

MicroRNA-298 and MicroRNA-328 Regulate Expression of Mouse β -Amyloid Precursor Protein-converting Enzyme 1*[§]

Received for publication, September 29, 2008, and in revised form, November 5, 2008. Published, JBC Papers in Press, November 5, 2008, DOI 10.1074/jbc.M807530200

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MicroRNAs (miRNAs) are key regulatory RNAs known to repress mRNA translation through recognition of specific binding sites located mainly in their 3'-untranslated region (UTR). Loss of specific miRNA control of gene expression is thus expected to underlie serious genetic diseases. Intriguingly, previous post-mortem analyses showed higher β -amyloid precursor protein-converting enzyme (BACE) protein, but not mRNA, levels in the brain of patients that suffered from Alzheimer disease (AD). Here we also observed a loss of correlation between BACE1 mRNA and protein levels in the hippocampus of a mouse model of AD. Consistent with an impairment of miRNA-mediated regulation of BACE1 expression, these findings prompted us to investigate the regulatory role of the BACE1 3'-UTR element and the possible involvement of specific miRNAs in cultured neuronal (N2a) and fibroblastic (NIH 3T3) cells. Through various experimental approaches, we validated computational predictions and demonstrated that miR-298 and miR-328 recognize specific binding sites in the 3'-UTR of BACE1 mRNA and exert regulatory effects on BACE1 protein expression in cultured neuronal cells. Our results may provide the molecular basis underlying BACE1 deregulation in AD and offer new perspectives on the etiology of this neurological disorder.

Alzheimer disease (AD)⁴ is a neurodegenerative disorder that currently affects nearly 2% of the population in industrialized countries. The risk of AD dramatically increases in indi-

viduals beyond the age of 70, and it is predicted that the incidence of AD will increase by 3-fold within the next 50 years (1). This progressive disease is characterized by the accumulation of plaques formed of short β -amyloid (A β) peptides (1–5). These peptides are obtained upon proteolytic cleavage of the β -amyloid precursor protein (APP), a type 1 transmembrane protein (6), by a β -secretase known as the β -site APP-cleaving enzyme (BACE) (7–10). This reaction liberates a soluble APP fragment and a 99-amino acid fragment (C99) that remains attached to the membrane (7–10). This latter fragment is further processed in its intramembrane domain by the γ -secretase to produce C-terminal fragment γ and A β peptides (1), whose levels have been correlated with those of BACE1 (11).

Interestingly, *bace1*-null mice do not demonstrate any developmental problems or aberrant behavioral phenotypes, rendering BACE1 a potential and attractive target for therapies against AD (12, 13). However, the structure of BACE1 reveals an active site that is more open and less hydrophobic, as compared with other aspartyl proteases, which may severely hamper the development of small inhibitory molecules (14). On the other hand, targeting of BACE1 mRNA by small interfering RNAs has proven to be effective in down-regulating BACE1 protein levels and activity in cultured primary cortical neurons (15) as well as in a mouse model of AD (16). Although these findings suggest a potential therapeutic use for small interfering RNAs in treating AD, they also imply a certain degree of accessibility of BACE1 mRNA to the endogenous RNA silencing machinery, which is based on microRNAs (miRNAs).

miRNAs are key regulatory RNAs known to initially repress mRNA translation through recognition of specific binding sites (BS) located mainly in their 3'-untranslated region (UTR) (for a recent review, see Ref. 17). Encoded in the genome of almost all living eukaryotes, miRNA genes are transcribed by RNA polymerase II (18) into primary miRNA transcripts, which are then processed by Drosha (19) into miRNA precursors (pre-miRNAs). After being exported to the cytoplasm by the Ran-GTP-dependent nuclear transporter Exportin-5 (20), these imperfectly paired stem-loop precursors are trimmed by the ribonuclease III Dicer into miRNA:miRNA* duplexes (21, 22). In most cases, although the nonfunctional miRNA strand (miRNA*) is encountered much less frequently and is presumably degraded (23), the miRNA strand is loaded into the effector ribonucleoprotein complexes, guiding them toward the target mRNAs to be regulated (24, 25).

miRNAs have been implicated in tissue morphogenesis and in various cellular processes such as cell differentiation and pro-

* This work was supported by NARSAD, The World's Leading Charity Dedicated to Mental Health Research, young investigator award (to P. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

¹ Supported in part by a studentship from the Alzheimer Society of Canada.

² Holds a Canada Research Chair in Neuroimmunology.

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⁴ The abbreviations used are: AD, Alzheimer disease; APP, β -amyloid precursor protein; BACE, β -amyloid precursor protein-converting enzyme; BS, binding site; miRNA, microRNA; mRNA, messenger RNA; UTR, untranslated region; EMSA, electrophoretic mobility shift assays; WT, wild type; FMRP, fragile X mental retardation protein; DMEM, Dulbecco's modified Eagle's medium; ISH, *in situ* hybridization; A β , β -amyloid; nt, nucleotide; KO, knock-out.

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liferation, apoptosis, and major signaling pathways (26). Emerging evidence suggests a direct link between miRNAs and diseases, some of which may be caused by impairment of the miRNA-guided RNA silencing machinery itself. This may be the case for the fragile X syndrome, which is the most frequent cause of inherited mental retardation. The fragile X mental retardation protein (FMRP), whose loss represents the etiologic factor of the related syndrome (27), has been reported to be part of a ribonucleoprotein complex harboring miRNAs. Our studies unveiled that FMRP has the ability to accept miRNA products derived from Dicer and to facilitate miRNA assembly on specific target RNA sequences (28). These findings led us to propose that suboptimal utilization of miRNAs, *i.e.* miRNA:mRNA assembly and/or disassembly, may account for some of the molecular defects in patients with the fragile X syndrome (29).

Disease may also arise from the loss of miRNA control of a specific gene. Although up to 92% of the genes in mammals could be subjected to miRNA regulation (30), only a few miRNA:mRNA target pairs have been studied in detail. Among the disease-related genes that we examined, we were intrigued by BACE1 and the described features of a possible loss of post-transcriptional control in the brain of deceased AD patients. Indeed, previous studies had reported that BACE1 protein levels and activity were up-regulated in brains from patients suffering from AD, as compared with brains from unaffected patients, with BACE1 mRNA levels remaining unchanged (31–33). These observations prompted us to hypothesize that BACE1 expression is under the control of miRNAs.

EXPERIMENTAL PROCEDURES

Animals and Histology—Transgenic animals harboring the human presenilin 1 (A246E variant) and a chimeric mouse/human β -amyloid precursor protein (APP_{Swe}) were originally obtained from The Jackson Laboratory (B6C3-Tg(APP695)3Dbo Tg(PSEN1)5Dbo/J; The Jackson Laboratory, Bar Harbor, ME). This colony is maintained in a C57BL/6J background. WT and transgenic mice were acclimated to standard laboratory conditions (14-h light, 10-h dark cycle; lights on at 06:00 and off at 20:00 h) with free access to rodent chow and water. All protocols were conducted according to the Canadian Council on Animal Care guidelines, as administered by the Laval University Animal Welfare Committee.

To collect the brain tissues at different ages, mice were deeply anesthetized via an intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde, 3.8% Borax in sodium phosphate buffer (pH 9.0 at 4 °C). Brains were rapidly removed from the skulls, post-fixed overnight, and then placed in a solution containing 10% sucrose diluted in 4% paraformaldehyde, 3.8% Borax buffer, pH 9.0, overnight at 4 °C. The frozen brains were mounted on a microtome (Reichert-Jung, Cambridge Instruments Co., Deerfield, IL), frozen with dry ice, and cut into 25- μ m coronal sections from the olfactory bulb to the end of the medulla. The slices were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, 20% glycerol) and stored at –20 °C.

BACE1 protein and mRNA were detected via immunohistochemistry and *in situ* hybridization (ISH), respectively (34). We used a full-length BACE1 cDNA (cloned by PCR) and antibody (PC529, 1/1000 dilution, Calbiochem) for ISH and immunohistochemistry, respectively. For the determination of mBACE1 mRNA and protein levels of expression, we used the ImageJ software (rsb.info.nih.gov) to select the hippocampal region, including the dentate gyrus, and we determined both optical densities and area of expression.

miR-298 and miR-328 were detected by ISH by using the same protocol with probes that were of perfect complementarity to either miRNAs. The optical densities on film were determined using the ImageJ software.

