred to a positively charged nylon membrane, and fixed to the membrane by UV crosslinking. A 699-bp DNA fragment corresponding to the nucleotide positions 868-1566 of the mouse *Ntrk2* cDNA (GenBank NM_008745) was amplified by PCR and cloned into the pGEM-T vector (Promega). Antisense *Ntrk2* transcripts were obtained from the expression construct and digoxigenin-labeled with DIG RNA Labeling Mix (Roche) to use as a probe. The membrane after blotting was hybridized with the RNA probe overnight at 68℃ in DIG Easy Hyb (Roche). Excess probe was washed away twice at 68℃ with 2 × SSC/0.1%SDS twice, and then twice with 0.1 × SSC/0.1%SDS. The probe on the membrane was

incubated with alkaline phosphatase-conjugated anti-DIG antibody and CSPD substrate (Roche) to detect chemiluminescent signals. For re-probing, the probe was stripped from the membrane at 75℃ in Strip Buffer (Roche).

Animal experiments

All experiments were conducted at Utsunomiya Institute of PhenixBio Co., Japan, in accordance with national and institutional experimental guidelines, and were approved by the institutional animal care and use committee of this corporation. Purified BAC transgene was microinjected into 320 pronuclei of fertilized oocytes from C57BL/6N mice, 300 of which were subsequently implanted into the oviducts of foster mothers. All animals used in this study were C57BL/6N mice housed at standard temperature (21℃) and in a light controlled environment (12:12 h light-dark cycle) with *ad libitum* access to the food and water under pathogen-free conditions.

3 Results

Screening for brain-specific genes

The first step in designing BAC transgenic mice was to identify genes suitable for the BAC driver gene showing an expression mode that satisfied our requirements. Since our interest is focused on the functional significance of miR-29 in the CNS and, in addition, miR-29 is astrocytic in the adult brain, expression of the driver gene should be regionally restricted to the brain tissue, preferably to astroglia at high levels. A global gene expression analysis of the mouse CNS using DNA microarray technology[47] suggested several candidate genes that met these criteria. Among these, the *Ntrk2* gene, also known as *trkB*, encoding neurotrophic tyrosine kinase receptor type2, was found to be expressed preferentially in astroglia and more abundantly than the *Gfap* gene, a representative astrocyte-specific marker gene. We hence examined the tissue specificity of the *Ntrk2* expression by Northern blotting.

We isolated the total RNAs from brain, heart, lung, liver, spleen, thymus, kidney, testis, ovary, and adrenal gland of adult C57BL/6N mice, and compared the abundance of *Ntrk2* transcripts among these RNA preparations. The RNA blot after probing exhibited strong signals specific to *Ntrk2* almost exclusively in the brain sample (Fig. 1). Accordingly, we chose the *Ntrk2* gene as the BAC driver gene used in this study. The several species of transcripts with different sizes appearing in this electrophoretogram were diagnostic of alternative splicing events[48,49] and alternative promoter usage[50] of this gene. Besides, a trace amount of smaller transcripts were observed in total RNAs from ovary and adrenal gland. This result fundamentally agreed with a previous study that analyzed mRNA preparations derived from multiple tissues of adult mice for the specificity of *Ntrk2*

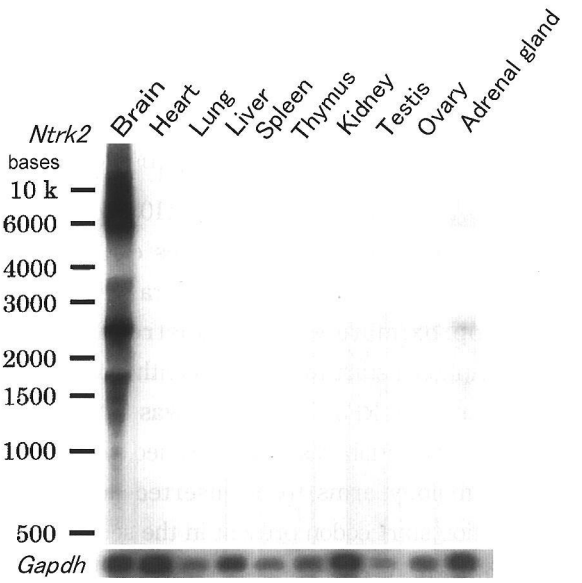

Fig. 1. Northern blot analysis of *Ntrk2* expression in adult mouse tissues.

