Analyses of Cell Type-Specific Effects of MicroRNA-298 on Native Protein Expression Via Truncated 3'UTR Hold Translational Promise

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Research Article

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Abstract

Alzheimer's disease (AD) is marked by neurofibrillary tangles and senile plaques comprising amyloid β (Aβ) peptides. However, specific contributions of different cell types to Aβ deposition remain unknown. Non-coding microRNA (miRNA) play important roles in AD by regulating major proteins involved, like Aβ precursor protein (APP) and β-site APP-cleaving enzyme (BACE1), two key proteins associated with Aβ biogenesis. MiRNAs typically silence protein expression via binding specific sites in 3'- untranslated region (3'UTR) mRNA. MiRNA regulates protein levels in a cell-type specific manner; however, mechanism of miRNA's variable activities remains unknown. We developed "miRNA-associated native protein expression" (miRnape) assays to determine a *natural* "UTR limit" for a miRNA's function in a particular cell type. We report that miR-298 treatment reduced native APP protein levels in an astrocytic but not in a neuronal cell line. From miR-298's effects on APP-3'UTR activity and native protein levels, we infer that APP 3'-UTR length could explain the differential miR-298's activity. Such truncated, but natural, 3'-UTR found in a specific cell type provides an opportunity to regulate native protein levels by particular miRNA. Thus, miRNA's effect tailoring to a specific cell type bypassing another undesired cell type with a truncated 3'-UTR would potentially advance translational research.

Introduction

Alzheimer's disease (AD) is the leading cause of dementia globally and has no effective disease modifying therapy [1]. AD is a progressive neurodegenerative disease, pathologically characterized by extracellular deposition of amyloid plaques, intracellular neurofibrillary tangles, synaptic loss, dystrophic neurites, neuroinflammation and glia activation in vulnerable brain regions, hippocampus and cortex. [2] The co-existence of amyloid plaques, which mainly consist of amyloid β (Aβ), and neurofibrillary tangles, which primarily comprise hyperphosphorylated tau protein, remains the fundamental requirement for pathological diagnosis. [3]

Aβ peptide is derived from its precursor molecule, Aβ precursor protein (APP) via sequential cleavage by β-secretase enzyme (β-site APP-cleaving enzyme, BACE1) and γ-secretase complex. Dysregulation of APP and BACE1 proteins during disease progression have long been the focus of AD research.[4–6] However, several clinical trials tested with the Aβ plaque cascade hypothesis trying to stop or reverse disease progression result in unsatisfactory results; hopefully, new research would pave the way for new treatment options. [7] Indeed, renewed optimism has been fueled by aducanumab's recent approval by the FDA as probably the first disease-modifying anti-amyloid treatment.[8] Several BACE1 inhibitor clinical trials using small pharmacological molecules including verubecestat, lanabecestat and LY2886721 found significant adverse effects in multiple organs besides brain.[9–13] Non-specific overall BACE1 inhibition might account in part for the various adverse effects. Thus, regulation of APP and BACE1 in an organ or cell type specific manner is very important, a goal that can be achieved by microRNA (miRNA) regulation of mRNA translation.

AD transcriptomes shows progressive changes in hippocampal functions, epigenetics and miRNA regulation.[14] It is known that AD is marked by cerebrovascular deposition of Aβ peptides; however, specific contributions of different tissue and cell types to Aβ deposition remain unknown. We try to bridge this knowledge gap by studying the role of specific miRNAs that play key roles in AD by regulating key proteins involved.

MiRNAs are a group of short non-coding RNAs with mature length around 22 nucleotides. MiRNAs function as important regulators of mRNA translation, modulating gene expression post-transcriptionally. Typically, miRNAs are generated from longer double-stranded RNA precursors via sequential cleavage of the primary miRNA (pri-miRNA) transcripts by two RNase III proteins, Drosha and Dicer.[15] First, pri-miRNAs are transcribed by RNA polymerase II in various genomic contexts. In humans, most pri-miRNAs reside in introns of coding or non-coding transcripts. However, miRNA genes also exist within larger gene exons or independent genomic transcription units.[16] Pri-miRNAs are usually over 1kb long and contain secondary stem-loop structures, where mature miRNA sequences are embedded. After transcription, pri-miRNAs are cropped by Drosha and its essential cofactor DGCR8 to release pre-miRNAs that are around 60 nucleotides long and have typical hairpin structures. Following Drosha processing, pre-miRNAs are transported out of the nucleus via exportin-5 for further maturation.

In the final processing step, pre-miRNAs in the cytoplasm are bound by Dicer, which cleaves the stem loop structure and releases a miRNA-miRNA duplex typically of 21–25 nucleotides in length. The small RNA duplex then binds to Argonaut protein (AGO) to take part in a large ribonucleoprotein complex called RNA-induced silencing complex (RISC). Upon AGO loading, the miRNA duplex will unwind, leaving one strand of miRNA from the duplex to serve as the guide strand. The other strand (aka “passenger strand”) is cleaved or removed. Guide strand selection is not very strict and mainly depends on thermodynamic stability of AGO-miRNA binding.[17]

MiRNAs within the RISC complex generally inhibit target mRNA expression by either transcription inhibition or mRNA degradation. MiRNA binding to target mRNA 3’UTR usually depends on its seed sequence located at 2–8 nucleotide from the miRNA 5’ end. Seed sequence base pairing with target mRNA 3’-untranslated regions (UTR) is exactly complementary though base pairing on other sites may not be perfect.[18,19]

MiRNA regulation of APP and BACE1 mRNA has recently been studied. APP mRNA can be regulated by multiple miRNAs including miR-20b, miR-101, miR-153, miR-31, miR-346, miR-106a, miR-520c[20–27]. BACE1 can be regulated by miR-124, miR-339-5p, and miR-29c[28,29]. Notably, miR-298 reduced both APP and BACE1 levels.[30,31]

In this context, we have recently reported [31] that miR-298 targets APP and BACE1 mRNA 3’UTRs. Notably, miR-298 treatment reduced two major potentially toxic forms of Aβ (Aβ 1–40 and Aβ 1–42) by reducing APP and BACE1 proteins in primary human mixed cell culture derived from human fetal brain samples.

Surprisingly, miR-298 also reduced one specific isoform of tau protein. However, specificity of miR-298 functions regarding cell types including neurons, microglia and astrocytes has been poorly studied.

Several miRNA profiling studies have demonstrated that miRNA levels vary greatly in different cells, tissues and organs.[32] But differences in miRNA function and targeting in cell and tissue specific manners has not been well studied, especially the mechanism. MiRNA and mRNA transcript interaction can be extensively regulated via multiple potential mechanisms, which can be leveraged to translational advantages. For example, alternative polyadenylation could shorten the length of 3'UTR mRNA that deletes the miRNA binding sites. Further, the presence of a single nucleotide polymorphism (SNP) within or around miRNA binding sites might alter the folding energy and hence binding affinity. For example, miR-298 miRNA levels and SNPs associated with AD progression as well. Moreover, the endogenous miRNA levels vary greatly among various cell types. The presence of high level of miRNAs could saturate the target. Finally, mRNA binding proteins that interfere with miRNA binding can also be expressed in a cell-type specific manner.

In the present study, we attempted to address miRNA's activity at the cellular level. Although miRNA regulates protein levels in a cell-type specific manner, the mechanism of miRNA's variable activity in different cell types remains unknown. We posit that although miRNA binds a specific seed sequence in the 3'-UTR of a target mRNA, the variation in UTR length, with the seed sequence absent, could prevent a miRNA's binding and, thus, its activity in a particular cell type. The *natural* 3'- UTR could be a full length (e.g., 1.12kb for APP-3'UTR) in a particular cell type or truncated (undefined) in another. We have considered several methods to determine the exact length and sequences of target mRNA 3'-UTR to account for differential miRNA activity. For example, the 'Rapid amplification of cDNA ends' (RACE) is a common technique to obtain the full length sequence of an RNA transcript found within a cell. However, sequence information *alone* would not be sufficient to understand the activity of miRNA and hence its *function* in regulating cellular protein.