Cell Culture—Neuroblastoma N2a murine cells were grown in complete DMEM, *i.e.* supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. NIH 3T3 murine cells were grown in complete DMEM containing 10% calf bovine serum, whereas mouse embryonic *Fmr1* KO (STEK TSV-40) and WT (Naives) fibroblasts (28) were grown in complete DMEM containing 2 mM L-glutamine. All cell lines were grown and maintained in tissue culture plates and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were kept in the exponential growth phase and subcultured every 3–4 days.

Plasmid Constructs—The sequence encoding the precursors of mmu-miR-298 (pre-miR-298), mmu-miR-328 (pre-miR-328), and mmu-miR-105 (pre-miR-105) were cloned in the psiSTRIKE vector (Promega), according to the manufacturer's protocol. The sequences of the complete 3'-UTR of mouse BACE1 (nt 1932–3855, NCBI accession number BC048189), the partial 3'-UTR of mouse BACE1 (nt 2175–2374; miRNA BS module), and the complete 3'-UTR of mouse BACE2 (nt 2784–3614, NCBI accession number NM_019517) were amplified by PCR and introduced downstream of the *Rluc* reporter gene in the XhoI/NotI cloning sites of the psiCHECK vector (Promega). Mutations in the miRNA BS module of BACE1 were introduced by whole plasmid amplification in the seed region of both miR-298 and/or miR-328 BS (298mut, 328mut, and 298mut + 328mut). A reporter construct bearing a downstream miR-328 target sequence was also engineered by introducing a single copy of a sequence perfectly complementary to miR-328 in the XhoI/NotI cloning sites of psiCHECK. PCR fragments containing one or three copies of miR-298 or miR-328 natural BS were blunt-ligated downstream of the *Rluc* coding region in psiCHECK reporter vector. All the constructs were confirmed by restriction analysis and DNA sequencing.

Cell Transfection and Dual-Luciferase Assay—For Dual-Luciferase assays, cells were cultured in 24-well plates and transfected at 70–80% confluency using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Cells were transfected with psiCHECK alone (200 ng of DNA), psiCHECK (400 ng of DNA), and psiSTRIKE (250 ng of DNA) or psiCHECK (400 ng of DNA) and miRNA duplexes (120 pmol), respectively. For Western blot analysis of BACE1 expression, cells grown in 6-well plates to at least 60% confluency were transfected with either miRNA duplexes (600 pmol) or 2'-OMe oligoribonucleotides (500 pmol). Cells confluent at 90% were transfected with psiSTRIKE (20 μ g) by the calcium

phosphate method. Cells transfected with the psiCHECK vector were lysed in 100 μ l of passive lysis buffer (Promega), and the samples were analyzed on a luminometer, according to the manufacturer's instructions. Rluc values were normalized to *Firefly* luciferase (Fluc) readings, and the results were expressed as means \pm S.E.

Electrophoretic Mobility Shift Assays (EMSA)—Fragments harboring the natural miR-298 or miR-328 BS, either WT or mutated in their miRNA seed region (mut), were synthesized using T7 promoter-driven *in vitro* transcription (Megashortscript kit, Ambion). The DNA oligonucleotides were annealed to obtain the transcription modules. Five μ g of each deoxyribonucleotide were solubilized in 50 μ l of DNA annealing buffer (10 mM Tris·HCl, 100 mM NaCl, and 1 mM EDTA), heated to 95 °C for 5 min, and cooled down gradually to room temperature. The annealed oligonucleotides were precipitated with ethanol, resuspended in water, and used as templates for *in vitro* transcription reactions. RNAs were purified on a 10% polyacrylamide gel containing 7 M urea, eluted in elution buffer (0.5 M sodium acetate, 1 mM EDTA, and 0.2 M SDS), and ethanol-precipitated. The miRNA BS or tRNA control was incubated in the absence or presence of synthetic ³²P-labeled miR-298 or miR-328 in reaction buffer composed of 20 mM Tris·HCl, 30 mM NaCl, 0.1 mM MgCl₂, 0.2 mM ZnCl₂, 5 mM dithiothreitol, and 5% Superase-in, pH 7.0. miRNA-miRNA BS complex formation was monitored by 7.5% nondenaturing PAGE and autoradiography.

miRNA Duplexes—The oligoribonucleotides miR-298, miR-328, and miR-196, and their miRNA* strands, were purchased from either Dharmacon or Integrated DNA Technologies. miRNA duplexes were reconstituted by coincubating equimolar ratios of mature miRNA and miRNA* strands in RNA annealing buffer (10 mM Tris·HCl, 20 mM NaCl, pH 8.0), heating at 95 °C for 5 min, and gradually cooling down to room temperature. miRNA duplex formation was ascertained by 7.5% nondenaturing PAGE and ultraviolet shadowing.

Protein Extraction and Western Blot Analysis—Proteins were extracted by lysing cells in protein extraction buffer (40 mM Tris·HCl, 275 mM NaCl, 20% glycerol, 2% Igepal, 1 mM phenylmethylsulfonyl fluoride, 1 \times protease inhibitor mixture mix, pH 8.0) on ice for 15 min, prior to the addition of gel loading buffer and boiling of the samples for 5 min. Protein extracts (100 μ g) were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, which was incubated in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% dry milk at room temperature for 1 h. The membrane was then incubated in the presence of the primary antibody recognizing BACE1 (PC529, from Calbiochem) or actin (AC-40, from Sigma) for 1 h, washed three times with TBST, incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h, and washed again three times with TBST. The signal was visualized by ECL (Amersham Biosciences).

RNA Extraction and Northern Blot Analyses—Small RNA (< 200 nt) fractions were isolated by using the mirVana miRNA isolation kit (Ambion) from N2a and NIH 3T3 cells. RNA was separated on a 10% polyacrylamide gel containing 7 M urea, transferred to a nylon membrane followed by detection using a ³²P-labeled probe complementary to miR-298 or miR-328, fol-

lowing the Northern blot procedure improved for the detection of small RNAs described previously by Pall *et al.* (35). 5 S RNA was probed as a control.

RESULTS

Loss of Post-transcriptional Regulation of BACE1 Expression in the Brain of APP_{Swe}/PS1 Mice—To initiate our investigation as to whether miRNA-mediated translational repression could be involved in the regulation of BACE1 expression *in vivo*, BACE1 mRNA and protein levels were determined in the brain of aging mice. As depicted in Fig. 1, *A* and *B*, age-related variations in BACE1 mRNA levels were detected in the brain of APP and WT mice. Interestingly mRNA levels were higher in the brains of APP_{Swe}/PS1 mice than those of WT littermates at 4 months of age, but these levels upturned in mice sacrificed 15 months later (Fig. 1, *A* and *B*). The switch in *BACE1* gene expression in the hippocampus of aging APP and WT mice was not translated to the same tendency of changes at the protein level, which were measured in the same regions of adjacent sections (Fig. 1, *C* and *D*). Indeed, BACE1 protein levels increased with age in APP mice, whereas BACE1 mRNA levels declined. BACE1-immunoreactive regions also generally overlapped with those exhibiting senile plaques in the cerebral cortex and hippocampus of APP mice at 10 months of age. These anatomical features, which are similar to those observed in human (31–33), are consistent with a possible loss or deregulation of translational repression of BACE1 mRNA mediated by miRNAs.

Presence of Regulatory Elements in the 3'-UTR of Mouse BACE1—Because the regulatory elements recognized by miRNAs are usually located in the 3'-UTR of mRNAs, we examined the gene regulatory properties of BACE1 3'-UTR. For that purpose, this region was amplified and cloned downstream of a *Renilla* luciferase (Rluc) reporter gene, and assayed in transiently transfected N2a or NIH 3T3 cells. Incorporation of BACE1 3'-UTR reduced expression of the Rluc reporter gene by ~70–80%, as compared with empty psiCHECK vector (set at 100%) (Fig. 2). No regulatory effects were conferred upon insertion of the corresponding region of BACE2 3'-UTR downstream of *Rluc*. These data suggest the presence of down-regulatory elements in the 3'-UTR of BACE1 mRNA.