Upper panel: 10 μg each of total RNA extracted from brain, heart, lung, liver, spleen, thymus, kidney, testis, ovary, and adrenal gland (from left to right) of 8-week-old mice were electrophoretically separated on a denaturation agarose gel, immobilized onto a blotting membrane, and hybridized with a labeled RNA probe specific to the mouse *Ntrk2* gene. The migration positions of co-electrophoresed RNA size standards are indicated to the left of the panel.

Lower panel: The same RNA blot was re-probed for the mouse glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) transcript as loading control.

expression[51].

Construction of *Ntrk2*::anti-miR-29 BAC transgene

The outline of preparation processes of the BAC targeting construct used here was illustrated in Supplementary Fig. 2. We accessed to the University of California at Santa Cruz (UCSC) Genome Browser (http://www.genome.ucsc.edu) and located BACs that carry the *Ntrk2* locus as the driver gene fraction (Supplementary Fig. 3). To achieve a high rate of success in reproducing the expression pattern of a gene of interest, it is necessary for the BAC insert to accommodate the entire transcription unit and all of its associated regulatory elements. Because the endogenous transcription region of *Ntrk2* encompasses more than 300 kb in size, covering all of the genomic information required for its optimal expression within a single BAC is practically impossible. As such, to obtain a larger functional domain of this gene, we planned to fuse two BAC clones, RP32-391J8 and RP32-192I4, by a two-step recombineering method[43]. The RP32-391J8 BAC spans approximately 110 kb of the 5'-flanking portion and twelve exons of the *Ntrk2* gene, while the RP32-192I4 BAC ranges from a site approximately 30 kb upstream of the transcriptional start to the fourteenth exon of this gene. First, the RP32-391J8 BAC was tagged with a counter-selection cassette, *Rpsl*-neo, flanked by two homology arms to be inserted across the translation start codon present in the second exon (exon2) of *Ntrk2*. The BAC DNA and the counter-selection cassette were co-transformed into electro-competent *E. coli* host cells expressing Red recombination machinery. Cells were selected on agar culture media supplemented with chloramphenicol and kanamycin for transformants that underwent recombination between the counter-selection cassette and its target site on the driver gene *Ntrk2*. The marker-tagged *Ntrk2*::*Rpsl*-neo BAC was subsequently transformed in parallel with a linear form of a retrieving vector into fresh Red-proficient bacterial host cells. The retrieving vector consists of the *SacB* gene[52], an ampicillin resistance gene, and two terminal sequences for recombination, one from the 5'-breakpoint of the BAC vector backbone and another from a stretch of nucleotide residues immediately downstream of the *Ntrk2* ATG start codon. In the resulting ampicillin/kanamycin-resistant cells, homologous recombination has occurred to excise a DNA segment 80 kb upstream from the ATG codon and integrate it into the retrieving vector. After the cloning of the 80 kb DNA segment, the retrieving vector was linealized by restriction enzyme and electroporated into bacterial cells harboring RP32-192I4 and the Red-encoding plasmid. Another homologous recombination was induced between the retrieved 80 kb DNA segment and RP32-192I4 through the BAC breakpoint and the overlapping sequence 3' to the *Ntrk2* start codon to yield kanamycin/sucrose-resistant transformants. The fusion BAC thus obtained accommodates the 5'-flanking segment of *Ntrk2* extended to 80 kb further upstream relative to RP32-192I4. In the final step, the counter-selection cassette inserted into the driver gene was replaced by the modification cassette to express anti-miR-29 RNAs. The positive recombinants, *Ntrk2*::anti-miR-29, were identified by a counter-selection for loss of streptomycin sensitivity. DNA sequencing for the final construct confirmed the correct sequence and insertion of the modification cassette.

Establishment of *Ntrk2*::anti-miR-29 BAC transgenic mouse lines

To generate BAC transgenic mice that express antisense RNA against miR-29, the recombinant BAC thus prepared was digested with PI-*Sce*I, purified by pulsed-field gel electrophoresis, and used for pronuclear injection into C57BL/6N mouse zygotes. A total of 300 eggs were transferred into pseudo-pregnant females and 57 pups were born. These potential founder mice were genotyped by Southern blotting of genomic DNA from their tails using a probe specific for

the anti-miR-29 insert sequence, and 16 pups were found to carry the BAC construct. Out of these, six transgenic founders were backcrossed with wild-type mice for germline transmission. The resulting F1 mice were also screened for transgenesis by Southern blot analysis of genomic DNAs collected from tail biopsy. As a result, about two-thirds of the offspring were positive for genomic integration of the BAC transgene, and these transgenic mice were detected to integrate one to ten copies of the transgene into their genome (Supplementary Fig. 4). A preliminary assessment of transgene expression by Northern analysis using probes for anti-miR-29 sequences revealed that a successful expression and processing of anti-miR-29 transcripts from the BAC construct occurred in the brain tissue of five independent transgenic lines (not shown, see below). As expected, all lines of transgenic mice overexpressing anti-miR-29 in the brain were born normal and did not display any gross anatomical and behavioral abnormalities from birth to weaning, and also no significant differences in the mortality of pups was noticed during puberty between the transgenic and non-transgenic animals. Based on the expression level of the antisense transgenes, we chose one transgenic founder line, the highest expressing line which incorporated ten copies of the transgene, for a more detailed phenotypic analysis.