Instead of RACE, we developed "miRNA-associated native protein expression" or "miRnape" assay by RNA transfection and cellular protein expression studies (including mRNA and protein levels). We present data that miRnape assay can determine a *natural* "UTR limit" for a miRNA's function in a cell type. Briefly, we selected several miRNAs that are located at different locations within APP-3'UTR. We checked the function of each miRNA on APP-3'UTR activity using the full-length UTR and dual reporter assay. Since miRNAs bind to target sequence on mRNA 3'-UTR and inhibit protein, we checked protein expression too. In short, by selecting a range of miRNAs with different binding sites on the same target mRNA 3'-UTR, we could map the biologically active 3'-UTR. The miRnape-based technique would be useful in understanding a miRNA's cellular function.

Herein, we show the effects of miR-298 in different cell lines. MiR-298 reduces APP and BACE1 in one type of human astrocytes U373 but not in human differentiated neurons or microglia cells. Our results suggest that miR-298 regulates its targets in a cell type specific manner by leveraging full-length or truncated 3'-UTRmRNA.

Materials & Methods

Cell cultures

Different cell lines, such as human glioblastoma (U373), neuroblastoma cells (SK-N-SH), microglia (HMC3) and HeLa cells, were obtained from ATCC. Cells were grown in Eagle's modified minimum essential media (EMEM) containing 10% FBS and penicillin/streptomycin solution at 37°C in 5% CO2 humid incubators as described.[57]

Cloning of APP 3'-UTR and BACE1 3'-UTR into a dual reporter vector.

We performed the cloning of APP 3'UTR (gene accession NM_000484.3) in the following way. APP 3'-UTR (1.1 kb) was cloned into a dual luciferase assay reporter vector pEZX-MT05 (GeneCopoeia), downstream of Guassia luciferase (GLuc) gene, which is driven by SV40 promoter. A synthetic poly(A) tail follows APP 3'-UTR sequence. The pEZX-MT05 plasmid is 8.6 kb long containing a separately transcribed gene, Secreted Alkaline Phophastase (seAP) luciferase gene, with its own CMV promoter and poly(A) tail served as an internal control.

Likewise, we cloned BACE1 3'-UTR (3.9 kb) (gene accession NM_012104.4) into a dual luciferase assay reporter vector pEZX-MT05 as described above.

Reporter assay to measure UTR activity

GLuc and seAP luciferase activities were separately measured following the manufacturer's instructions (GeneCopoeia). Briefly, cell transfection media supernatants were collected and mixed with GLuc or seAP substrates. The luciferase intensities of mixes were then measured by a Veritas microplate luminometer (Turner Biosystems).

Transfection

Transfections were performed when cells reached around 80% confluence. Culture media were replaced with Opti-MEM media with 1% FBS and antibiotics were omitted from transfection media. For miRNAs and siRNAs transfection, Lipofectamine RNAiMax was applied 2µl per well in a 24 well plate format. Then 75nM miRNA or 50nM siRNA were premixed with RNAiMax according to the protocol. The newly formed RNA mimics-transfection complexes were added into each well and kept for 72 hours or otherwise indicated in figure legends. For co-transfection of miRNAs with plasmid, cells were treated with Lipofectamine RNAiMax and reporter vector alone or along with 75nM miRNAs. Cell culture media were harvested for luciferase assay after 72 hours.

Lysis of cells

After washing with PBS, cells were lysed on-plate with vigorous shaking using 100µl RIPA buffer containing 1x Halt Protease Inhibitor Cocktail (Thermo Scientific). Protein concentration was determined by BCA (ThermoFisher Scientific) assay according to the manufacturer's instructions, and then Laemmli

sample buffer (LSB) was added to each tube of lysate. Lysate and LSB mixes were boiled for 10 minutes and cooled down on ice or kept in freezers for further studies.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting

An equal amount of protein lysate was loaded onto 26 lane BisTris XT denaturing 4–12% polyacrylamide gels and run with XT MOPS or XT MES buffer at 200 V for 1 hour. Proteins were separated with SDS-PAGE and then transferred overnight onto PVDF membranes. Membranes were stained with 0.1% Ponceau S solution to confirm transfer success. After three times of washing using TBS with 0.05% Tween 20 (TBST), membranes were incubated with 5% nonfat milk in TBST for 1 hour at room temperature. Primary antibodies were incubated at either room temperature for 3 hours or 4°C overnight. Primary antibodies used in this study are APP (Millipore MAB348), BACE1 (Cell Signaling D10E5) and β-actin (Sigma A-5441). Either goat anti-rabbit or mouse secondary antibodies was applied for 1 hour at room temperature. Protein bands were visualized using ECL and autoradiography. Films were scanned for densitometry analysis.

Autorads were exposed at different times, and the most optimum images were selected. Time of exposures varied from 10 sec to 60 sec, according to the experiments. They were explicitly mentioned in the image of each raw western blot as shown separately in Supplemental file. The brightness and contrast of western blot images were adjusted only for presentation and not for quantification. Samples from all treatment groups were analyzed in a same gel, transferred onto a single membrane, and presented here without cropping. The same blot was cut horizontally into two parts. The top part was probed anti-APP and anti-BACE1 antibodies sequentially and process as described above. The bottom part of the blot was primarily used to probe against be-actin, which was used to normalize protein loading in each gel. This reprobing in each blot was explicitly shown in the Supplemental file.

RNA isolation and real-time quantitative PCR

Total RNA was isolated with mirVana miRNA isolation kit (ThermoFisher Scientific) following the manufacturer's protocol. RNA concentration was assessed by Nanodrop instrument (ThermoFisher Scientific). Equal amount of RNA per sample was reverse transcribed with High Capacity RNA-to-cDNA kit (Applied Biosystems) for mRNA quantification. For miRNA quantification, total RNA was extracted and reverse transcribed with TaqMan microRNA Reverse Transcription kit (Applied Biosystems). cDNA was subjected to real-time qPCR analysis on QuantStudio 6 Flex instrument (Applied Biosystems). Relative quantification was achieved by $\Delta\Delta C_T$ normalization with the geometric means of housekeeping genes GAPDH and β-actin.

Data and Statistical Analysis

Western blots were scanned, and target band densitometry were determined by ImageJ software. Statistical analysis was performed with JMP software (SAS Institute). For comparing means of two groups, two-tailed student's t test was applied. For comparison of means across more than two groups

with one or two variable(s), analysis of variance (ANOVA) was applied; Tukey's honest significant difference (HSD) test was followed to compare means between each two groups. Statistical significance threshold was set at 0.05.

Results

Analyses of APP- and BACE1 3'-UTR reveal potential miRNA binding sites and polyadenylation sites. APP and BACE1 mRNA 3'-UTRs have multiple potential polyadenylation sites[33], which could interfere with or even prevent miRNAs from binding to their target sequences (Fig. 1A-B). The seed sequence-binding site of miR-101 (242–249 bp counting from the beginning of 3'-UTR) on APP 3'-UTR is upstream of miR-298 binding sites (549–555 bp, and 780–786 bp). In addition to APP, we also studied BACE1 regulation. The seed sequences of binding sites of miR-339 (484–490, and 610–617 bp) on BACE1 3'-UTR are also much upstream of miR-298 binding sites (4225–4231 bp). Potential alternative polyadenylation could truncate the APP- or BACE1 3'-UTR, deleting miR-298 binding sites. APP 3'-UTR can be either truncated, mutated, or bound by some cis-acting elements. Based on the above, we propose a possible mechanism of APP 3'-UTR modification as shown in Fig. 1C.