Putative miRNA-binding Sites Are Located in the 3'-UTR of BACE1 mRNA—Considering a recent report by Miranda *et al.* (30) suggesting that up to 90% of the genes in mammals may be subjected to miRNA regulation, we inspected the 3'-UTR of BACE1 mRNA for the presence of putative BS for specific miRNAs. To this end, we utilized two different algorithms designed to identify miRNA-mRNA target interaction pairs that exhibit favorable free energies. As depicted in Fig. 3*A*, analysis of BACE1 mRNA 3'-UTR sequence with the DIANA-microT program identified two potential BS for miRNAs as follows: one for miR-298 (nts 2205–2230) and another for miR-328 (nts 2321–2346). This computational program uses a combination of bioinformatics and experimental approaches to define important rules that govern miRNA recognition of animal target mRNAs (36). Noticeably, both miR-298 and miR-328 showed perfect pairing of their seed region, *i.e.* nts 2–8 from their 5' extremity, to their respective BS. Complementary pair-

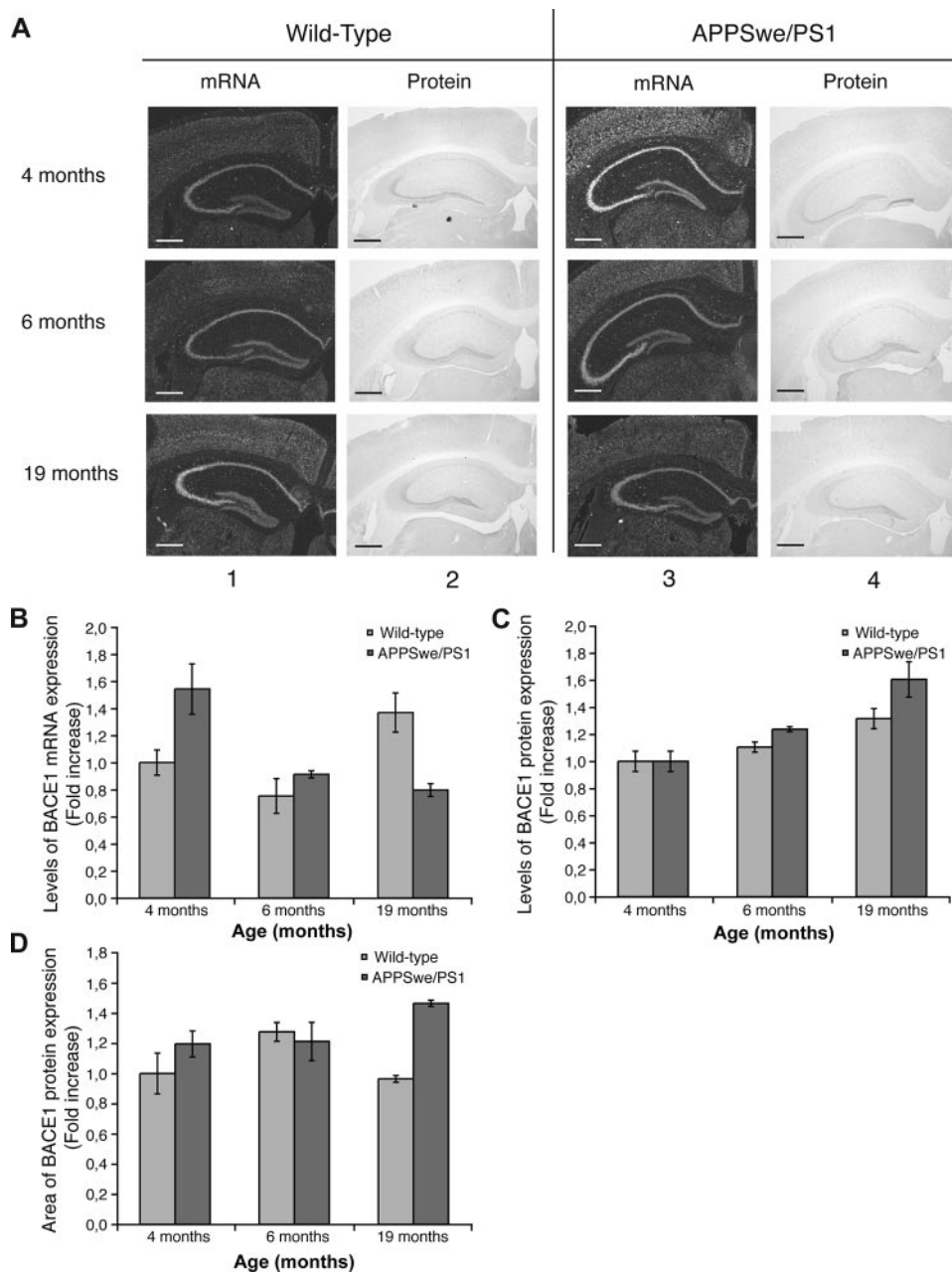


FIGURE 1. Deregulation of BACE1 mRNA and protein expression patterns in the hippocampus of APP_{Swe}/PS1 versus WT mice. A and B, BACE1 mRNA levels were determined by *in situ* hybridization on brain sections taken from WT (lane 1) or APP_{Swe}/PS1 (lane 3) at 4, 6, and 19 months of age ($n = 4$ for each time point). A, C, and D, protein levels were assessed on adjacent brain sections by means of immunohistochemistry using BACE1 PC529 antibody and 3,3'-diaminobenzidine tetrahydrochloride (Sigma) staining. Please note the different patterns of BACE1 mRNA and protein in aging APP mice, although the mRNA decreased, the protein levels increased in the hippocampus of the mouse model of AD at 19 months of age. Scale bars represent 500 μm .

ing of the miRNA 3' sequence further stabilizes target recognition, yielding highly favorable free energies for miR-298 ($\Delta G = -48.2$ kcal/mol) and miR-328 ($\Delta G = -44.6$ kcal/mol), respectively. As a comparison, these interactions are predicted to be of higher stability than that observed between the known let-7 miRNA:lin-41 mRNA pair ($\Delta G = -31.0$ kcal/mol), which has been experimentally validated in the nematode *Caenorhabditis elegans*. These results have been confirmed by using another algorithm, RNAhybrid, which was initially conceived to find the minimum free energy hybridization between a long and a short RNA, and is primarily intended as a means for miRNA target

prediction (37). Using this tool, highly favorable free energies were again obtained for miR-298 ($\Delta G = -36.0$ kcal/mol) and miR-328 ($\Delta G = -32.6$ kcal/mol), respectively. Neither of these computational strategies could predict miRNA BS in the BACE2 mRNA 3'-UTR sequence.

Next, to validate these predictions experimentally, we initially assessed whether miR-298 and miR-328 could interact with their respective BS *in vitro* by adapting and using EMSA, an established method commonly used to monitor perfectly paired oligonucleotides. For that purpose, synthetic ^{32}P -labeled miR-298 and miR-328 were incubated in the absence or presence of their putative, *in vitro*-transcribed BS, and miRNA-miRNA BS complex formation was analyzed by EMSA. Although partially complementary, both miR-298 and miR-328 bound to their respective BS, as demonstrated by the formation of a slowly migrating complex (Fig. 3, B and C, left panels, lanes 2). No such complex was observed when the miRNA BS were swapped (Fig. 3, B and C, left panels, lanes 4). Moreover, mutation of the miRNA seed region disrupted miRNA: miRNA BS complex formation (Fig. 3, B and C, left panels, lanes 3). Although they did not interact with tRNA, used as a negative control, miR-298 and miR-328 bound to their respective BS in a dose-dependent manner (Fig. 3, B and C, right panels, lanes 1–4). In addition, to demonstrate the suitability of EMSA to visualize imperfectly paired miRNA-miRNA BS complexes, as slower migrating bands on nondenaturing polyacrylamide gels,

these results indicate that miR-298 and miR-328 are able to recognize their respective BS in the 3'-UTR of BACE1 mRNA.

