Published in final edited form as:

Biochem Biophys Res Commun. 2011 January 28; 404(4): 889–895. doi:10.1016/j.bbrc.2010.12.053.

MicroRNA-101 downregulates Alzheimer's amyloid- β precursor protein levels in human cell cultures and is differentially expressed

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Abstract

The full repertoire of regulatory interactions utilized by human cells to control expression of amyloid- β precursor protein (APP) is still undefined. We investigated here the contribution of microRNA (miRNA) to this regulatory network. Several bioinformatic algorithms predicted miR-101 target sites within the APP 3'-untranslated region (3'-UTR). Using reporter assays, we confirmed that, in human cell cultures, miR-101 significantly reduced the expression of a reporter under control of APP 3'-UTR. Mutation of predicted site 1, but not site 2, eliminated this reporter response. Delivery of miR-101 directly to human HeLa cells significantly reduced APP levels and this effect was eliminated by co-transfection with a miR-101 antisense inhibitor. Delivery of a specific target protector designed to blockade the interaction between miR-101 and its functional target site within APP 3'-UTR enhanced APP levels in HeLa. Therefore, endogenous miR-101 regulates expression of APP in human cells via a specific site located within its 3'-UTR. Finally, we demonstrate that, across a series of human cell lines, highest expression of miR-101 levels was observed in model NT2 neurons.

Keywords

Aging; Amyloid; Alzheimer; Dementia; Neurodegeneration; Non-coding RNA; Post-transcriptional

1. Introduction

Amyloid- β precursor protein (APP) is a transmembrane protein that undergoes proteolytic processing by secretase enzymes to liberate soluble fragments. Sequential cleavage by β -secretase (BACE1) and the γ -secretase complex releases both the secreted beta-form of APP (sAPP β) and the amyloid- β (A β) peptide, the major component of insoluble amyloid plaques found in Alzheimer's disease (AD) [1]. Given the hypothesized centrality of A β to

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AD pathogenesis [2], understanding the processes that govern expression of its parental molecule (APP) is an essential task.

The regulation of APP expression has been extensively characterized. Cis-elements and trans-acting factors controlling APP expression have been identified in the gene promoter, and mRNA 5'-untranslated region (5'-UTR), coding sequence (CDS), and 3'-UTR [3–11]. However, the complete regulatory network governing expression of APP is yet to be fully elucidated.

MicroRNAs (miRNAs) are short (~22 nucleotides), non-coding RNAs that act to inhibit protein expression by interacting with specific recognition elements in the 3'-UTR of target transcripts. These recognition elements demonstrate near perfect complementarity to the 5' end of miRNA, termed the seed sequence, while interaction with the 3' end of miRNA requires less stringency [12]. The miRNA guides a ribonucleoprotein complex termed RNA-induced silencing complex (RISC) to the target site in the 3'-UTR where RISC mediates either translation repression or mRNA destabilization [13]. Dysregulation of miRNAs is known to contribute to disease. Indeed, specific miRNAs have been shown to be either up or down-regulated in AD [14–17]. Certain miRNAs also participate in physiological regulation of APP levels [18–22]. However, the regulatory roles of many predicted miRNA target sites in APP 3'-UTR and quantification of miRNA in different cell types have yet to be studied.

With this in mind, we sought to further characterize regulation of APP expression by miRNAs. Here we report that endogenous miR-101 regulates APP levels in human cell cultures through a specific site located in the APP 3'-UTR. We suggest that modulating miR-101 may represent a novel strategy for attenuating A β secretion and downstream pathogenic mechanisms underlying AD.

2. Materials and Methods

2.1. Cell Culture

HeLa, HEK293T, U373, SK-N-SH and PC12 cells were obtained from ATCC, and cultured as described [23]. Preparations of differentiated NT2/D1 neurons (NT2N) were prepared as described [24]. NT2N were used after two weeks mitotic inhibitor treatment. Human neuroblastoma SK-N-SH cells were differentiated by exposure to 10 μ M retinoic acid (Sigma) for approximately three weeks.