Local miR-29 knockdown in *Ntrk2*::anti-miR-29 BAC transgenic mouse

The activity of anti-miR-29 transcribed from the BAC transgene in the transgenic founder line was assayed by monitoring the amount of miR-29 using qRT-PCR that specifically amplified the mature form of miR-29. Since expression of the anti-miR-29 is under the control of the *Ntrk2* promoter, its inhibitory effect on PCR amplification of the signal for miR-29 would

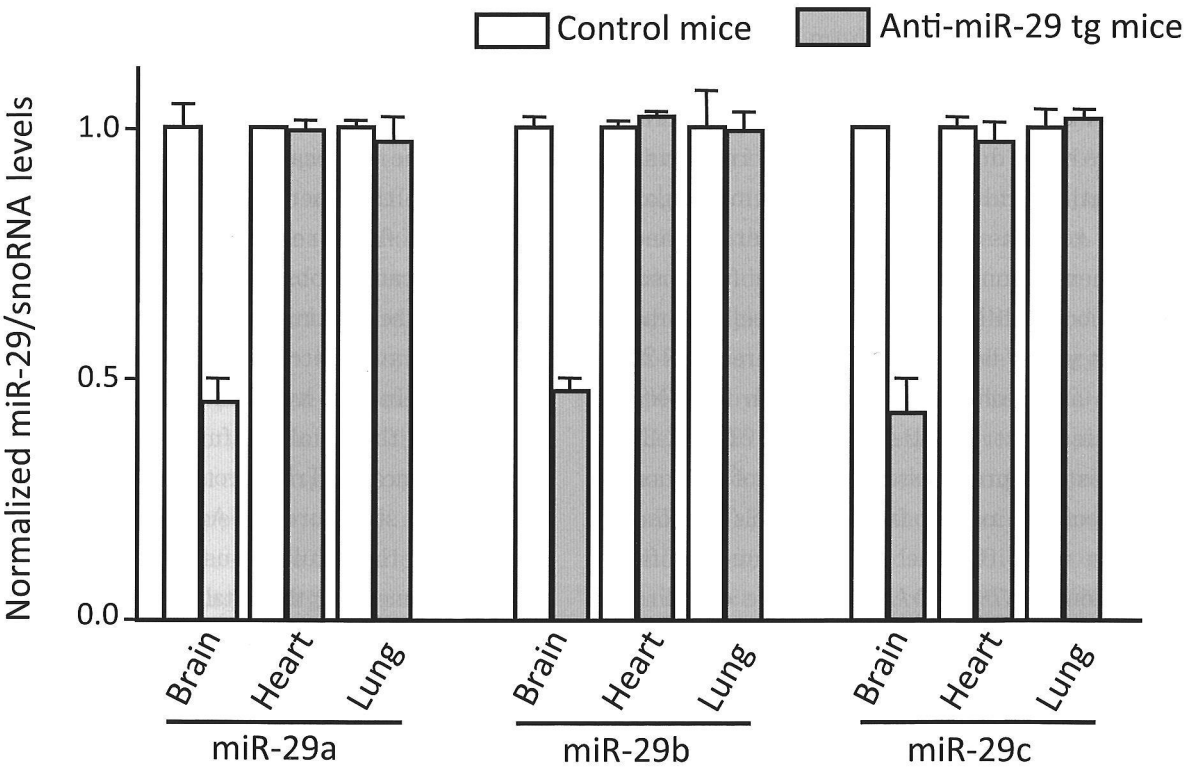

Fig. 2. Quantitation of *in vivo* miR-29 knockdown.