Pre-miR-328 Expression Exerts Gene Down-regulatory Effects in Cultured Neuronal Cells—To study the gene regulatory role of miR-298 and miR-328 *in vivo*, we engineered vectors aimed at expressing pre-miR-298 and pre-miR-328 in cultured neuronal N2a and NIH 3T3 cells. Insertion of the pre-miRNA sequences in psiSTRIKE implied their adaptation to the U6 promoter and terminator sequences, *i.e.* substitution for a G at the 5' end of the pre-miRNA and a UU pair at its 3' end, respectively (supplemental Fig. S1A). Vector-based expression of pre-

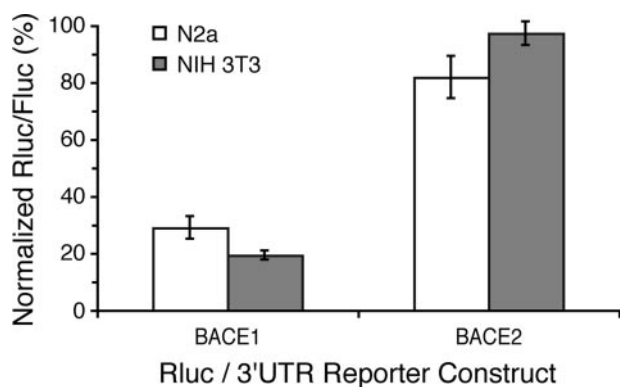


FIGURE 2. Mouse BACE1 3'-UTR harbors a negative gene regulatory element. Cultured murine neuronal (N2a) and fibroblastic (NIH 3T3) cell lines were transfected with a reporter construct expressing Rluc coupled with the 3'-UTR of either BACE1 or BACE2. The results of Rluc activity were normalized with Fluc reporter activity and expressed as a percentage of the results obtained with the empty reporter vector (set at 100%) ($n = 6$ experiments).

miR-298 and pre-miR-328 was verified in transiently transfected N2a cells by Northern blot. Analysis of small RNAs isolated from these cells revealed detectable levels of pre-miR-328 and mature miR-328 (supplemental Fig. S1B), whereas neither pre-miR-298 nor miR-298 could be detected (data not shown). These observations indicate that the pre-miR-328 construct, in contrast to that encoding for pre-miR-298, is suitable for our studies and U6-mediated expression in cultured mammalian cells.

We then focused and utilized the pre-miR-328 expression vector to confirm the functionality of an miRNA-guided RNA silencing machine in our cells. To this end, we monitored Rluc activity of the corresponding reporter constructs in cotransfected N2a and NIH 3T3 cells. Expression of the Rluc reporter gene coupled with a single BS perfectly complementary to miR-328 was decreased by more than 80% upon coexpression of pre-miR-328, as compared with that of an unrelated precursor control (Fig. 4A). The down-regulatory effects conferred by the single BS were not increased further by the presence of two additional BS (data not shown). These results support the existence of a functional miRNA-guided RNA silencing machinery in cultured N2a and NIH 3T3 cells.

Functionality of the miRNA-binding Sites—miRNAs act in synergy, and mRNAs regulated by miRNAs often contain more than one miRNA BS. Furthermore, in mammalian cells, miRNA BS located in target mRNAs are recognized by miRNAs mainly through imperfect complementarity. To get closer to this situation, we generated reporter constructs in which the Rluc reporter gene is coupled with one or three copies of the miR-328 natural BS. Expression of Rluc coupled with one BS was down-regulated by ~60% upon coexpression of pre-miR-328 in N2a or NIH 3T3 cells (Fig. 4B). Gene expression was further decreased by an additional 50% when increasing the number of BS copies from one to three, as compared with a control reporter lacking miRNA BS (Fig. 4B). These results confirm the functionality of the miR-328 natural BS and its ability to mediate down-regulation of gene expression induced by miR-328.

To attest if such a regulation was dependent on the RNA silencing pathway, the same transfections were made in mouse

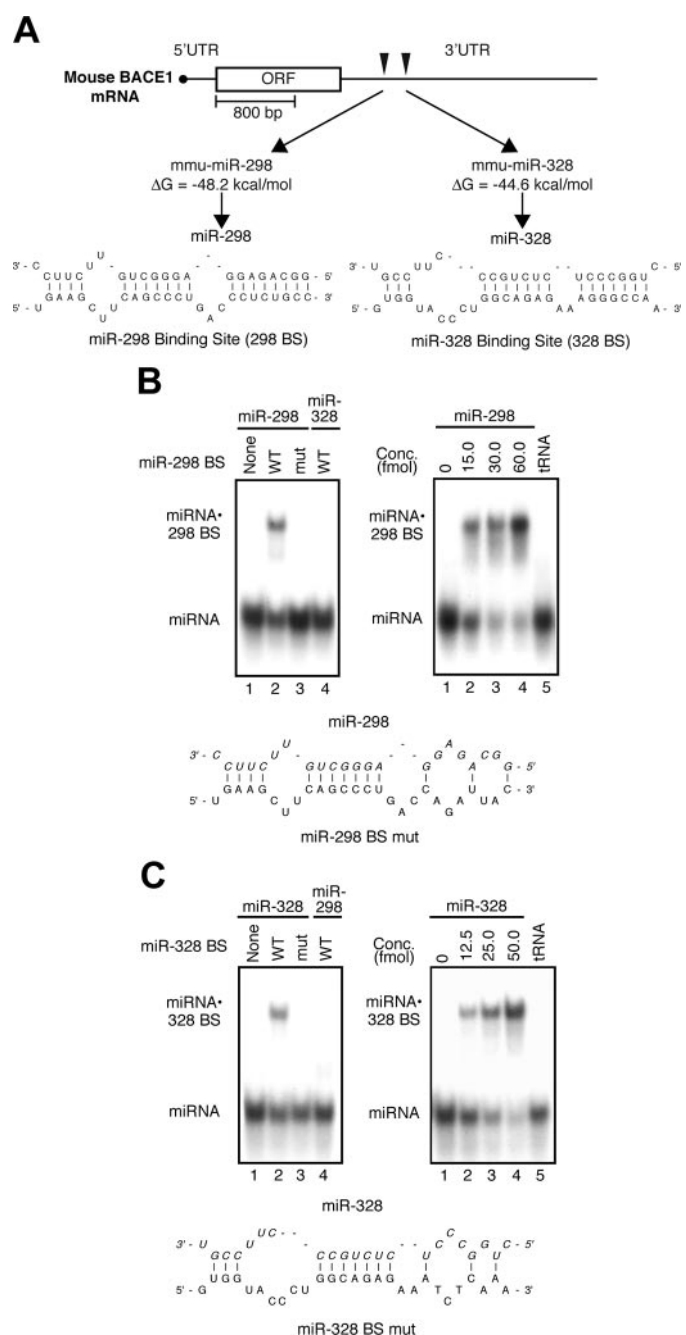


FIGURE 3. Binding sites for miR-298 and miR-328 are present in BACE1 3'-UTR. A, computational prediction of BS for miR-298 and miR-328 in BACE1 3'-UTR and their respective binding free energies (ΔG), as determined by the algorithm RNAhybrid. B and C, EMSAs were performed to validate miRNA recognition of the putative BS for miR-298 (298 BS) (B) or miR-328 (328 BS) (C), using wild-type (WT) and mutated (mut) BS RNA sequences. tRNA was used as control RNA. miRNA:298 BS and miRNA:328 BS duplex formation was monitored by nondenaturing PAGE and autoradiography. Conc., concentration; ORF, open reading frame.

