Global profiling of gene expression in mouse astrocyte in response to the potential longevity determinant miR-29

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Synopsis

MicroRNAs (miRNAs) are an evolutionarily conserved large class of small non-coding RNAs that mediate post-transcriptional silencing of genes and influence a broad spectrum of biological processes ranging from embryonic development to organismal death. Our previous study identified the miR-29 family, three paralogous species of miR-29a/b/c, as the most predominantly expressed small RNA in aged mouse brain compared to the neonate one. Mouse brain miR-29 is highly astrocytic. Its expression is quiescent during early brain development, and then steadily increases to a plateau state around reproductive maturity. To explore the functional relevance of miR-29 expression in early life to the neural physiology of the mouse brain from the mechanistic perspective of mammalian species-specific lifespan, we here undertook a gain-of-function approach through exogenous expression of miR-29 in astrocyte from mouse fetus and surveyed the resulting alteration in both the transcriptional and translational levels. DNA microarray analyses retrieved a total of 5,589 genes showing temporal significant expression changes in the miR-29-transfected fetal astrocytes, and classified them into two gene groups: positively or negatively regulated by miR-29. Mass spectrometry (MS)-based quantification of translational products of miR-29-responsive genes identified 18 species of miR-29 target candidates. We performed functional enrichment analyses utilizing bioinformatics resources to characterize the gene sets thus identified, and found their expression trend that favor the processes for facilitating cell differentiation while supporting normal cell proliferation /survival, which is somewhat different from the functional signatures of miR-29 as observed at adult stages, implying a pleiotropic property of miR-29 depending on the developmental context. Our present results strongly suggest that miR-29 in the developing mouse brain serves as the central coordinator to shift the global gene expression toward adult phenotypes, through which ensure the programmed transition in the life course to the post-developmental/reproductive stage which has inherently been set to delimit the mouse life potential.

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

1 Introduction

We hold that organismal lifespans are specified in a species-specific manner by a biological program set late in the life cycle, in which an as-yet-unidentified molecular entity that presides over a long run of downstream cellular processes contributing to the completion of the life program[1]. Since the brain is the dictator of all the physiological functions of higher animals, it is reasonable to assume that the life-controlling factor for advanced ages plays the majority of its

role in the central nervous system. Recent evidence in a primate brain study indicated that the dynamics and regulatory interaction of gene expression during post-natal development and aging are functionally connected: there are few gene expression changes that can be exclusively associated with aging, and instead, many expression change patterns that have previously been attributed to aging, such as down-regulation of genes involved in neuronal functions, already start to emerge in childhood[2]. This continuity observed between the developmental and age-related expression trends strongly supports our notion. We speculated that some development-associated small non-coding RNA plays leading roles in delimiting the mammalian lifetime by analogy to the case of ciliated Protozoans, and performed an in-depth survey of small-RNA expression by a high-throughput sequencing on libraries derived from young and old mouse brains[1]. This screening detected several species of microRNA (miRNA) as the major expression alterations between the two age cohorts. Among these miRNAs, we identified the miRNA-29 family composed of three paralogous members, miR-29a, miR-29b and miR-29c, to be the most robustly up-regulated in the aged brain. In the mouse brain, expression of miR-29, which is minimal throughout the embryonic and perinatal period, becomes progressively prominent from the juvenile stage[3, 4]. After reaching the highest level in early adulthood, its abundance remains essentially unchanged until the termination of life[1, 5]. miR-29 expression in adult brain is almost unique for astrocyte[6,7], but is widespread the entire regions of this organ system[8-10]. These transcriptional signatures of miR-29 fit the working model presented above for the hypothetical central player that programs the later passage of the life course well, making miR-29 the most likely causal determinant of the murine life potential.

miRNAs are small regulatory RNAs that are expressed in animals and plants, and affect the translation or stability of target mRNAs. The biogenesis of miRNA is rather diverse. The 19-24 nt single-stranded miRNAs are derived from longer primary transcripts termed pri-miRNAs[11, 12]. The pri-miRNAs, which can be more than 1000 nt, contain multiple hairpin-like structures in the case of about the half of mammalian miRNAs. The hairpins, each of which typically comprises 60-120 nt, are cleaved from the pri-miRNA in the nucleus by the double-strand-specific ribonuclease Drosha[13, 14]. The resulting precursor miRNA, pre-miRNAs, are transported to the cytoplasm via a process that involves Exportin-5[15,16]. The pre-miRNAs are further processed by a second ribonuclease named Dicer[17] to generate short, partially double-stranded RNAs in which one strand is the mature form of miRNA. The mature miRNAs are taken up by a protein complex similar to the RNA Induced Silencing Complex (RISC), termed miRISC or miRNP, that supports RNA interference, and the miRNA-bound complex functions as a post-transcriptional gene regulator to degrade or translationally repress its mRNA target[18, 19]. Although animal miRNAs mainly inhibit translation, they can also induce degradation of their mRNA targets[20]. The molecular function of miRNA can be characterized by using tools like those for analyzing protein-encoding genes. Overexpression or underexpression of the miRNA is performed to obtain gain-of-function or loss-of-function phenotypes, respectively, through which we can identify genes and cellular processes that are modulated by this small functional RNA[21, 22].