Total RNA was prepared from dissected brain, heart, or lung of 2-month-old F1 female mice derived from a representative anti-miR-29 BAC transgenic founder and its non-transgenic female littermate (control). Expression levels of mature miR-29 in each sample were assayed by miR-qRT-PCR and represented relative to those in the control mice. Shown are mean and SEM, n=3.

depend on the regional specificity of endogenous *Ntrk2* expression. As shown in Fig. 2, we confirmed the down-regulation of all members of the miR-29 family in brain samples from the adult transgenic founder, with values for miR-29a, miR-29b and miR-29c being 2.2-, 2.1- and 2.4-fold decreased, respectively, when compared with non-transgenic littermate controls. We assessed in parallel the miR-29 repression by miRNA antagonism in the adult heart and lung that also express a high level of miR-29, but the results revealed no changes between the transgenic and non-transgenic mice for every member of the miR-29 family. These data demonstrated significantly reduced levels of mature miR-29 in the BAC transgenic mouse brain, whereas its levels in RNA samples from heart and lung were unaffected, consistent with the result of Northern analysis (Fig. 1) that indicates the brain specificity of endogenous *Ntrk2* expression.

4 Discussion

Astrocytes are the most abundant cells in CNS. Although astrocytes are essential in normal CNS function and are involved in most neuropathlogical conditions, their molecular identity remains largely uncharacterized and comprehensive understanding of their functional interaction with local neighboring cells in the CNS has yet to be resolved. Since three miR-29 paralogs are localized to astrocytes in the CNS, an efficient astroglial expression of miR-29 antagonists is prerequisite for loss-of-function analyses of this miRNA family. For this purpose, we here exploited the neural-tissue-specific expression of *Ntrk2* (*trk*B), the gene encoding neurotrophic tyrosine kinase receptor type2, or tropomysin-related kinase B (TrkB). TrkB is a tyrosine kinase receptor with high affinity for brain-derived neurotrophic factor (BDNF) and neurotrophin (NT) -4/5[53, 54]. The Trk family of tyrosine protein kinases, TrkA/B/C, are signal transducers that mediate the biological effects of nerve growth factor (NGF), BDNF, NT-3, and NT-4/5, which are collectively named the NGF family of neurotrophins. Neurotrophins and their Trk receptors play pivotal roles in the development and maintenance of the vertebrate nervous system, where they regulate neuronal survival, axonal growth and guidance, synaptic plasticity, and long-term potentiating events. Two types of TrkB isoforms with distinct signaling capacities are known to result from alternative splicing of the same pre-mRNA: one is the full-length form with a classical tyrosine kinase activity and another lacks most of the cytoplasmic region including the kinase catalytic domain. The *Ntrk2*gene is broadly expressed in a wide variety of neural structures during serial passage of the life course[55]. During embryonic development, *Ntrk2* expression can be first detected at a time when the central and peripheral nervous systems are in the process of differentiating into anatomically recognizable structures. In later-stage embryos, the pattern of *Ntrk2* expression parallels the developing structures of the central and peripheral nervous systems. In the adult brain, *Ntrk2* transcripts can be found in most structures, including the cortical layers, the thalamus, the hippocampus, the lateral choroid plexus, and the ependymal lining of certain ventricles. A more restricted pattern of expression can be observed in the cerebellum, where only the Purkinje cell layer and the caudal peduncle have detectable levels of *Ntrk2* expression. In situ hybridization studies have shown that the catalytic full-length and non-catalytic truncated TrkB proteins are expressed in different structures[48]. Analysis of coronal sections of the adult mouse brain revealed transcripts encoding the catalytic isoform in the cerebral cortex, the thalamus, and pyramidal cell layer of the hippocampus, therefore suggesting a primary role in neurons. In contrast, messages for non-catalytic isoform appear to be most prominent in structures predominantly comprising non-neuronal cells such as the choroid plexus and ependymal cell layer of the ventricles, which raises the possibility that the non-catalytic

receptor may play a role in the transport and/or clearance of TrkB ligands. Other observations quite agree with such a rather distinct pattern of expression for the splice variants of *Ntrk2* mRNA in neuronal and non-neuronal cell types[56-59]. Although the molecular mechanisms that control the differential spatial expression of *Ntrk2* have not fully been clarified, these findings favor our strategy that selected the *Ntrk2* locus as the BAC driver gene for neural expression of the miR-29 antagonist.