WT and *FMRI* KO embryonic fibroblasts. We have previously demonstrated that *FMRI* KO cells are impaired in their RNA silencing efficiency (28). Gene repression induced upon pre-miR-328 expression averaged 33% in the Naives cell line and was less efficient in RNA interference-deficient cell line STEK TSV-40, averaging less than 21% ($p < 0.05$, unpaired Student's t test). The defect in miRNA-guided RNA silencing observed in the *FMRI* KO cells was relatively modest, which is probably

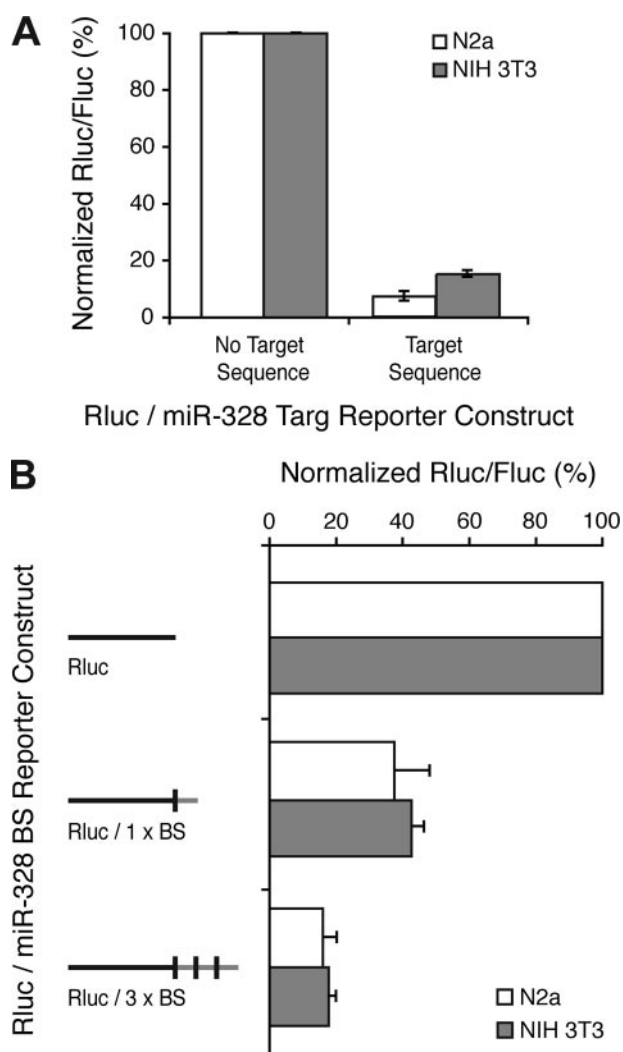


FIGURE 4. The miR-328-binding site element is functionally regulatable upon expression of pre-miR-328. A, N2a and NIH 3T3 cell lines were cotransfected with the pre-miR-328 expression construct and a reporter construct expressing *Rluc* coupled with a single copy of a target sequence perfectly complementary to miR-328 ($n = 3$ experiments). psiNEG, vector encoding an inactive *Rluc* short hairpin RNA; *Targ*, target. The results of *Rluc* activity were normalized with *Fluc* reporter activity and expressed as a percentage of the results obtained with a reporter vector lacking the regulatory sequence in the 3'-UTR of its *Rluc* reporter gene (*Rluc*) (set at 100%). B, N2a and NIH 3T3 cell lines were cotransfected with a reporter construct expressing *Rluc* coupled with 1 ($1 \times BS$) or 3 ($3 \times BS$) copies of the miR-328 natural binding sites (*BS*) and the pre-miR-328 expression construct ($n = 3$ experiments).

due, at least in part, to a compensatory mechanism involving FXR1p, a paralog that has been shown to share the properties of FMRP in miRNA function (28). These findings suggest that the regulatory effects of miR-328 require an integral miRNA-guided RNA silencing machinery.

Specific Binding Sites for miR-298 and miR-328 Mediate Repression of Gene Expression—To investigate the functionality of the miR-298 BS, we circumvented the lack of a suitable pre-miR-298 expression construct by using synthetic miR-298 duplexes. N2a and NIH 3T3 cells were cotransfected with the miR-298 duplex and an *Rluc* reporter construct harboring one or three copies of the BS for miR-298. Introduction of miR-298 duplexes in N2a or NIH 3T3 cells inhibited coexpression of *Rluc* coupled with one copy of its natural BS by 40–55%,

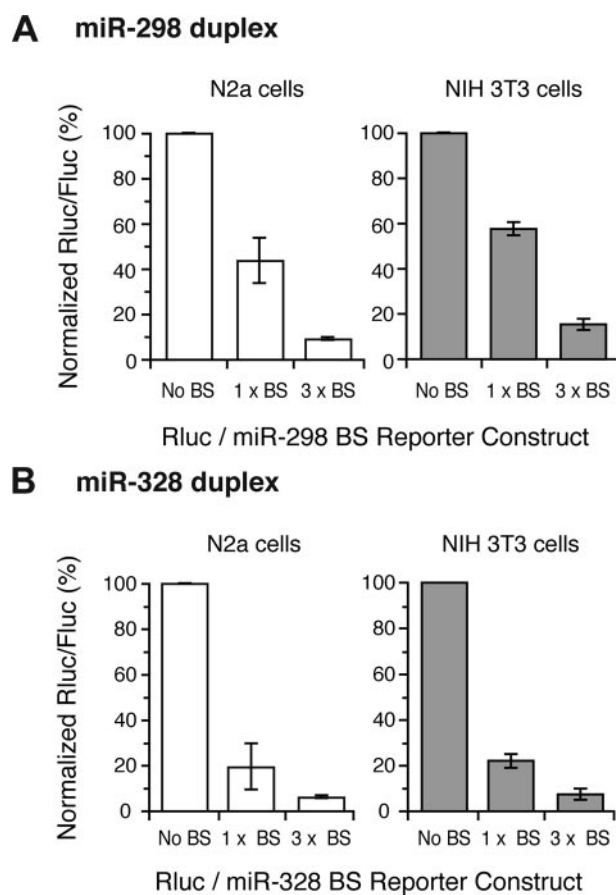


FIGURE 5. The miRNA binding sites for miR-298 and miR-328 mediate repression of gene expression. A and B, N2a and NIH 3T3 cell lines were cotransfected with synthetic duplexes of miR-298 (A) or miR-328 (B) and a reporter construct expressing *Rluc* coupled with 1 ($1 \times BS$) or 3 ($3 \times BS$) copies of their respective natural binding sites (*BS*). The results of *Rluc* activity were normalized with *Fluc* reporter activity and expressed as a percentage of the results obtained with a reporter vector lacking miR-298 or miR-328 BS in the 3'-UTR of its *Rluc* reporter gene (*no BS*) (set at 100%) ($n = 3$ experiments).

whereas the extent of inhibition reached up to 90% in the presence of three contiguous miR-298 BS (Fig. 5A).

miR-328 duplexes exerted similar, albeit more pronounced, gene inhibitory effects, with a 77–80% inhibition conferred by the presence of a single BS, which was less than the 92–94% inhibition observed in the case of three miR-328 BS copies (Fig. 5B). These findings demonstrate that specific BS for miR-298 and miR-328 mediate the suppressive effects of these miRNAs on gene expression in cultured mammalian cells.

Functional Validation of the Binding Sites for miR-298 and miR-328 within BACE1 3'-UTR—Next, we wished to verify if the regulatory properties conferred by the BS could be found within a 200-nt fragment encompassing both BS. To this end, we amplified and cloned this miRNA BS module downstream of *Rluc* in psiCHECK. This construct was cotransfected in N2a or NIH 3T3 cells with miR-298 and/or miR-328 duplexes, and luciferase activity was measured. The miR-196 duplex was used as a negative control. In N2a cells, miR-328 was more efficient than miR-298 in down-regulating *Rluc* expression, and their effects appeared to be additive, as shown in Fig. 6A (left panel). The gene inhibitory effect of the miRNA duplexes was superior in NIH 3T3, with both miRNA duplexes showing similar poten-