Numerous biological phenomena such as organismal development, cell fate determination and even diseases involve the activity of a large subset of miRNAs working in a spatiotemporally restricted manner[23-25]. Brain development and aging are also thought to progress in association with highly dynamic waves of miRNA levels, with specific groups of miRNA being expressed only at limited time-points in serial passages of the processes, which induce dramatic shifts in mRNA and protein profiles[26-29]. A detailed gene

expression map of mouse astrocyte depicted by miR-29 would offer insight as to whether this miRNA serves as the upstream integrator of physiological responses appearing at a specific phase in the murine life cycle, and if it holds true, how miR-29 programs molecular events contributing to the life design. Microarray technology has increasingly been exploited as a powerful tool to tabulate the transcriptional alterations of thousands of genes simultaneously and address the decoding of their complex biological implications[30]. Ectopic miRNA expression in conjunction with subsequent DNA array assessment is now routinely applied to high-throughput screening for miRNA-targeted genes. These putative miRNA targets have been observed to be enriched among the population of mRNAs down-regulated by the exogenously added miRNA[31]. Admittedly, the microarray-based transcriptomic approach is well established in the study of unraveling gene regulatory networks on a genome-wide scale, but its combination with proteomics, which implicates all the cellular proteins functionally in the genetic networks, would provide further information on miRNA-guided post-transcriptional control of biological processes. As such, here we explored the overall pattern of gene expression regulated by the miR-29 family using gene array analysis of mRNA transcripts in parallel with proteomic cataloging of translation products.

2 Materials and Methods

Cell culture and miRNA transfection

Primary astrocyte cultures prepared from cerebral cortexes of fetal C57BL/6 mice (embryonic 18-day old) were purchased from ScienCell Research Laboratories. Cells were always cultured and maintained in poly-L-lysine-coated plastic wares (AGC Techno Glass, Japan) supplied with Astrocyte Medium (ScienCell) following the supplier's recommendations. Astrocytes were transfected in six-well plates (AGC) containing the medium supplemented with 80 nM of miR-29 (Ambion Pre-miR hsa-miR-29a/b/c Precursors) or control miRNA (Ambion Pre-miR Negative Control1) each, using RNAiMax (Invitrogen) in accordance with manufacturer's protocols. The miR-29 sample used for transfection was prepared by mixing the three miR-29 isoforms in a 1:2:1 molar ratio of Pre-miR-29a:-29b:-29c Precursors. The transfection was conducted three weeks after the initial plating. After 48 hr posttransfection, cells were harvested and processed for preparation of RNA and protein samples. The transfection efficiency was above 90% as determined using a fluorochrome-conjugated miR-29 precursor (TAKARA BIO).

miRNA array experiments

RNA extraction was performed in triplicate for each cell type. Total RNA was isolated from transfected astrocyte cells using an RNAiso Plus (TAKARA BIO). The quality of RNA was assessed using an Agilent 2100 Bioanalyzer, which is indicative of the integrity of every RNA sample being sufficient for downstream assays. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer. miRCURY LNA microRNA Array 5th generation-mmu (Exiqon) was used as an array platform. Total RNA (1 μg) derived from miR-29 transfectants (test sample) was directly labeled with Hy-5 (Oyster-656) with a miRCURY LNA Power Labeling kit (Exiqon). As a reference, total RNA prepared from cells transfected with the control miRNA (control sample) was used and was labeled with Hy-3 (Oyster-556) by the same procedure. Hybridization and washing of the array slides were carried out as recommended by Exiqon. We used a GenePix 4000B scanner (Molecular Device) to scan the microarrays, and then Array-Pro Analyzer ver.4.5 software (Media Cybernetics) to extract, background-correct, LOWESS-normalize and summarize spot intensities. Fold changes in the intensity of the hybridization signal were calculated with respect to the test sample relative to the control sample for three pair wise comparisons between the two

samples, and were averaged to generate a mean value.