MiR-298 reduced exogenous, full-length, defined APP 3'-UTR activity. APP 3'-UTR was cloned into a dual luciferase assay reporter vector, downstream of Guassia luciferase (GLuc) gene (Fig. 2A-B). Secreted Alkaline Phophastase (seAP) luciferase gene served as an internal control (Fig. 2A). Also, miR-101 and miR-298 binding sites are shown in Fig. 2B. Separate treatment of miR-298 and miR-101 significantly reduced APP 3'UTR activities as compared to APP 3'-UTR constructs alone and APP 3'-UTR with miR-298 inhibitors. (Fig. 2C-D) They did not significantly alter the activities of empty vector. Both miR-101 and miR-298 are able to bind full-length APP 3'UTR and reduce its activity.

MiR-298 administration reduced APP and BACE1 mRNA in astrocytes but not in neurons. MiR-298 significantly reduced APP and BACE1 mRNA levels in astrocytes (Fig. 3A-B) but not in differentiated neurons. (Fig. 3C-D) It indicates that miR-298 associated with degrading target mRNA transcripts in astrocytes. Endogenous miR-298 level was significantly higher in differentiated neurons than in astrocytes. (Fig. 3E) The miR-298 level was quantified by normalization with several other small non-coding RNA miR-16, RNU48, and RNU6B validated and applied in various studies as reference small RNAs. [34–36]

MiR-298 significantly reduced endogenous native APP and BACE1 protein levels in human astrocyte cells. First, we mapped appropriate miRNA binding sites (e.g., miR-339 and miR-298) that should be present in endogenous, native, undefined APP and BACE1 3'-UTRs (Fig. 4A-B). Notably, miR-298 overexpression significantly reduced (~ 80–85%) levels of APP and BACE1 protein in human astrocyte cells *vs.* mock transfection. When miR-298 and antagomiR (anti-miR-298) were co-transfected, protein reduction was significantly reversed, though partially. The antagomiR alone did not alter levels of APP or BACE1 (Fig. 4C, E, F), suggesting that interaction between the antagomiR with endogenous miR-298 is incomplete. Alternatively, endogenous miR-298 levels were too low that there was no functional binding on its target

mRNAs and that further inhibition by the antagomiR could not significantly alter target protein levels. Besides reduced APP and BACE1 protein levels, cell viability was also significantly reduced by miR-298 in astrocytes, suggesting the essential roles of APP and BACE1 protein in cellular viability (Fig. 4D).

MiR-298 did not reduce endogenous APP protein in differentiated neurons. MiR-298 treatment did not significantly reduced APP protein *vs*. mock transfection; and it was not significantly different from miR-298 inhibitor (antagomiR) treatment and non-toxic to the cultures (Fig. 5A-D). This is the significant difference between astrocytic and neuronal cell lines used here. Further, we noted different profiles of APP bands between these cell types, which could be due to splicing variants, and posttranslational modifications (Figs. 4–5). MiR-298 treatment did not alter BACE1 protein levels (Fig. 5E). We asked the question of whether transfection of small RNA is hindered in differentiate NB cells. Independent transfection by APP and BACE1 siRNA resulted in significant reduction of APP and BACE1 levels, respectively, suggesting that siRNA transfection was unhindered in these neurons (Fig. 5). MiR-298 treatment did not change neuronal cell viability in contrast to the significant reduction in U373, where both APP and BACE1 levels were significantly reduced (Fig. 4).

MiR-339-5p but not miR-298 changed endogenous BACE1 protein levels in differentiated neurons. We mapped miRNA binding sites (e.g., miR-339 and miR-298) in BACE1 3'-UTR that should be either present or absent in endogenous native BACE1mRNA depending on the cell type (Fig. 6A). Like in APP3'UTR described before, specific potential alternation or variation could interfere with miR-298 binding. (Fig. 6A) Notably, miR-339-5p transfection but not miR-298 significantly reduced native BACE1 protein (Fig. 6B-C). Likewise, cell viability was not changed by miR-298. (Fig. 6D)

MiR-298 treatment's effect on APP and BACE1 proteins depends on the intrinsic property of each cell type.

We recognize that each cell type has not only its specific 3'UTR, either full-length or truncated, but also other cellular 'factors' intrinsic to each cell type. We asked whether or not cell-type specific factors, such as secreted proteins, cytokines and small molecules, would explain the differential miR-298 results discussed previously (Figs. 5 and 6). Interestingly, some of these factors are secreted in the respective cell culture media. Since cells are allowed to grow 72 hours prior to harvest, could such secreted products influence the outcome of miR-298 in a cell-type manner? Since we cannot take intracellular proteins/factors from one cell type and add them to another, we reasoned that secreted products that are usually selected out and collected could be a good proxy. For this, we divided the experiment in the following way.

One set of astrocytes (U373) were transfected with miR-298 and cultured in fresh opti-MEM media for 3 days, as one usually would do. The second set of astrocytes were similarly transfected with miR-298 but cultured in opti-MEM media that were derived from differentiated neurons (NB) for 3 days. Such media exchange, from 'negative-effect' NB cells to U373 cells to, did not significantly change the otherwise positive-effects observed in U373 cells. (Fig. 7A-B) Therefore, miR-298's effect remained the same irrespective of post-transfection media change in astrocytes, and we attribute it to intrinsic property of the

cell type used herein. To confirm it further, we did the reverse (cell type) experiment similarly. The first batch of neuronal (NB) cells were transfected with miR-298 and cultured in fresh opti-MEM media. The second set of NB cultures were transfected with miR-298 but cultured in opti-MEM media derived from U373 cells for 3 days. Like the previous time, such media exchange did not significantly change the results. (Suppl. Figure 1A-B) In neurons, miR-298's "negative-effect" was not altered in the second set even though the cells received media from the "positive" U373 cells at the post-transfection stage.

Discussion

The central nervous system (CNS) is a remarkably complex organ system, requiring an equally complex network of molecular pathways controlling the multitude of diverse cellular activities. Gene expression is a critical node at which regulatory control of molecular networks is implemented. As such, elucidating the various mechanisms employed in the physiological regulation of gene expression in the CNS is important both for establishing a reference for comparison to the diseased state and for expanding the set of validated drug targets available for disease intervention. MicroRNAs (miRNAs) are an abundant class of small RNA that mediates potent inhibitory effects on global gene expression. Recent advances have been made in methods employed to study the contribution of these miRNAs to gene expression.[37] Here we present a methodological workflow from the perspective of an investigator studying the physiological regulation of a gene of interest. We discuss methods for identifying putative miRNA target sites in a transcript of interest, strategies for validating predicted target sites, assays for detecting miRNA expression, and approaches for disrupting endogenous miRNA function. We consider both advantages and limitations, highlighting certain caveats that inform the suitability of a given method for a specific application. Through careful implementation of the appropriate methodologies discussed herein, we are hopeful that important discoveries related to miRNA participation in CNS physiology and dysfunction are on the horizon.