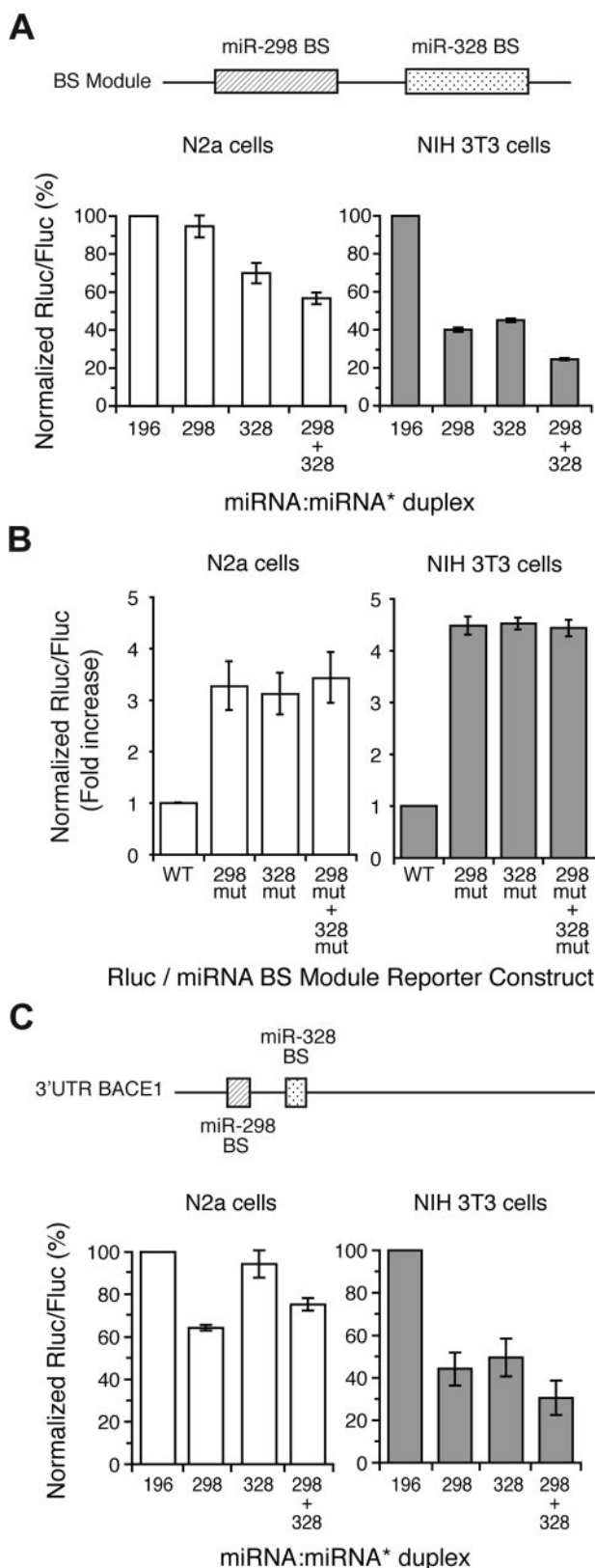


FIGURE 6. Binding sites for miR-298 and miR-328 are functional and act as negative regulators of gene expression. A, N2a and NIH 3T3 cell lines were cotransfected with miR-298 and/or miR-328 duplexes, or miR-196 control duplex, and a reporter construct expressing *Rluc* coupled with the 200-nt miRNA-binding site (BS) module comprising both miRNA BS. The results of *Rluc* activity were normalized with *Fluc* reporter activity and expressed as a percentage of the results obtained with a duplex of miR-196, used as a control (set at 100%) ($n = 3$ experiments). B, N2a and NIH 3T3 cell lines were

cies (Fig. 6A, right panel). Less than 25% residual *Rluc* activity was measured in lysates of NIH 3T3 cells transfected with both miRNA duplexes.

To confirm the functionality of the miRNA BS, we introduced mutations in the BS sequences recognized by the seed region of miR-298 and/or miR-328, transposing in the 200-nt miRNA BS module the mutagenesis design used for the EMSA experiments (see Fig. 3, B and C). Taking advantage of the endogenously expressed miR-298 and miR-328 in N2a and NIH 3T3 cells, we observed a 3–4.5-fold increase in *Rluc* activity from the reporter genes carrying mutated BS for both miRNAs, as compared with the wild-type (WT) sequence (Fig. 6B, left and right panels). Moreover, disruption of each miRNA BS individually did not restore gene repression to WT levels. These findings support the notion that miR-298 and miR-328 may act in concert to functionally regulate gene expression.

Whether the regulatory roles of miR-298 and miR-328 BS are preserved within the entire 3'-UTR of *BACE1* mRNA was assessed using a construct in which this element was inserted downstream of the *Rluc* reporter gene in psiCHECK. As observed in Fig. 6A with the BS module, cotransfected duplexes of miR-298 and miR-328 exerted down-regulatory effects on *Rluc* expression (Fig. 6C). These results demonstrated the regulatory role of miR-298 and miR-328 in the context of the full-length 3'-UTR of *BACE1* mRNA.

miRNA-mediated Modulation of Endogenous BACE1 Levels—Whether endogenous *BACE1* protein levels can be regulated by miR-298 and/or miR-328 in N2a and NIH 3T3 cells was our next objective. For that purpose, miRNA duplexes were transfected in N2a cells, which were harvested 24 h later. Western blot analysis of protein extracts showed that cells transfected with a combination of miR-298 and miR-328 duplexes express lower levels of *BACE1*, as compared with N2a cells treated with the miR-196 control duplex (Fig. 7A). Inhibition of *BACE1* expression was less pronounced when duplexes of either miRNAs were transfected individually into N2a cells.

These findings prompted us to examine if *BACE1* protein expression is regulated endogenously by miR-298 and miR-328 in neuronal N2a and NIH 3T3 cells. It is worth noting that both miRNAs were first cloned and identified in mouse undifferentiated and differentiated embryonic stem cells (38) and rat cortical neurons (39), respectively. Nevertheless, our initial attempts to confirm miR-298 and miR-328 expression in N2a and NIH 3T3 cells by Northern blotting were not successful, presumably because of their relatively low levels. However, the use of a more sensitive Northern blot protocol (35) allowed us to detect both miR-298 and miR-328, as well as their precursors, in N2a and NIH 3T3 cells (Fig. 7B).

transfected with a reporter construct expressing *Rluc* coupled with the miRNA BS module wild-type (WT) or harboring mutations in the miRNA BS sequences recognized by the seed region of miR-298 (298mut) and/or miR-328 (328mut). The results of *Rluc* activity were normalized with *Fluc* reporter activity and expressed in fold increases as compared with the results obtained with the WT 3'-UTR of *BACE1* (set at 1) ($n = 3$ experiments). C, N2a and NIH 3T3 cell lines were cotransfected as described in A, but with a reporter construct expressing *Rluc* coupled with the 3'-UTR of *BACE1*.