DNA array experiments

Sample labeling, hybridization and washing were performed following the standard protocol detailed in the Agilent One-Color Microarray-Based Gene Expression Analysis ver.5.7. Briefly, a One-color Spike-Mix was diluted 1000-fold and a 5-μl aliquot of the diluted mix was added to every 0.5 μg of total RNA samples (three each of extraction replicates of the test and control samples) prior to labeling reaction. The labeling reaction was carried out separately for these RNA samples using a Quick Amp Labeling Kit one-color, in the presence of cyanine 3-CTP. The dye-labeled target (1.6 μg as cRNA) was fragmented and hybridized on an Agilent Whole Mouse Genome 4X44K microarray at 65° C for 17 hr with a Gene Expression Hybridization Kit. The hybridized slide was washed in Gene Expression Wash Buffer 1 at room temperature for 1 min, which was followed by a wash for 1 min in Gene Expression Wash Buffer 2. The processed microarrays were scanned using an Agilent DNA Microarray Scanner. Data extraction from raw image files was done with Agilent Feature Extraction software ver.10.7. Statistical comparison between two independent sample data was made using the Student's t-test, and multiplicity correction was followed to control the false discovery rate (FDR) by a step-up approach as described[32], with an FDR of 5%. The DNA array dataset has been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) and is accessible through GEO Series accession number GSE27035.

MS analysis of proteins

Cellular proteins were extracted with M-PER Mammalian Protein Extraction Reagent (PIERCE). Protein concentration was determined by the Bradford method[33]. For quantitative proteomic comparison, stable-isotope-labeled profiling was approached utilizing iTRAQ reagents[34, 35]. Total cellular protein (120 μg) was precipitated by the addition of five volumes of acetone at −20°C for 2 hr. After being dissolved, the protein was denatured with SDS, reduced with TCEP, alkylated with MMTS, and digested with trypsin as described in the iTRAQ protocol (AB SCIEX). Thereafter, isobaric tagging iTRAQ reagent was added to label the protein digest. Test samples from miR-29-treated cells prepared in duplicate were labeled using mass 117 and 118 isobaric tags, while the reagents of mass 119 and 121 were used to label control samples from duplicated control-miRNA transfection. The resulting four labeled peptide pools were mixed together in a 1:1:1:1 ratio. The mixture was charged on a PolySulfoethyl A strong cation exchange (SCX) column (PolyLC) equilibrated with 10 mM potassium phosphate, pH 4.5, 20% CH_3CN, and fractionated with a stepwise gradient from 40 to 350 mM KCl in the equilibration buffer. The SCX fractions were desalted using a Sep-Pak Light C18 cartridge (Waters) isocratically with a loading solution (5% ACN. 0.1% formic acid) and an eluting solution (80% ACN, 0.1% formic acid). A DiNa System equipped with a HiQ sil C18P-3 column (KYA TECH) was interfaced to a QSTAR Elite Hybrid mass spectrometer (AB SCIEX), which was used for electrospray analysis of the peptide samples. MS TOF scans were acquired from m/z 400 to 1800 with up to two precursors selected for MS/MS from m/z 100 to 2000. Analyst QA software ver.2.0 (AB SCIEX) was used for data acquisition, and then each peptide was identified and quantified using ProteinPilot software ver.4.0 (AB SCIEX). The sequence data were searched against the IPI mouse protein database (release 3.75) with a total of 113,730 entries. The search parameter was set to a confidence limit of 95%, which corresponds to a ProteinPilot cut-off score of 1.3. The average iTRAQ signal ratios from different runs were calculated for each peptide using the Paragon Algorithm. The statistical significance was determined by a one-sample t-test. Fold-change values were expressed as the

overall average signal ratio of the test samples relative to the control samples.

Computational searches

Programs and datasets used to search for potential mouse miRNA targets were downloaded from the miRecords website at http://miRecords.umn.edu/miRecords. This informatics resource consists of an integration of predicted miRNA targets produced by 11 established algorithms such as miRanda and a collection of experimentally verified miRNA-target gene pairs[36]. As, in general, wobblingly paired and loosely conserved interactions are known to occur between animal miRNAs and their targets, our correlations made in this work were not restricted to only stringent target predictions and provided a wider range of target candidates.