Our lab has studied the mechanisms of cell-type-specific regulation of APP, APOE, and BACE1 genes, focusing primarily on promoters and 5'-flanking regions[38–43]. The present novel findings of miRNA's role on protein expression, focusing on the 3'-UTR of APP and BACE1, add significantly to our previous studies on gene regulation. Herein, we posit that although miRNA binds a specific seed sequence in target mRNA 3'- UTR, the variation in UTR length might prevent a miRNA's binding and, thus, affects its activity in a particular cell type. In this study, we demonstrated that miR-298 significantly reduced APP and BACE1 levels in human astrocytes at both protein and mRNA levels. However, miR-298 does not alter APP or BACE1 levels in other human cell lines. Surprisingly, the effects of one miRNA could be so diversified and even contrasting in different cells. The cell-type-specific regulation by miRNAs has not been well studied. Few cases have been reported, but the exact mechanism of the phenomenon remains unclear.[44]

We embarked on this study to answer a fundamental question. It is known that miRNA regulates protein levels in a cell-type specific manner but how miRNA functions differently in various cell types remains elusive. We postulated that although miRNA binds a specific seed sequence in the 3'-UTR of a target mRNA, the variation in UTR length could prevent a miRNA's binding and thus affect its activity in a

particular cell type. We reasoned that the natural 3'- UTR could be a full length (e.g., 1.12kb for APP-3'UTR and 4.36kb for BACE1-3'UTR) in a particular cell type or truncated (undefined) in another. The main objective of our work was to address this issue as far as miRNA's function is concerned.

We deliberated other methods to determine the exact sequences of target mRNA 3’-UTR in order to account for differential miRNA activity. For example, the RACE technique is generally used to obtain the full-length sequence of an RNA transcript found within a cell. RACE produces a cDNA copy of the RNA sequence of interest, generated through reverse transcription, followed by PCR amplification of the cDNA copies. It involves using one common primer that takes advantage of mRNA transcript poly(A) tail and another customized primer. In essence, the PCR amplification is one-sided PCR with single-sided specificity. Further, such a sequence is not sufficient to understand the activity of miRNA and hence its function in regulating cellular protein.

Instead of RACE, we utilized a miRnape to determine a natural "UTR stop" in a cell type. In essence, we selected several miRNAs that are located at different locations within APP 3'-UTR. We checked the function of each miRNA on UTR activity using the full-length UTR and by dual reporter assay. Then we checked the function of each miRNA on native protein levels. Transfection results of known miRNA on native protein expression would determine whether UTR is fully active (positive results) or truncated (negative results) in a particular cell type. We then matched the results by doing transfection experiment with a known full-length UTR. Using a range of miRNAs with binding sites spread across the same target mRNA transcript 3’-UTR, we could map the functional miRNA’s site within 3’-UTRmRNA. Our miRnape technique is useful and may prove to be complementary to RACE in understanding cellular function of a miRNA.

To explain our work, we also propose several potential hypotheses and additional explanations.

Scenario 1: Endogenous miR-298 levels vary in various cells. It is possible that in some cells miR-298 levels are sufficiently high that its targets are already saturated. Additional exogenous miR-298 mimics transfected into cells could not further reduce endogenous APP and BACE1 levels. However, this hypothesis is not favored in our case based on the two pieces of evidence. First, endogenous miR-298 levels vary a little compared to exogenous miR-298 transfected. An about 5-fold elevation in neurons is not likely to saturate its target. Second, even if endogenous miR-298 had already saturated to its targets, exogenous miR-298 inhibitors should disengage that interaction and significantly increase APP and BACE1 levels, which is not the case.

Scenario 2: Alternative polyadenylation sites or SNPs are present within mRNA 3’-UTR targeted by miR-298. Our results suggest that endogenous APP protein was reduced only in U373 but not HMC3 (Suppl. Figure 2A-C) or neurons, and that a full-length APP 3’-UTR activity reporter activity was reduced in neurons when co-transfected with miR-298 mimics. These results indicate that endogenous APP 3’-UTR is not identical in all cell types. We consider two possibilities, either alternative polyadenylation site makes the APP 3’-UTR shorter in some cells or APP 3’-UTR has some SNPs in miR-298 binding region that reduces miR-298 binding affinity. However, the SNPs reported in the NCBI SNP database are located

within or close to miR-101, miR-298, and miR-339 seed sequence binding sites on APP and BACE1 mRNA 3’-UTR, are of very low frequency (< 0.03%). The presence of such a low frequency of related SNPs would unlikely explain the miRNA effect differences observed by these rare SNPs, some of which are listed in Table 1.

Table 1
SNPs located within or close to miRNA seed sequence binding sites*

miRNA	Target	SNP	Frequency
miR-101	APP 3’-UTR	rs1568997318	0.000007
miR-101	APP 3’-UTR	rs1439565783	0.000057
miR-101	APP 3’-UTR	rs1260407227	0.000007
miR-101	APP 3’-UTR	rs2036975815	0.000007
miR-298	APP 3’-UTR	rs1479517600	0.000043
miR-298	APP 3’-UTR	rs191651536	0.0002
miR-298	APP 3’-UTR	rs2036942563	0.000004
miR-298	APP 3’-UTR	rs2036942344	0.000004
miR-298	APP 3’-UTR	rs1216676820	0.000007
miR-339	BACE1 3’-UTR	rs570503330	0.000091
miR-339	BACE1 3’-UTR	rs2034340128	0.000004
miR-339	BACE1 3’-UTR	rs2034340051	0.00006
miR-339	BACE1 3’-UTR	rs1159500293	0.000004
miR-339	BACE1 3’-UTR	rs1053615732	0.000008
miR-339	BACE1 3’-UTR	rs755195807	0.00015
miR-298	BACE1 3’-UTR	rs910101318	0.000004
miR-298	BACE1 3’-UTR	rs1037052324	0.000004
miR-298	BACE1 3’-UTR	rs922820472	0.000019
* SNP information was obtained from dbSNP of National Center for Biotechnology Information.			
https://www.ncbi.nlm.nih.gov/snp/rs1568997318?vertical_tab=true			

Scenario 3: Some cis-acting elements bind to APP and BACE1 mRNA 3’-UTR and may prevent miR-298 from binding. Indeed, miRNA machinery in different cells work differently, even miR-298 and its target mRNA 3’-UTR are identical, its results would differ in different cells. In the case of exogenous APP 3’-UTR transfection experiments, since APP 3'-UTR are over-expressed transiently into the cells compared to

endogenous APP mRNA, the abundance of exogenous APP 3'-UTR may make endogenous cis-acting elements, which bind native APP mRNA, unavailable. In other words, exogenous APP 3'-UTR might not be as tightly controlled as native APP mRNA.

To move the project further, sequence of native APP and BACE1 mRNA3'-UTRs would be important to check whether polyadenylation sites exist. Likewise, sequence of genomic DNA containing APP and BACE1 genes would confirm whether SNPs near miR-298 binding site might interfere with the binding. Further, transfection of miR-298 mimics in primary or induced pluripotent stem cell (ipsc) derived astrocytes and microglia as well as neurons derived from same patient would be an important model. The role of miR-298 in these cells would have critical biological significance.

In addition to Aβ generation, APP also plays critical roles in multiple biological and pathological processes, including synaptic pruning, inflammation, iron regulation and mild traumatic brain injury (mTBI).[45–47] Indeed, insufficient pruning could be a potential cause of autism spectrum disorder.[48,49]

Likewise, BACE1 serves more than an APP cleaving enzyme and a pathogenic role in AD. BACE1, an aspartic protease, has many other native substrates in the brain, such as neuregulin[50], seizure protein 6[51] and sodium gated voltage channel β2 (Navβ2)[52], which are important for neuron function and biology. Other BACE1 substrates are involved in cell signaling and immunity including Golgi localized membrane-bound α−2,6−sialyltransferase (ST6Gal I)[53], interleukin-1 type II receptor (IL1R2),[54] P−selectin glycoprotein ligand−1 (PSGL-1)[55] and low density lipoprotein receptor−related protein (LRP).[56] By reducing BACE1 levels in astrocytes instead of neurons, we could possibly inhibit pathological Aβ production while saving the physiological functions BACE1 has in neurons. Same line of reason, APP is also not just amyloid producing. APP is involved in multiple signaling pathways by interacting with other proteins.