miR-298 and miR-328 Regulate BACE1 Expression

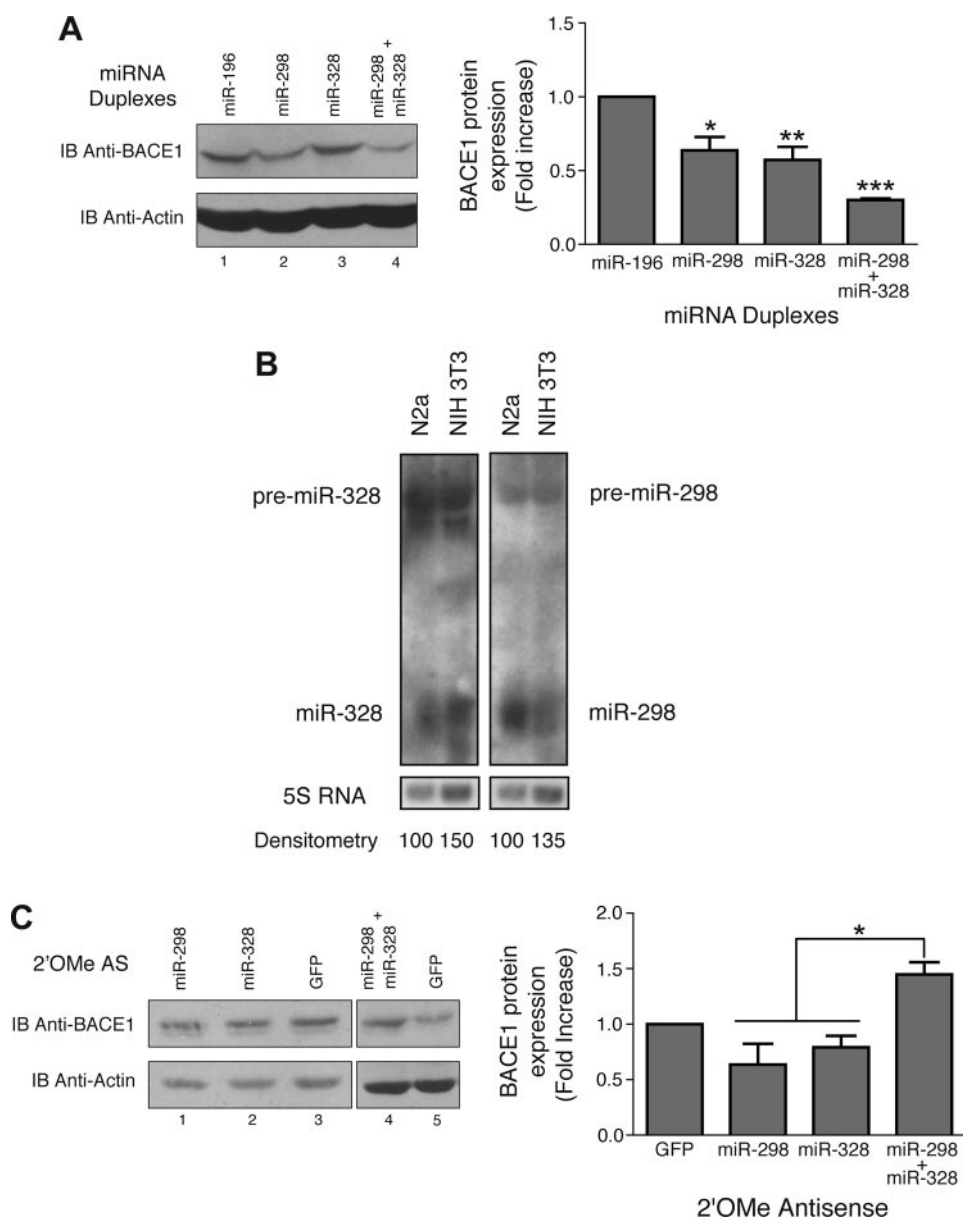


FIGURE 7. miR-298 and miR-328 regulate endogenous BACE1 expression in N2a cells. A and C, N2a cells were transfected with duplexes of miR-298 and/or miR-328, or miR-196 (used as a negative control) (A), or 2'-Ome oligoribonucleotide (2'-Ome) antisenses (AS) directed against miR-298 and/or miR-328, miR-324 or green fluorescent protein (GFP) (used as negative controls) (C). Protein extracts were analyzed by immunoblot analysis (IB) using the PC529 antibody recognizing BACE1 or AC-40 antibody recognizing actin, and the immunoreactive bands revealed by ECL (left panels). The intensity of the bands from three experiments was analyzed quantitatively by densitometry, and the results obtained with the anti-BACE1 antibody were normalized with the anti-actin signal (right panels). Results are expressed as mean \pm S.E. (analysis of variance with Bonferroni's post hoc test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). B, Northern blot analysis of small RNAs (<200 nt) isolated from N2a and NIH 3T3 cells. A 32 P-labeled probe complementary to miR-298 or miR-328 was hybridized following a Northern blot procedure improved for the detection of small RNAs (35) (upper panels). 5 S RNA was probed as a control (lower panels).

To determine whether miR-298 and miR-328 of endogenous origin are important regulators of BACE1 protein levels in neuronal N2a cells, we have used a 2'-Ome antisense approach. Immunoblot analysis showed that neutralization of both miRNAs by complementary 2'-Ome oligoribonucleotides increased the level of BACE1 protein (Fig. 7C). Single neutralization of miR-298 or miR-328 had no significant effect (Fig. 7C), thereby unveiling the coordinated nature of miRNA regulation of BACE1 expression in neuronal cells.

Decreased Expression of miR-298 and miR-328 in the Brain of Aging APP_{Swe}/PS1 Mice—To strengthen the possible link between miRNA regulation of BACE1 expression and AD, we monitored the levels of miR-298 and miR-328 in the brain of aging APP_{Swe}/PS1 mice. ISH experiments revealed the expression of both miRNAs in the granular neurons of the hippocampus (Fig. 8A). The intensity of the miRNA signals significantly decreased by more than 50% in the brain of 13-month-old APP_{Swe}/PS1 mice, as compared with 3-month-old animals (Fig. 8, A and B). Taken together with the results shown in Fig. 1, these data established an inverse correlation between the hippocampal levels of miR-298 and miR-328, and that of BACE1 protein in our mouse model of AD.

DISCUSSION

The major etiologic factor of AD in elderly patients relates to the specific accumulation of deleterious A β peptides in the cortex and hippocampal region of the brain. Because BACE1 is directly involved in A β peptide formation, it has been incriminated as a major player in neuronal degeneration associated with AD. Indeed, post-mortem analysis revealed a 2.7-fold increase in BACE1 protein expression in the cerebral cortex of patients suffering of AD, in parallel with a similar increase in A β peptide levels, when compared with age-matched controls (31). Intriguingly, the up-regulated BACE1 protein expression (31–33) and activity (32, 33), which were correlated (33), could not be transposed to the level of mRNA; in AD brains, BACE1 mRNA levels remain unchanged (31, 40, 41).

These features are faithfully recapitulated in APP_{Swe}/PS1 transgenic mice, a widely used model of AD in rodents. In fact, protein levels of BACE1 in WT mice were shown to slightly increase in the aging brains, consistent with mRNA levels. Moreover, in the APP_{Swe}/PS1 model, BACE1 protein levels increased as early as after the 4th month and remained elevated until the 19 month, whereas mRNA levels showed the opposite trend and were decreased. The apparent loss of the correlation between BACE1 mRNA and protein levels in AD, which is not observed in WT mice, occurs in a

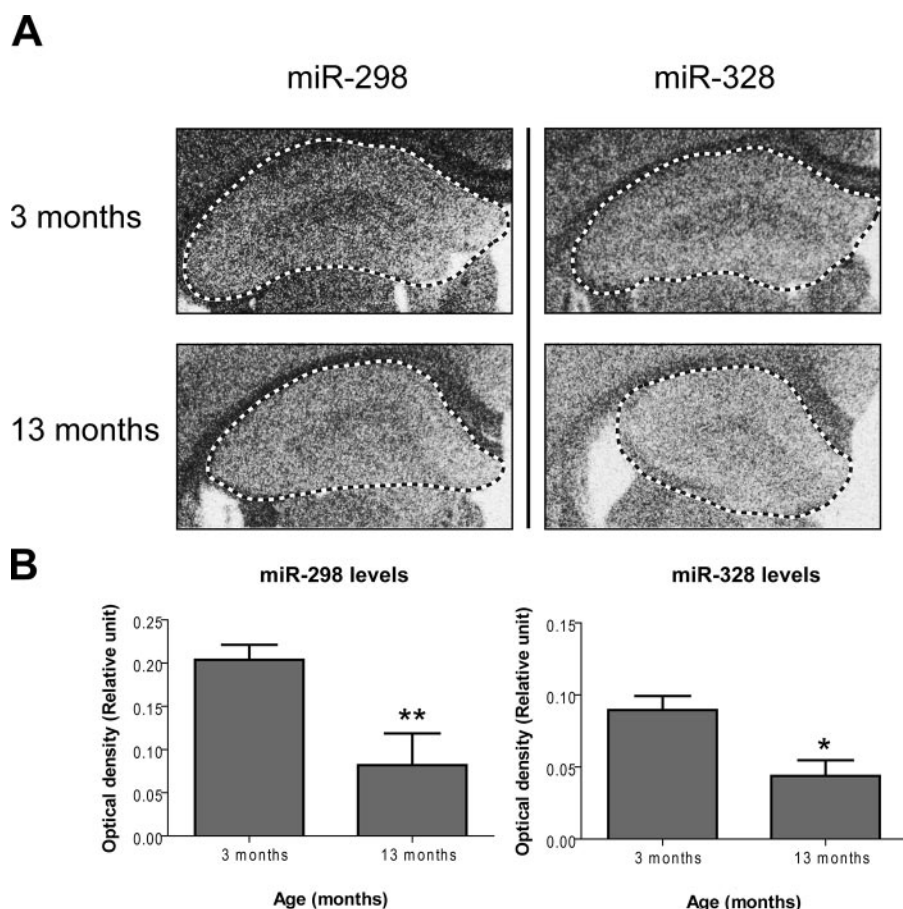


FIGURE 8. Decreased expression of miR-298 and miR-328 in the granular neurons of the hippocampus during aging of APP_{Swe}/PS1 mice. *A*, *in situ* hybridization for miR-298 and miR-328 on brain sections taken from APP_{Swe}/PS1 mice at 3 and 13 months of age. The granular part of the hippocampus is delimited by a dashed line. *B*, quantification of the RNA hybridization signals. Results are expressed in relative optical density (O.D.) units as mean \pm S.E. (Student's *t* test: *, $p < 0.05$; **, $p < 0.01$).

pattern expected for a loss of post-transcriptional repression. Knowing that endogenous miRNAs exert their regulatory role mainly through mRNA translational repression in mammals, an impaired regulation by some miRNAs or a deficient miRNA-mediated translational repression machinery could be responsible for such a variation. This could explain why, even with decreased mRNAs, BACE1 protein levels and area of expression are increased in APP_{Swe}/PS1 mice during disease progression. miRNA-mediated translational repression could exert some kind of fail-safe function by buffering BACE1 protein expression in case of variations at the mRNA level, as seen in the WT.