For all genes that showed a significant expression change, we conducted secondary tests for functional enrichment. We adopted here GO (Gene Ontology) terminology and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway mapping, and assigned the genes to broad functional categories. GO annotates gene products further for biological processes, cellular components and molecular functions, and describes how they behave in distinct cellular situations[37]. The KEGG program provides a reference knowledge base for linking genomes to biological systems, categorized as building blocks in the genomic space and the chemical space, and wiring diagrams of interaction networks and reaction networks[38]. For the category assignments, we calculated the number of times each gene associated with each of the GO functional terms and KEGG regulatory pathways, and then determined statistical significance (global enrichment p-value). A category is considered to be significantly enriched in a defined gene set if it contains a greater number of functionally related genes than expected by chance. The Ensemble gene-GO data were downloaded from the Ensemble database, and the Ensemble gene-KEGG data from the KEGG database.

3 Results

miRNAs affected by miR-29 overexpression in fetal astrocytes

We first assayed for miRNA expression varied when external miR-29 was taken up by astrocytes from perinatal mice in which endogenous expression of this miRNA is yet at basal levels. Total RNA including small-sized transcripts from the miR-29 transient transfectants (test sample) was fluorochrome-labeled and loaded on a miRNA array comprising 710 species of probes for all mouse miRNAs in release 14.0 of the miRBase microRNA Registry. As a control experiment, the total RNA from the cells transfected with control miRNA (control sample) was subjected to the same measurement. The normalized expression signal intensities were monitored against the 710 miRNA species and selected for miRNAs with a differential expression fold change of at least twofold.

The expression profile of the miR-29-derived test sample displayed more than 19-fold transcriptional increase for every miR-29 family member compared to the control sample. This result verifies the successful incorporation and subsequent maturation of the miR-29 precursor molecules delivered to the astrocyte culture. We did not consistently detect more than a twofold up-regulation for any other miRNA species present in the test sample. Conversely, of the down-regulated miRNAs in the miR-29 transfectants, only miR-22 showed a severely reduced expression, with about 48% of the control level. This transcriptional decline in miR-22 is speculated to be ascribable to the miR-29 overexpression and will be discussed later. A master data table of our full results is shown in Supplementary Table 1.

miR-29-induced transcriptional alterations in fetal astrocyte

We next identified the genes that undergo

expression changes in miR-29-transfected cells by a DNA microarray survey using the same RNA samples as in the preceding miRNA profiling. Transcripts in the test sample derived from miR-29 transfectants were screened for robust expression signals on a mouse genome array which houses a probe set of 41,000 species of oligonucleotide DNA.

By comparing with mRNA expression profiles of the mock-delivered control sample at a 5% FDR threshold, we consistently detected 5,589 unique genes that exhibited a greater than 1.5-fold significant (p <0.025) difference in expression between the two samples, of which 2,671 genes were up-regulated, whereas 2,918 genes were down-regulated in the test sample. All of these differential genes are listed with feature descriptions in Supplementary Table 2. As mRNA targets of several highly tissue-specific miRNAs are expressed at significantly lower levels in the tissues compared to expression in tissues where such miRNAs are absent[39], down-regulation caused in considerable numbers of the above 2,918 genes can be explained by miR-29-mediated destabilization of target mRNAs. A miRNA target search using the miRanda program resulted in a plethora of positive hits against the mouse mRNA repertoire with respect to every member of the miR-29 family (see Supplementary Table 3 for details). The up-regulation of the 2,671 genes is presumably ascribable to indirect effects of miR-29 overexpression, e.g., through the depression of miR-22 observed following the miR-29 transfection. All of the 5,589 genes identified here proceeded to the undermentioned functional annotation.

Screening for fetal astrocyte genes directly targeted by miR-29

We also searched miR-29-treated and -untreated fetal astrocytes for genes differentially expressed on protein level using an MS methodology coupled with isobaric tagging chemistry. Quantitative comparison of the translation profile between the two cell types allowed identification in miR-29 transfectants of 12 proteins significantly (p <0.04) showing preferential expression (>1.5-fold relative to control transfectants) and 18 proteins with attenuated expression (<0.66-fold relative to the control) at a 95% confidence level. The results of the comparative profiling are summarized in Supplementary Table 4.