We have also considered the translational implication of our work based on miRnape. We suggest that truncated but *natural*, 3'-UTR found provides an avenue to regulate native protein levels by a particular miRNA in a cell type-specific manner. In short, while a traditional chemical or drug would have access to any cells, miRNA's (e.g., miR-298) biological effects can be tailored to a specific cell type (e.g., astrocytic line) over another undesired cell type (e.g., differentiated NB) with a 3'UTR truncated enough to lack a miRNA binding site. Likewise, other miRNAs and their target 3'UTRs can be tested by the miRnape method. Future work is also warranted to study different scenarios and potential outcomes, as described above, to achieve optimal miRNA activity in regulating native protein expression.

Declarations

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Conflict of Interest Statement. None

Ethical approval. No Animal work and no human subjects involved in the present work

All other procedures were approved and overseen by the Institutional Biosafety Committee (IBC), Office of Research Compliance, Indiana University, Indiana, USA.

Author contribution. RW performed the experiments, analyzed the results, and wrote the initial manuscript draft. DL designed the experiments, help to analyze the data, revised the manuscript, and led the overall project.

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Figures

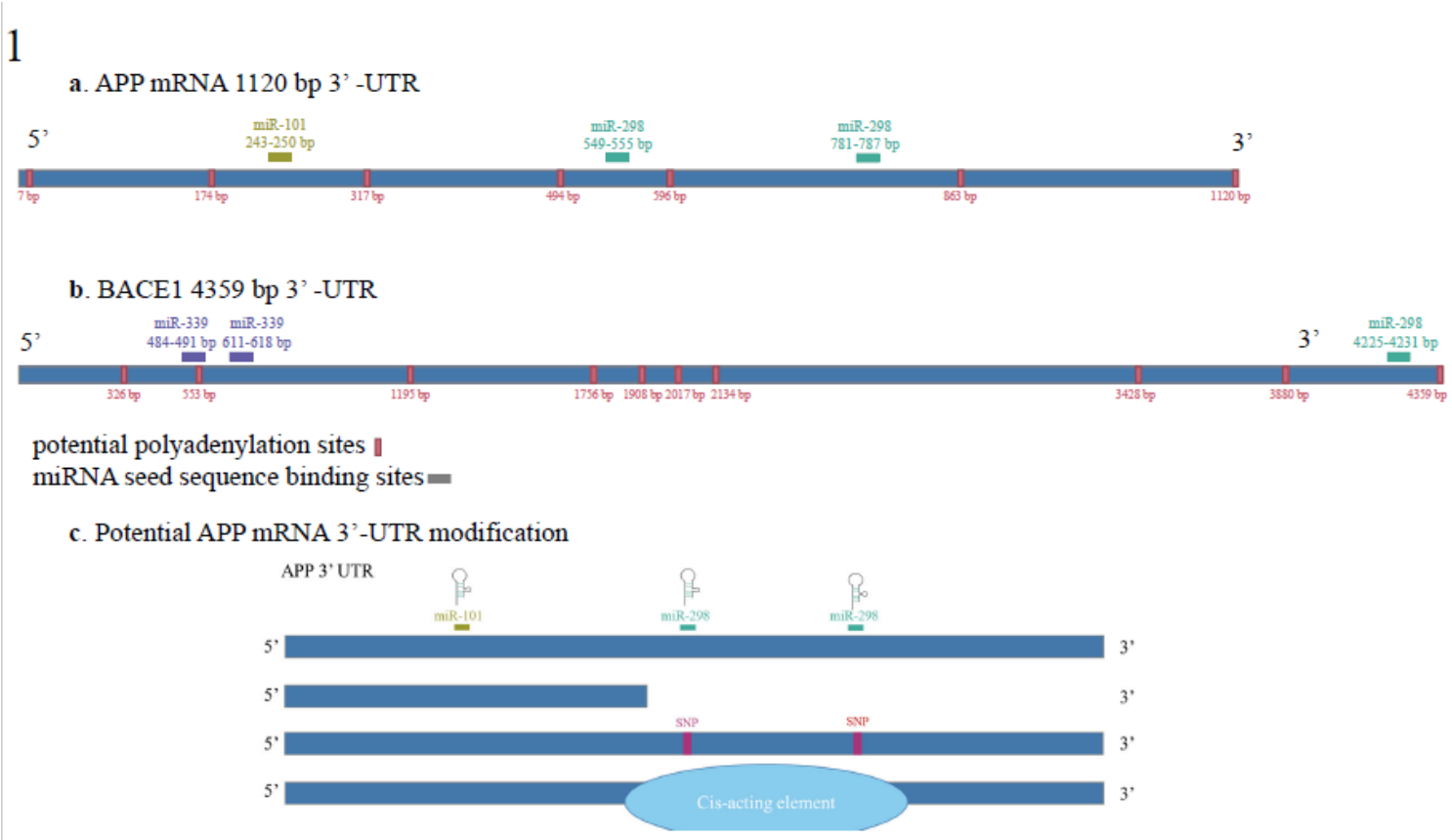

Figure 1

Schematic diagram showing APP and BACE1 mRNA 3'-UTR along with potential polyadenylation sites and miRNA binding sites. A and B. Scale drawing of APP and BACE1 mRNA 3'-UTR separately. Positions of miR-101, miR-298 and miR-339 published seed sequence binding sites are shown. Potential polyadenylation sites are inserted into 3'UTR as red rectangle. Schematic display of potential ways of regulation on APP mRNA 3'-UTR. C. The miR-298 binding sites on APP 3'-UTR are located downstream of

miR-101. Several scenarios that lead to truncated 3'-UTR and could interfere miR-298 binding include alternative polyadenylation sites, presence of SNPs, mutations in miR-298 binding sites and cis-acting elements.