Thus, in this study, we set out to use regulatory regions of the *Ntrk2* locus to create a mouse line allowing brain-specific antagonism of the miR-29 family, where expression of miR-29 antagonists is driven by the *Ntrk2* promoter. However, isolation of specific regulatory elements is a significant scientific challenge. *cis*-Active sequences that control the proper gene expression may be positioned far upstream of the transcription start site or far downstream of the polyA addition signal. If there is very little 5'- or 3'-flanking DNA present, then expression may not be accurate. The conventional transgenic approach has widely been employed to study gene expression and its regulation *in vivo*, but it also has its limitation: position effects, that is, expression of the transgene is influenced by its integration site, which may manifest in different forms such as lack of transgene expression, ectopic expression, and mosaic expression. The major cause for position effects is lack of important regulatory domains in the transgene construct. Therefore, transgene expression that mirrors the expression of the native gene requires not only the entire transcription unit but also its surrounding intergenic portions to be present on the targeting construct used to produce transgenic animals. For BAC transgenic studies, BAC clones that contain at least 50 kb flanking genomic sequence both 5' and 3' to the transcribed unit of diver gene are desired for accurate reproduction of its endogenous expression pattern[37,38]. For a mammalian gene that is smaller than 100 kb in size, which is typical of most genes, one can easily obtain BAC clones that satisfy this criterion. For the small number of mammalian genes that are larger than 200 kb such as *Ntrk2*, reports thus far indicate that single BAC targeting construct is relatively inconsistent in achieving faithful transgene expression *in vivo*. Accordingly, we selected two overlapping BAC clones of *Ntrk2*, and combined them into a larger unified BAC carrying more than 100 kb of the 5'-leader region in addition to the *Ntrk2* coding exons. Although the fusion BAC construct lacks almost of the 3'-flanking sequence, the present results show that this BAC is likely to confer an endogenous-like expression pattern and is less susceptible to position effects. Presumably, because of its large size, the BAC encodes locus control elements sufficient to prevent silencing and misregulation of the transgene by local chromatin.

Induction of stable loss-of-function phenotypes for individual miRNAs is required to explore the miRNA function. Previous studies using chemically modified oligonucleotides complementary to a particular miRNA such as antagomir[60] and LNA-antimiR[61,62] demonstrated that functional antagonism of miRNA by the cognate antisense oligonucleotide specifically relieves the repression of the miRNA targets, which is due to intracellular formation of stable heteroduplexes between the miRNA and its RNA antagonist. By analogy, our data suggest that specific hybridization of the anti-miR-29 RNA to the miR-29 members before reverse transcription hampered the amplification of mature miRNA by miR-qRT-PCR. While the molecular mechanism underlying the reduced miR-29 detection in the presence of the BAC-derived antisense transcripts is currently not well-known, combined data are in line with a model proposing that a competition occurs between the stem-looped RT primer and the antisense molecule for binding to miRNA as their common target, which interferes with primer annealing for subsequent reverse transcription. The present study indicates the utility of the BAC-based methodology in

functional silencing of a particular miRNA. The conditional knockdown mouse for miR-29 that we have generated here would provide a powerful *in vivo* tool for identifying endogenous target genes of miR-29 and for studying the biological role of miR-29 and miR-29-associated gene-regulatory networks in the adult mouse brain, thereby serving as a valuable model animal in which the function of miR-29 in CNS can be specifically dissected from the mechanistic perspective of mammalian species-specific lifespan.

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脳特異的 miR-29 ノックダウンマウス

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要　約

miR-29 は哺乳類生物種固有の寿命決定プログラミングの中心的存在として働いている可能性を有するマイクロ RNA の 1 種であるが，遺伝子の in vivo 機能の解明において安定な機能損失型表現形質の構築は不可欠である。今回，生物寿命の決定機構と脳組織での miR-29 の機能的役割との関係解明の手段として，miR-29 の機能阻害性アンチセンス鎖を脳内で特異的に発現する BAC tg マウスを作製した。まず，脳特異的に発現し，かつ miR-29 が主に存在するアストロサイト内での発現性が特に高い遺伝子として神経栄養因子受容体の 1 種 TrkB の遺伝子 *Ntrk2* を特定した。つぎに，*Ntrk2* の遺伝子座が挿入されている 2 種の BAC クローンを Web 検索により選択し，これらの *Ntrk2* 遺伝子領域間の組換えにより 100 kb 以上の上流発現調節領域を包含した融合 BAC を作製した。一方で，miR-29 の機能阻害活性を有するアンチセンス配列をデザインした後，この配列を含む遺伝子カセットを相同組換えにより融合 BAC 内の *Ntrk* プロモーター下流に組込んだ BAC 型トランスジーンを構築した。このトランスジーンを C57BL/6 系マウスの受精卵に注入して得られた産仔の中から，染色体にトランスジーンが導入されたファウンダー個体をサザン解析によりスクリーニングした。さらにこのファウンダーと野生型マウスを交配して F1 個体を得た。この F1 個体の脳，心臓，肺の各組織中の成熟体 miR-29 を RT-PCR で定量したところ，脳のみで miR-29RNA コピーの増幅抑制が認められた。