As aforementioned, the major effect of miRNA action in animal is an inhibition of translation, so the enhancing of miRNA levels could be inferred to generally induce a translational block and a concomitant decline of protein synthesis from the target mRNAs (assuming the mRNA transcription/abundance to be constant). Correspondingly, the reversal mode of expression change between miR-29 and its target gene products should be denoted in these profiles, and hence we focused on the 18 down-regulated genes as candidates of the direct target for miR-29 (Table 1). Scanning of the 3'-UTR sequence of mouse mRNAs by the miRecords miRNA target resource raised the result that, although experimentally validated interactions with miR-29 have not been recorded in this database for any of the 18 genes, at least one of the 11 target prediction programs detected putative miR-29 binding sites within the individual 3'-UTR region of all the genes (Supplementary Table 4).

Among the 18 genes, toward the genes coding for Mcfd2, Calml, Ptma, Plecl and Isg15, the negative effect of miR-29 is likely direct. Despite substantial reduction in protein abundance in miR-29 transfectants, the copy number of mRNA was almost comparable to (for Mcfd2, Calml and Ptma) or even higher than (Plecl and Isg15) that in the control cells. This disparity in expression change between the gene message and its protein product is consistent with miRNA-mediated regulation caused exclusively at the translation level, suggesting that miR-29 directly interferes with translation of these genes by binding to their respective targeting sites without affecting their steady-state mRNA levels. In addition to regulating gene expression by

Table 1. Translationally down-regulated genes in miR-29-overexpressing fetal mouse astrocyte

NCBI RefSeq accession	Encoded protein	GO annotation	Protein expression		mRNA level change* (test/control)
			Level change (test/control)	*p*-Value	
NM_009468	**dihydropyrimidinase-like 3 (Dpysl3)**	nervous system development	0.62	2.6E-05	0.54
NM_009242	**secreted acidic cysteine rich glycoprotein (Sparc)**	extracellular matrix binding	0.29	5.2E-05	0.37
NM_007422	**adenylosuccinate synthetase, non muscle (Adss)**	purine nucleotide biosynthesis	0.61	0.00045	0.67
NM_024427	**tropomyosin 1, alpha (Tpm1)**	structural constituent of cytoskeleton	0.63	0.0014	0.71
NM_173011	**isocitrate dehydrogenase 2 (NADP+) , mitochondrial (Idh2)**	tricarboxylic acid cycle	0.62	0.0015	0.56
NM_013632	purine-nucleoside phosphorylase (Pnp)	nucleoside metabolism	0.59	0.0015	
NM_007952	protein disulfide isomerase associated 3 (Pdia3)	positive regulation of apoptosis	0.57	0.0019	
NM_009825	**serine(cysteine) peptidase inhibitor, clade H, member1 (Serpinh1)**	collagen fibril organization	0.53	0.0025	0.48
NM_027959	**protein disulfide isomerase associated 6 (Pdia6)**	cell redox homeostasis	0.64	0.0041	0.65
NM_201386	**plectin 1 (Plec1)**	actin filament binding	0.59	0.0061	1.44
NM_023625	phospholipase B domain containing 2 (Plbd2)	lipid metabolism	0.56	0.0065	
NM_015783	**ISG15 ubiquitin-like modifier (Isg15)**	modification-dependent protein metabolism	0.64	0.012	1.71
NM_007591	**calreticulin (Calr)**	negative regulation of neuron differentiation	0.43	0.019	0.51
NM_139295	**multiple coagulation factor deficiency 2 (Mcfd2)**	carboxylic acid metabolism	0.59	0.021	0.98
NM_009790	**calmodulin 1 (Calm1)**	calcium-dependent protein binding	0.63	0.023	0.96
NM_011313	**S100 calcium binding protein A6 (calcyclin) (S100a6)**	calcium-dependent protein binding	0.65	0.026	0.77
NM_019698	aldehyde dehydrogenase 18 family, member A1 (Aldh18a1)	amino acid biosynthesis	0.63	0.034	
NM_008972	**prothymosin alpha (Ptma)**	anti-apoptosis	0.62	0.039	1.10

Genes consistently detected on the DNA microarray are designated in boldface.

* Data from the DNA microarray profiling.

canonical translation arrest, which is thought to be the predominant action pattern shared by animal miRNAs, they also regulate expression of a set of their target genes by lowering mRNA stability[40, 41]. This case may be applicable to the genes of Dpysl3, Adss, Idh2, Serpinh1 and Pdia6. There is a parallel relationship between transcriptional and translational decline. As for the genes of Sparc, Tpm1, Calr and S100a6, we observed a greater extent of attenuation in the protein level compared to the mRNA level, which is diagnostic of the concurrence of knockdown at both expression levels. Thus, the results indicate that miR-29 regulates expression of the 18 genes through decreased translation, increased degradation of target messages or both. To what extent these two regulation processes are coupled is not yet known, but whatever the regulation mode of miR-29, the present findings are regarded as supportive evidence of direct interaction between miR-29 and these genes, and its validation must await experimental confirmation by luciferase/eGFP reporter assays, Northern/Western blotting analyses, etc.