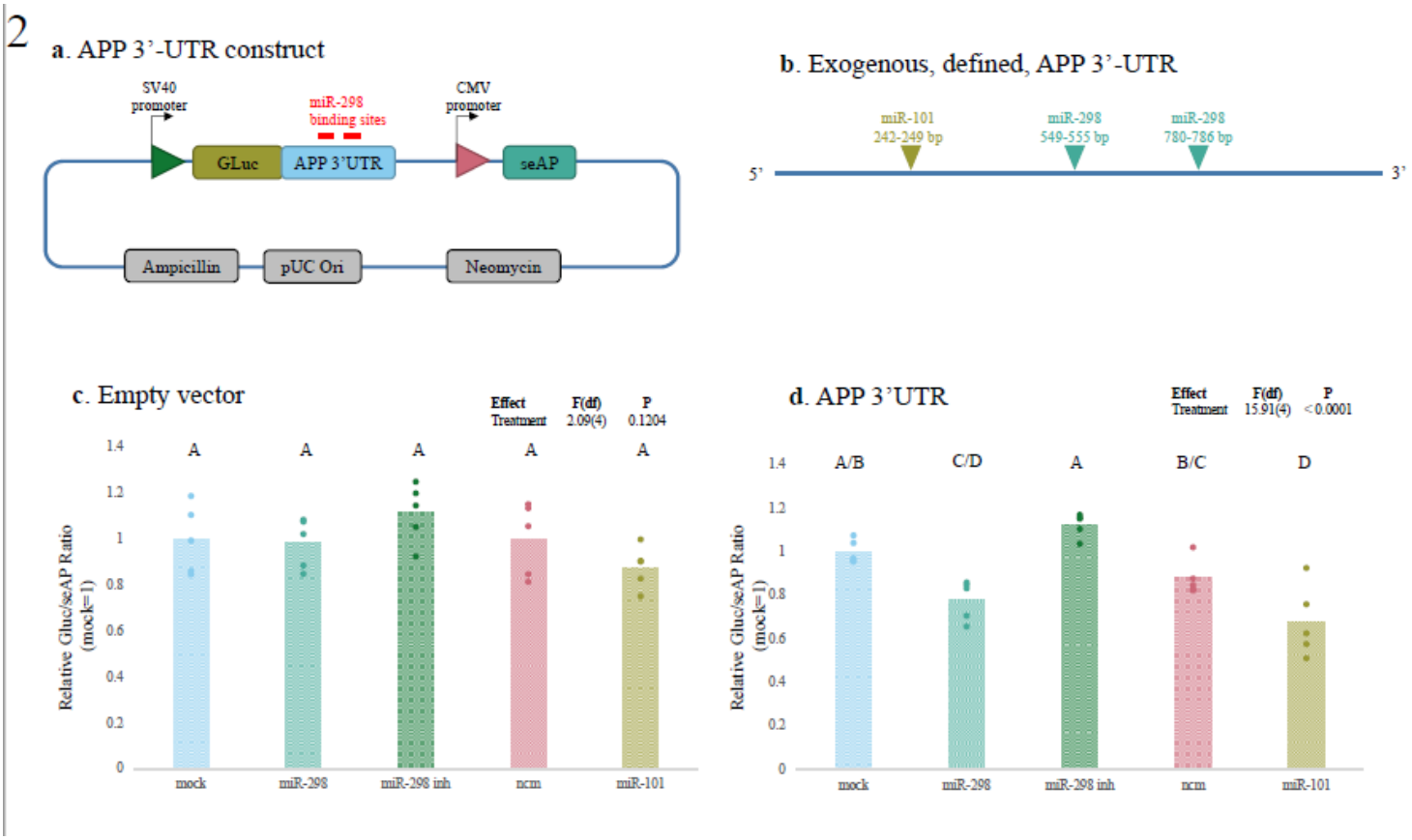

Figure 2

Effects of miR-298 on APP 3'-UTR activity in differentiated neurons. A. APP mRNA 3'-UTR was inserted into pEZX-MT05 plasmid downstream of secreted Gaussia luciferase (GLuc) gene. Another reporter gene in the plasmid, secreted Alkaline Phophastase (seAP), is independently transcribed and used as an internal control. B. Scale drawing of exogenous APP mRNA full length 3'-UTR with miR-101 and miR-298 binding sites shown. C and D. Plasmid pEZX-MT05 (empty vector or APP-3'UTR cloned plasmid) and miRNAs were co-transfected in neuronal cells, and thereafter processed as described in the 'Material & Methods'. Different treatment groups are as indicated in the figure. Briefly, cells were harvested and conditioned media were split into two parts to do separately GLuc and seAP luminescence assays. The GLuc/seAP ratio represents the target 3'-UTR activity, and data were analyzed. MiR-298 and miR-101 significantly reduced APP 3'UTR activity but not empty vector vs. mock transfection (C and D).

3

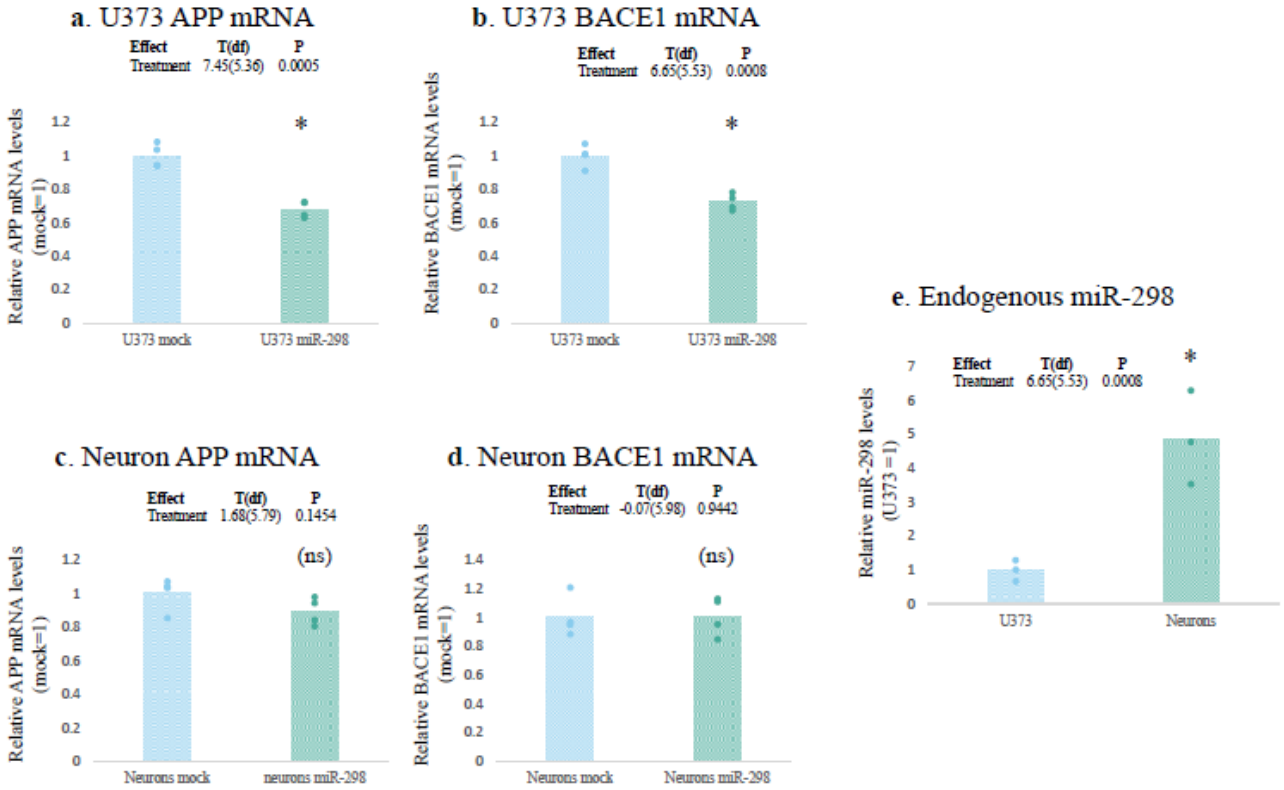

Figure 3

Effects of miR-298 treatment on APP and BACE1 mRNA in astrocytes and differentiated neurons. A and B. Astrocytes were transfected with miR-298 for 3 days. RNA was harvested for reverse transcription and following quantification by real-time qPCR. APP and BACE1 mRNA showed significant reduction when transfected with miR-298. C (APP mRNA) and D (BACE1 mRNA). Real-time qPCR analysis in differentiated neurons transfected with miR-298. E. Quantification of endogenous miR-298 in two cell lines of astrocyte and neuroblastoma origins, respectively. MiR-298 levels were normalized with the geometric means of miR-16, RNU48 and RNU6B.