The scenario involving miRNAs was supported initially by computational analyses predicting the presence of putative BS for miR-298 and miR-328 in the 3'-UTR of BACE1 mRNA. We validated these predictions and obtained experimental evidence establishing a regulatory role for miR-298 and miR-328 in BACE1 expression in mammalian cells, thereby advocating for a causal link between dysfunctional miRNA-based regulation of BACE1 and the etiology of AD. Identification of regulatory elements recognized by miRNAs in the 3'-UTR of BACE1 mRNA adds to the complexity of BACE1 regulation in mammals. A previous study reported the presence of down-regulatory elements in the long, GC-rich 5'-UTR of BACE1 mRNA

(42). Both mechanisms may thus contribute to maintain low levels of BACE protein expression *in vivo*. Discovery of new miRNAs as well as improvement of target prediction algorithms may possibly unveil additional miRNAs involved in regulating BACE1 expression.

Most experimental models designed to study miRNA regulation are based on the recognition of a single complementary BS which, in a sequence of events, leads to cleavage of the mRNA target and down-regulation of its expression. In mammals, however, miRNAs are known rather to recognize BS of imperfect complementarity, to act in synergy, and to repress translation of specific mRNAs through the occupation of a various number of BS. In this study, we showed that the extent of mRNA repression induced by miR-298 and miR-328 was dependent on the number of regulatory BS found in the target 3'-UTR, as reported previously (43). Vector-based expression of pre-miR-298, however, was found not to be suitable for these studies, as it failed even to inhibit expression of a reporter gene bearing a perfectly complementary sequence, probably because of the lack of miR-298

expression (data not shown). This may be related to the changes that needed to be made within the nucleotide sequence at the base of pre-miR-298 to adapt it for expression from the U6 RNA polymerase-driven promoter in psiSTRIKE. First, at position 63 of the precursor sequence, we introduced a U-to-C substitution that changed a G:U wobble for a thermodynamically more stable G:C base pair. Second, the last nucleotide (A) of pre-miR-298, which is part of the 3' overhang, was substituted by a U. The nature of these changes might have altered miRNA recognition and/or processing by the ribonuclease III Dicer, or interfered with strand selection through modification of the thermodynamic stability of the miR-298 duplex extremities. Notably, changes of that nature were not required for miR-328.

When comparing the importance of miR-298 and miR-328 in regulating gene expression in cultured murine cells, we noticed that the regulatory effects exerted by the miR-328 duplex via its isolated BS were more pronounced than those induced by miR-298 in both N2a and NIH 3T3 cells. These results may be explained by suboptimal levels of endogenous miR-328 capable of regulating the expressed reporter gene.

On the other hand, when the regulatory miRNA BS elements were studied within either the miRNA BS module or the complete 3'-UTR of BACE1 mRNA, the strongest repressive effects were observed in NIH 3T3 cells. In fact, the presence of addi-

miR-298 and miR-328 Regulate BACE1 Expression

tional contiguous sequences in these 3'-UTR elements may provide a niche for other miRNAs of fibroblastic origin and influence, to various extent, the gene regulatory effects of miR-298 and/or miR-328. This argument may also explain the decreased regulatory role of miR-298 and miR-328 in neuronal N2a cells when expanding from the isolated miRNA BS to the complete 3'-UTR of BACE1. Knowing that miRNAs act coordinately to repress gene expression, it is also possible that endogenously expressed miR-328 might have contributed, to various extent, to the relative potency of transfected miR-298, and vice versa.

miRNA regulation of BACE1 is further supported by the effects of the mutated BS for miR-298 and/or miR-328, which increased gene expression by more than 3-fold in N2a and NIH 3T3 cells. In these experiments, the inhibitory properties of these elements were equally abrogated regardless of whether miR-298 and/or miR-328 BS had been mutated. Considering that this BS mutagenesis strategy eliminates the contribution of the endogenous miRNAs specifically, we conclude that miR-298 acts together with miR-328 to regulate BACE1 protein expression. The magnitude of the changes observed following 2'-OMe oligonucleotide neutralization of miR-298 and/or miR-328, as compared with the BS mutagenesis strategy, was more modest. The discrepancies between the results obtained from these two different miRNA inactivating approaches may be explained by the miRNA/reporter gene ratio as well as by their relative efficiency to inhibit miRNA binding to their BS; site-directed mutagenesis inactivated the miR-298/miR-328 BS of all reporter mRNA transcripts that were assayed, whereas a 2'-OMe antisense approach is expected to neutralize a smaller proportion of miRNA-miRNA BS interactions. Nevertheless, the combination of these complementary experimental approaches, *i.e.* the ability of miRNA duplexes to decrease endogenous BACE1 expression and that of the 2'-OMe antisenses to increase it, allowed us to strengthen and extend our reporter gene data to neuronal cells endogenously expressing BACE1.

Together, these observations support the concept of a link between dysfunctional miRNA regulation of BACE1 expression and AD. Interestingly, both Drosha and Dicer mRNAs are expressed throughout the brain, with an enrichment in the hippocampus and the dentate gyrus (supplemental Fig. S2). The presence in mouse brain of the two major enzymes involved in miRNA biogenesis is consistent with the notion that these tiny regulatory RNAs are preferentially, actively synthesized in the regions that are the most severely affected by A β deposits in AD. This is supported by the detection of miR-298 and miR-328 in the hippocampus of APP_{swc}/PS1 mice, as well as by their decreased expression levels during aging.

In vivo, BACE1 mRNA is expected to be initially repressed translationally by the concerted action of miR-298 and miR-328. From a mechanistic point of view, whether BACE1 mRNA is translocated to the P-bodies, remains susceptible to be rescued from the P-bodies to be translated again, or is rapidly degraded or not merits further consideration, as well as the identity of the components involved and the dynamics underlying these processes. Whether mRNAs, other than BACE1, expressed in the hippocampus and the dentate gyrus are also

regulated by miR-298 and/or miR-328 also remains to be explored.

We propose a scenario in which the loss of miRNA control of BACE1 expression may deregulate BACE1 protein levels, leading to an increased A β formation and disease progression. Loss of miRNA control may take various forms, such as reduction in miRNA levels, mutational loss of miRNA or miRNA BS function, or a defect among the components of the miRNA pathway hampering optimal miRNA biogenesis or function. Regarding miR-298 and miR-328 BS, analysis of their evolution and conservation is rather complex and difficult because, in contrast to mice, human hosts four different BACE mRNAs harboring different 3'-UTR of various lengths and diverse composition. In addition, whereas the miR-328 sequence is perfectly conserved between mouse and human, that of miR-298 is only 72% identical. Therefore, the possibility that human BACE mRNAs are subjected to regulation by miRNAs other than miR-298 and miR-328 cannot be excluded and should be considered.

In support of our hypothesis, a recent study reported the abundance of specific miRNAs in the hippocampus, their differential regulation in aged brains, and changes in miRNA expression profile consistent with an altered miRNA-mediated mRNA regulation in AD brain (44). Our study offers a new perspective on the regulation of BACE1 expression, which may have important implications in the etiology and treatment of AD.

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