Functional categorization of miR-29-regulated genes in fetal mouse astrocyte

To characterize miR-29 as a gene expression controller, we required two conditions: enrichment of its target genes in a co-expressed gene set compared to other miRNAs and all other gene sets; and an excess of negative correlation in expression profiles with its targets in the enriched gene group compared to non-enriched miRNA. We first assigned the 2,670 up-regulated genes and 2,918 down-regulated genes isolated from miR-29 transfectants to GO functional categories and identified GO functional terms over-represented for each set of genes that were up- or down-regulated by miR-29. Instead of identifying functional groups enriched in the top significantly altered genes, in this analysis we counted the number of occurrences of all genes associated with a given GO term, and determined the probability of obtaining an equal or higher number of putative down- or up-regulated gene occurrences using the binomial distribution. The full results of our GO classification are summarized in Supplementary Table 5.

Although the GO annotation tool is very useful to quantify over- or underrepresentation of individual genes within the identified functional term relative to total genes on DNA microarray, it does not provide information about the regulatory contribution of individual genes to the entire reaction process defined by the identified term and their functional link to other gene members participating in the process. Therefore, to more systematically interpret the biological significance specifically found in hundreds of genes co-regulated by miR-29, we applied the same statistical strategy of testing for functional enrichment to the KEGG pathway collection. This approach is highly sensitive, as it can determine whether overall regulation of a functional pathway by each gene set is statistically significant even when expression changes of individual genes that comprise the gene set are not. The KEGG annotation here allowed us to significantly identify canonical signaling and metabolic pathways preferentially present in the two miR-29-regulated gene sets.

The top10 functional pathways most enriched in each co-regulated gene set are shown in Table 2 ranked according to p-values, and a full list of enriched pathways is given in Supplementary Tables 6 and 7 for the up- and down-regulated gene sets, respectively. The KEGG mapping data for miR-29-activated genes revealed a robust overrepresentation of a wide spectrum of metabolic pathways for energy production, cellular component turnover and RNA/protein synthesis. More noteworthy was the significant induction of a neural maturation process by miR-29 overexpression: axon guidance is a key step for the formation of the neural network. On the contrary, as is evident from the overrepresentation among the miR-29-silenced genes, pathways involved in synthesis of extracellular matrix (ECM) and its function (cell-

Table2. Top KEGG pathways enriched in up- and down-regulated genes in miR-29-overexpressing fetal mouse astrocyte

In miR-29-upregulated genes:			In miR-29-downregulated genes:		
Pathway	*p*-Value	% Count*	Pathway	*p*-Value	% Count*
Tight junction	0.0004	15	ECM-receptor interaction	0.0000	45
Fatty acid metabolism	0.0006	22	Focal adhesion	0.0000	27
Insulin signaling	0.0027	17	Pathways in cancer	0.0000	22
Basal transcription factor	0.0028	22	Small cell lung cancer	0.0000	35
Axon guidance	0.0035	14	Amoebiasis	0.0000	27
Lysine degradation	0.0089	19	p53 signaling	0.0000	32
Phosphatidylinositol signaling	0.010	15	Cell cycle	0.0000	21
Aminoacyl-tRNA biosynthesis	0.010	18	PG-mediated oocyte maturation	0.0001	24
Fructose/mannose metabolism	0.011	19	Dilated cardiomyopathy	0.0001	23
Glutathione metabolism	0.012	17	Renal cell carcinoma	0.0001	25

* Number of genes here identified to be associated with each functional patheway per total number of gene members of the pathway.

cell communication/adhesion, cell migration, etc.), as well as cancer-related signaling cascades, were consistently and strongly depressed with miR-29 transfection. ECM elements play dominant roles in cell differentiation and tumor progression as further mentioned below. Dilated cardiomyopathy is a heart muscle disease characterized by dilation and impaired contraction of ventricles, in which ECM-receptor interaction and intracellular Ca^{2+} homeostasis are intimately involved. Another observation that was unexpected to us was a negative effect of overexpressed miR-29 on p53-initiated cellular processes. miR-29 may obviate p53-dependent apoptosis and cell cycle arrest during embryogenesis and postnatal development, which is also discussed below. The result of the KEGG analysis is largely in accordance with the functional clustering datasets obtained from the GO term enrichment (Supplementary Table 5), and moreover, well correlates with the GO-assigned function of putative miR-29 target genes listed in Table 1. Impairment of Ca^{2+}-mediated signal transduction involving Ca^{2+}-binding proteins such as S100a6 can also be associated with decreased vulnerability to apoptotic cell death[42].