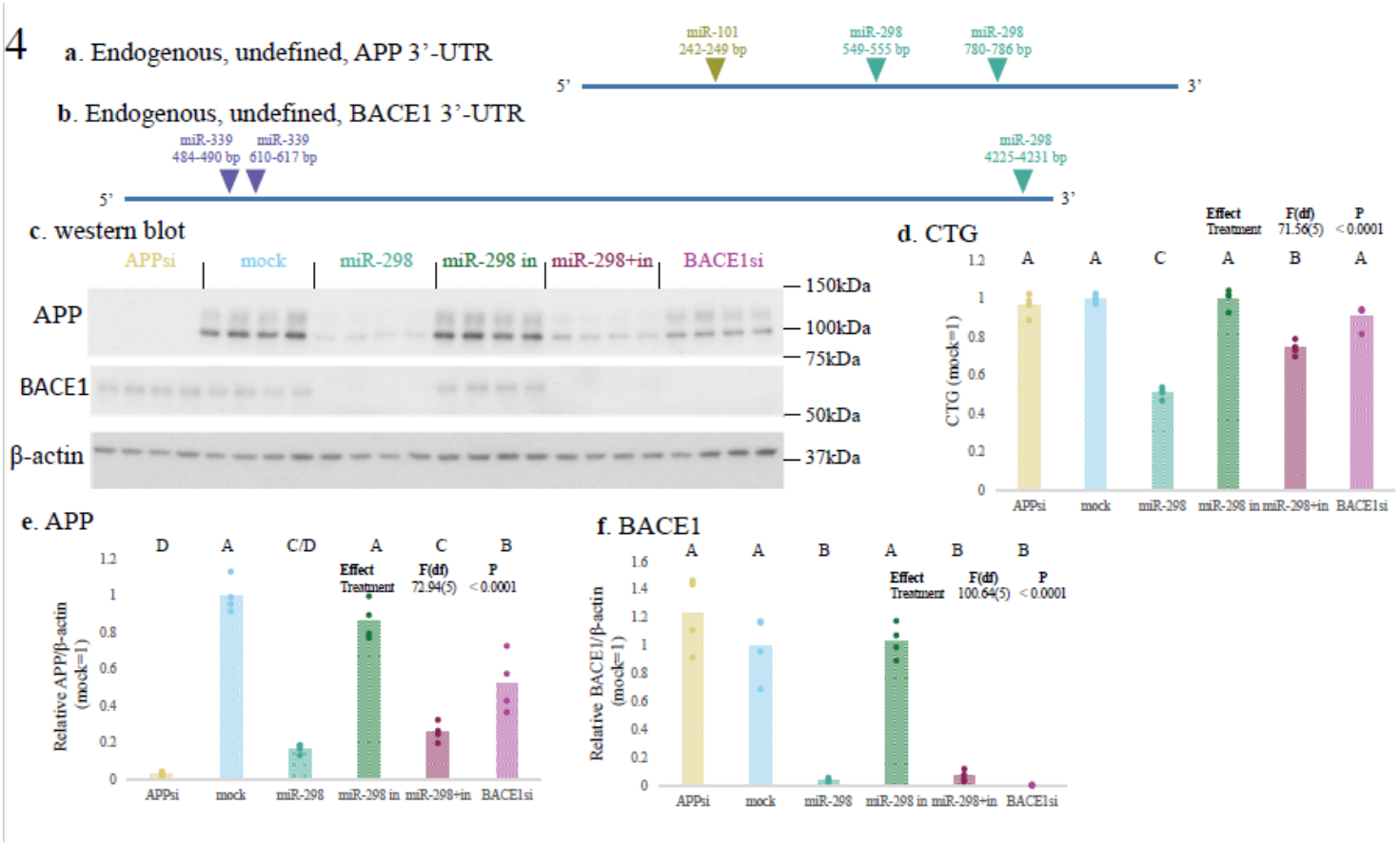

Figure 4

Effects of miR-298 on endogenous APP and BACE1 levels in human astrocyte cells. A and B. Scale drawing of endogenous APP and BACE1 mRNA undefined 3'-UTR with miR-298 binding sites. C. Western blotting of APP, BACE1 and β-actin. Different treatment groups are as indicated in the figure. D. Cell viabilty CTG assay. E (APP) and F (BACE1). Densitometric analysis of the specific protein bands as described in the 'Materials & Methods'. Each letter represents a different group.

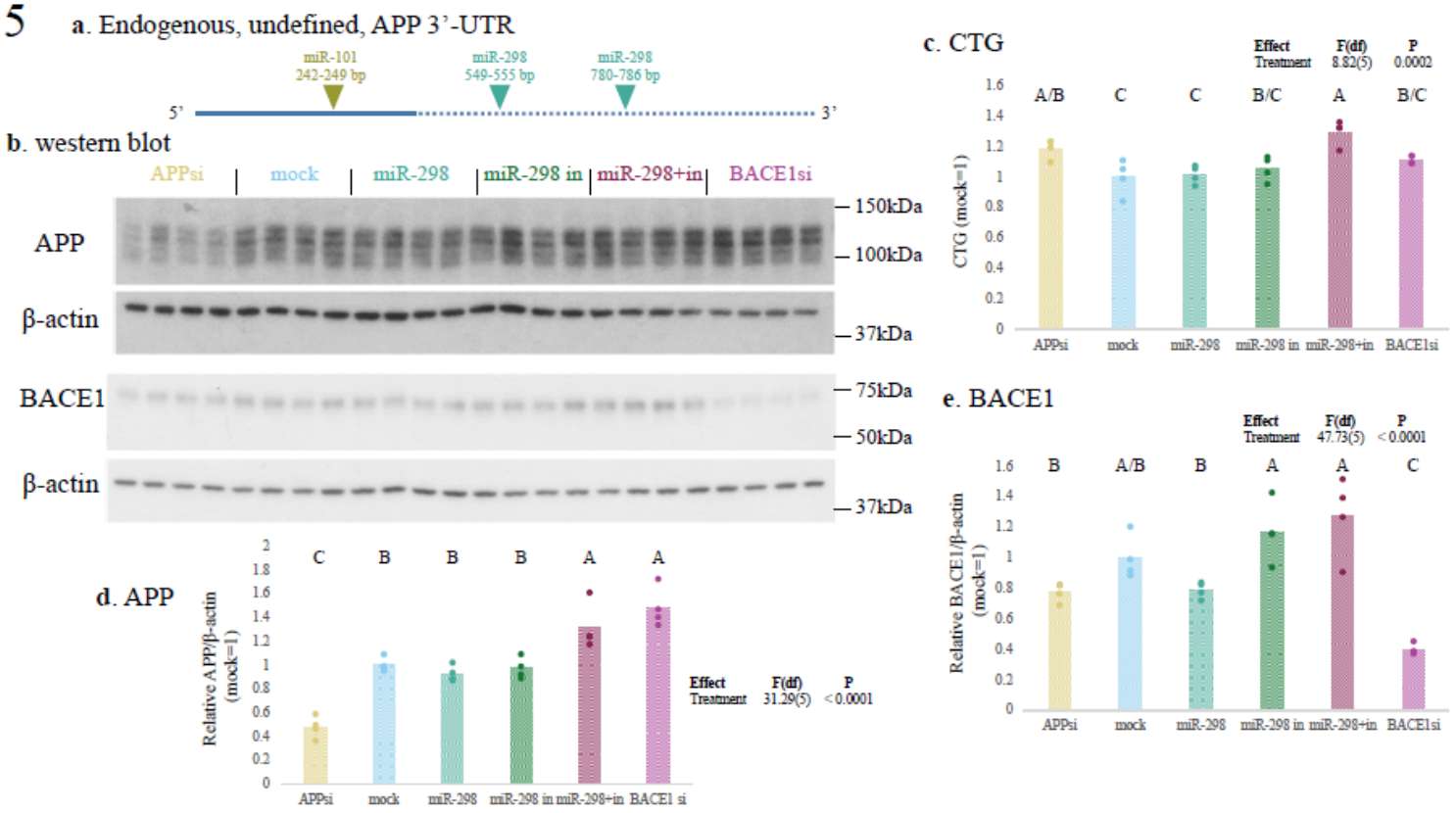

Figure 5

Effects of miR-298 on endogenous APP levels in differentiated neurons. A Scale drawing of endogenous APP mRNA undefined 3'-UTR with miR-101 and miR-298 binding sites. Dash line represents potential truncation or alternation of APP 3'-UTR. B. Western blotting of APP, BACE1 and β-actin protein. Treatment groups are as shown in the figure. C. Cell viability analysis by CTG assay. D (APP) and E (BACE1). Densitometric analysis of the blots for the specific protein bands as described in the 'Materials & Methods'.