4 Discussion

miRNAs offer a novel layer of regulation for gene expression via interfering with stability and/or translation of specific target mRNAs. The collective modulatory influence of a single miRNA on a wide array of its target genes might bring about drastic variations in cell physiology and certain pathological phenotypes. Consequently, a major challenge in understanding the biology of miRNA is to identify its target genes and their downstream effectors. In the current study, we profiled a genome-wide gene expression in developmentally immature mouse brain cells that suffered exogenous delivery of miR-29, and identified a cadre of genes co-regulated by the miR-29 overexpression. For better characterization of the miR-29-responsive genes, they were categorized into functionally relevant molecular groups by the GO and KEGG annotation tools. Interpretation of the read out is not straightforward, because some expression responses represent a compensatory mechanism. It is also feasible that some of these genes showed expression changes not due to their intrinsic roles but rather as a part of some other, or yet unknown, functions. Still, we are able to find among these gene sets a striking tendency to enhance differentiation-oriented housekeeping functions and signaling actions while preventing dysregulated cell growth and suicidal cell death. The miRNA expression profile in the miR-29 transfectants exhibited a strong reciprocal correlation between miR-29 and miR-22: miR-29 overexpression with concomitant miR-22 down-regulation. Since miR-22 has been validated to

target the Uqcrc2 gene coding for a cytochrome bc1 complex subunit[5], the miR-29-mediated repression of miR-22 will in turn relieve the knockdown of the mitochondrial ETC member, which could explain in part the signatures in favor of the basic cellular metabolism in the miR-29-upregulated gene set.

In line with our present findings, a pivotal role of miR-29 as a tumor suppressor has been suggested in previous studies, in which it was reporting that miR-29 expression enhances various apoptosis-stimulating signals and thereby represses cell proliferation and tumorigenesis in multiple extra-neural tissues including chronic lymphocytic leukemia[43, 44], lung cancer[45], prostate cancer[46], and invasive breast cancer[47]. Intriguingly in myoblasts, NF-κB directs YY-1, a member of Polycomb group that functions to silence transcription of a set of genes by chromatin modification, to epigenetically suppress miR-29b/c expression. Upon overexpression, miR-29 with a pro-myogenic activity inhibits growth of rhabdomyosarcoma cells and stimulates their differentiation likely through targeting its negative regulator YY-1[48]. Related studies have shown that miR-29 blocks global DNA methylation by targeting DNA methyltransferases, which re-express methylation labile tumor-suppressing genes in lung tumors[49] and myeloid leukemia[50]. Mcl-1, encoding an anti-apoptotic Bcl2 family protein, is also known to be a target of miR-29. By acting on the Mcl-1 3'-UTR, miR-29 sensitized cholangiocarcinoma cells to apoptosis when the cells were treated with tumor necrosis factorelated apoptosis-inducing ligand (TRAIL)[51]. miR-29 also induces apoptosis in a p53-dependent manner through targeting p85α, the regulatory subunit of PI3 kinase, and CDC42, a member of the Rho family of GTPases, the former reaction of which shuts down the PI3K-AKT-MDM2-p53 regulatory interplay and thereby rescues p53 from the proteolysis-inducing function of MDM2[52]. Recently, miR-29 was found to trigger the mitochondrial apoptosis pathway via knockdown of Mcl-1 as well as Bcl-2, another Bcl-2 family member[53]. Furthermore, miR-29 directly represses v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (B-Myb) by binding to its 3'-UTR, acting in conjunction with Rb-E2F complexes at the B-Myb promoter to mediate repression of B-Myb expression during Rb activation resulting in senescence[54]. miR-29 not only acts against the onset of tumors but also participates in tumor progression. In nasopharyngeal carcinomas, miR-29 miRNAs are thought to counteract cancer cell invasion and metastasis by targeting extracellular matrix proteins such as collagen and laminin[55]. In connection to this finding, miR-29 was reported to serves as a positive regulator of osteoblast differentiation again by silencing ECM components including osteonectin, or Sparc[56, 57] appearing in Table 1 of this article. Thus, miR-29 is likely to facilitate cell differentiation in other tissues as well through re-organization of deposited ECM followed by multifarious effects on differentiation-related signalings. In addition, miR-29 has been shown to contribute to the regulation of energy metabolism. Diabetic adipocytes treated with high levels of insulin and glucose stimulate miR-29a/b expression, which consequently blocks the insulin signaling pathway through a negative feedback control[58].