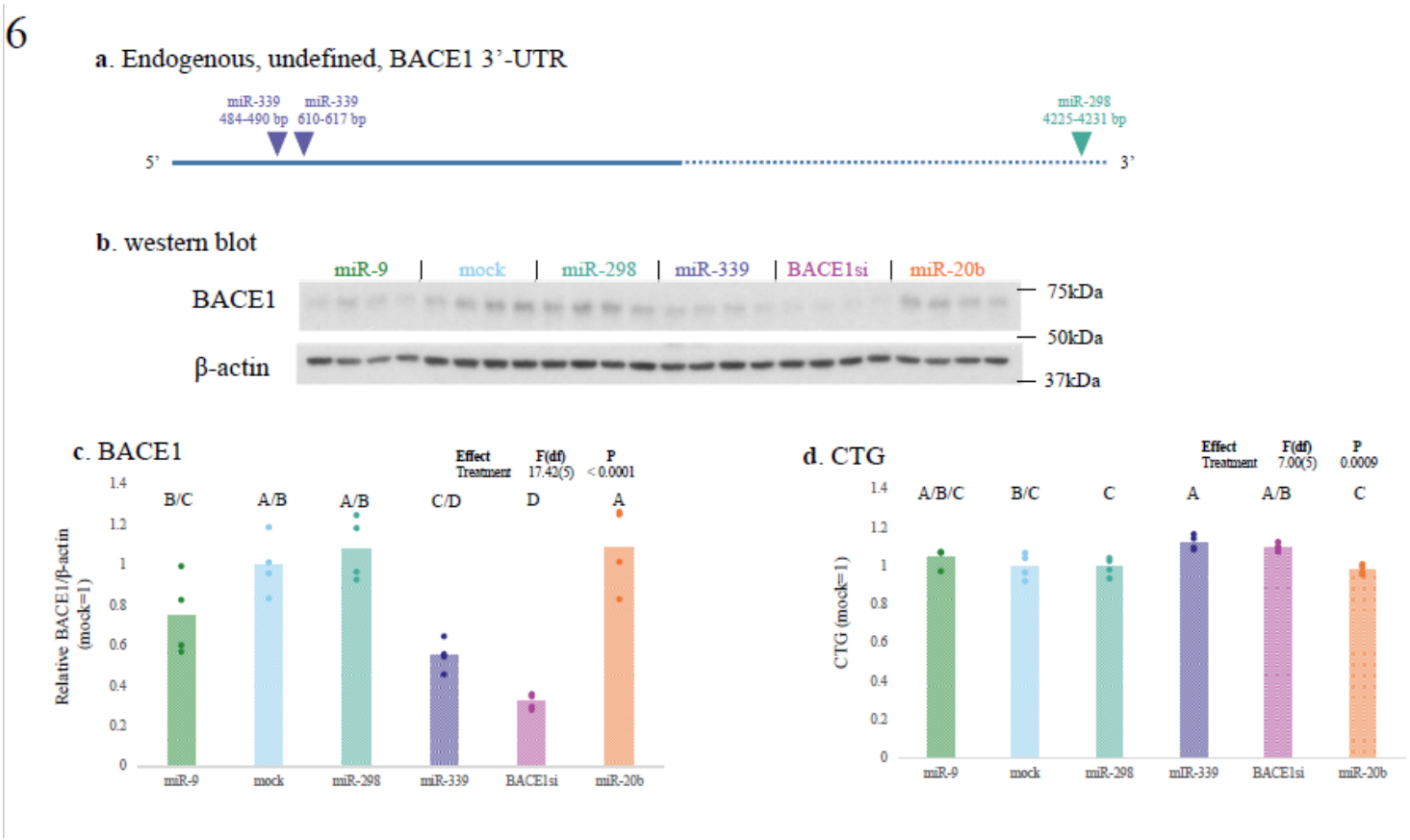

Figure 6

Effects of miR-298 and miR-339-5p on endogenous BACE1 levels in differentiated neurons. A. Scale drawing of endogenous BACE1 mRNA undefined 3'-UTR with miR-339 and miR-298 binding sites. Dash line represents potential truncation or alternation of BACE1 3'-UTR. B. Western blotting of BACE1 and β-actin. C. Densitometric analysis of BACE1 specific protein bands as described in the 'Materials & Methods'. D. Cell viability (CTG) assay. Each letter represents a different group.

7

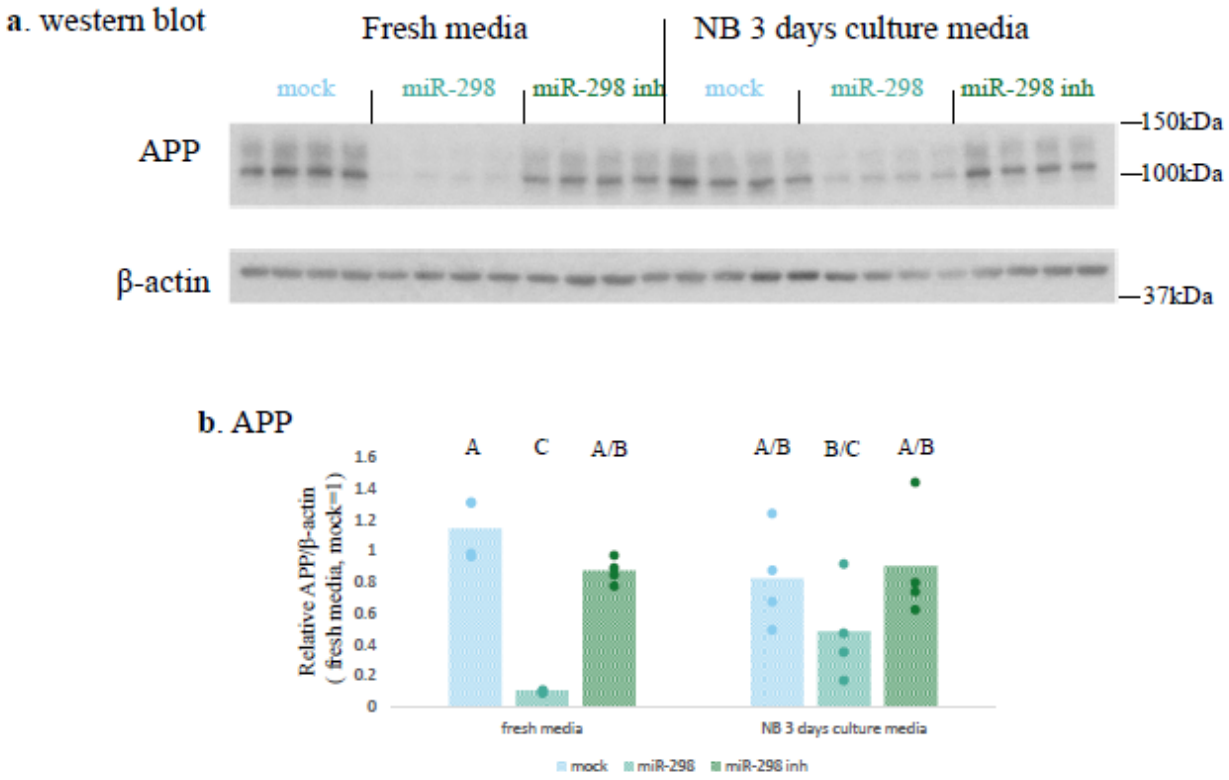

Figure 7

miR-298 treatment's effect on APP and BACE1 proteins depends on the intrinsic property of each cell type. A. One set of astrocytes were transfected with miR-298 and cultured in fresh opti-MEM media, as in Fig. 4. The second set of astrocytes were similarly transfected with miR-298 but cultured in opti-MEM media that were derived from differentiated neurons (NB) for 3 days. Western blot of APP and β-actin proteins. B. Densitometry analysis of APP specific protein bands as described earlier. Each letter represents a different group.

Supplementary Files

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