Although among the classical action modes of miR-29 is to maintain cells in a quiescent, non-proliferating state and induce apoptosis, our present data contradict the previous observations in this regard. As miRNA controls a large number of target genes with diverse nature, its action often might invoke opposing phenotypic effects in a context-dependent manner. Indeed, overexpressed miR-29a promotes metastatic growth of epithelial mammary tumorigenic cells in murine lung, and moreover, this miRNA is markedly induced in human breast cancer[59]. Another study reported that miR-29 is up-regulated in indolent human B-cell chronic lymphocytic leukemia (B-CLL) compared to aggressive B-CLL and normal CD19+ B-cells, while this miRNA is significantly down-regulated

in aggressive CLL relative to indolent CLL, suggesting that miR-29 can function as an oncogene as well as a tumor suppressor in the pathogenesis of CLL[60]. As such, we should note that a given miRNA does not always display identical behaviors spatiotemporally, and independent characterization of miRNA function is necessary for identifying its role in a specific cellular context. Together, the emerging picture in this study is one in which miR-29 elicits the leading signals that direct a profound shift in the populations of gene messages necessary for the transition from the immature to mature state in traversing the developmental stage of the mouse brain. There is a considerable overlap between putative miR-29 target genes (Table 1) and the top functional categories overrepresented among the gene set negatively regulated by miR-29 transfection (Table 2), which well reflects the overarching roles of this miRNA in the timing of the developmental transition. It is unlikely that miR-29 presents the same action pattern after the completion of brain maturation. miR-29 will programmatically start expressing its intrinsic functions based on the anti-survival, pro-death nature as manifested at later ages. Such a paradoxical role presented in a life-stage-dependent fashion is known as antagonistic pleiotropy[61]: beneficial for reproductive success early in life, but then harmful in late-life. We questioned what a temporal wave of gene expression under the control of miR-29 would appear after entering the post-developmental or the post-reproductive phase. Loss-of-function approaches to the mature mouse brain with miR-29 being fully expressed could answer this.

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マウス・アストロサイトでの miR-29 依存性の遺伝子発現の網羅的解析

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要　約

マイクロ RNA（miRNA）は多くの生物種の多岐にわたる生命現象において遺伝子発現の転写後制御機能を担っている小分子非コード RNA である。前回，著者らは加齢マウスの脳内において発現が最も顕著に上昇している小分子 RNA として miR-29 を同定した。miR-29 は脳組織では主にアストロサイトで発現する miRNA で，脳発達の初期にはほとんど発現は認められないが，若齢期から繁殖最盛期にかけて発現の上昇が続いた後，その最大発現レベルを寿命終末期まで維持するという発現パターンを示す。miRNA 本来の生物学的機能と miR-29 のこのような発現挙動を考え合わせると，miR-29 がその多面発現性を通して哺乳類生物種固有の寿命プログラミングの中心的存在として働いているのではないかとの期待がもたれる。今回，この寿命決定機構解明の観点から寿命プログラム初期のマウス脳内での miR-29 の機能的役割を明らかにする目的で，miR-29 が未発現のマウス胎児由来のアストロサイト内でこの miRNA を強制発現させ，そのゲノムワイドでの影響を転写と翻訳の両レベルで調べた。マイクロアレイから得られた転写レベルでの発現プロファイルでは miR-29 発現に応答した多数の遺伝子の発現変動が観察された。また，質量分析による翻訳レベルでのプロファイリングからは miR-29 の標的についての複数の候補遺伝子が特定された。これら miR-29 応答性の発現遺伝子の機能アノテーションをインフォマティックな手法により行った結果から，脳発達段階での miR-29 は細胞内代謝活性と正常な細胞増殖能は維持しながらも，成体としての完成状態への成熟を着実に誘導すべく多数の遺伝子の発現を統御しているという構図が想定された。このような機能的特徴は繁殖期以降の成体で従来報告されているものとは異なった側面をもち，miR-29 がライフステージ依存的に下流遺伝子の発現調節様式を巧妙に制御しながら寿命プログラムの進行役を果たしている様相が示唆された。