2.2. Generation of APP 3'-UTR and positive control reporter constructs and mutagenesis of predicted target sites

The APP 3'-UTR was PCR amplified from the pGALA construct [9] using custom primers (Invitrogen): forward 5'-TAGGCGATCGCTCGAGATAAAGGCCAAGAAGGGCGGAA-3', reverse 5'-AATTCCTCCGGGCTCGAGATCTTATCATGTCTGCTCGAAGCGGC-3'. Primer design included 5' extensions compatible with In-Fusion PCR cloning system (Clontech). The amplified fragment was cloned into the XhoI site of psiCHECK-2 (Promega), and integrity of the insert was confirmed by direct sequencing through our institutional core. A positive control construct containing a site perfectly complementary to miR-1 was constructed using oligonucleotides containing this miRNA recognition element (MRE). Oligos were annealed, directly cloned into XhoI-PmeI-linearized psiCHECK-2 using In-Fusion, and confirmed by sequencing. To introduce mutations in the APP 3'-UTR reporter construct, QuikChange Lightning Site-Directed Mutagenesis kit (Agilent) was used.

2.3 Transfection of plasmids, APP siRNA, miR-101 mimic, miR-101 inhibitor, and APP 3'-UTR-directed target protector

MiRNA mimics (Dharmacon) were introduced along with reporter constructs into HeLa cells using TransFectin lipid reagent (Bio-Rad). APP siRNA (Applied Biosystems), miRNA mimics, LNA inhibitors (Exiqon), and miScript target protectors (Qiagen) were introduced into HeLa, U373, and PC12 cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen).

2.4 Luciferase assay

HeLa cells were transfected with psiCHECK-2-derived reporter constructs. psiCHECK-2 contains both a Renilla luciferase gene under control of elements inserted into the 3'-UTR-positioned multiple cloning site and an independently-driven firefly luciferase gene that serves as internal control. Luciferase expression levels were assessed 48 hours post-transfection using DualGlo luciferase assay (Promega) [25]. Ratios of Renilla/firefly luminescence were calculated and scaled such that positive control was set to zero and APP 3'-UTR reporter alone was set to one.

2.5 RT-qPCR analysis of miR-101 and APP mRNA levels

For each cell type, one 100-mm plate was cultured to 80–90% confluence. Cells were harvested and RNA extracted as described below. MiRNA and mRNA level measurements were done in duplicate for each experiment. Total RNA was extracted using miRVana miRNA Isolation kit (Ambion) and converted to cDNA using TaqMan microRNA Reverse Transcription kit (Applied Biosystems) for miRNA assays or High Capacity RNA-to-cDNA kit (Applied Biosystems) for mRNA assays. RNA quantity was measured by Nanodrop (Thermo) and quality assessed on an Agilent Bioanalyzer. All samples with RIN values ≥ 8 were converted to cDNA and subjected to qPCR analysis using TaqMan assays on a 7300 Real-Time PCR instrument (Applied Biosystems). MiRNA and mRNA relative expression levels were quantified using the delta C_q method [26], normalized to the geometric mean of three reference genes [27]. For miRNA studies, RNU48, RNU6B, and hsa-miR-16 were used for normalization. For mRNA studies, GAPDH, B2M, and TBP were used. TaqMan assays (and IDs) used in this study: hsa-miR-101 (002253), RNU48 (001006), RNU6B (001093), hsa-miR-16 (000391), human APP [all splice variants] (Hs01552283_m1), human GAPDH (4333764T), human B2M (4333766T), and human TBP (4333769T).

2.6 Immunoblot detection of APP levels

Cells were harvested, protein extracted and concentrations estimated by BCA assay (Pierce). Lysates were resolved by SDS-PAGE and specific protein bands quantified by Western blotting techniques as previously described [28]. Blots were independently probed with primary antibodies against APP (22C11, Chemicon), α -tubulin (B-5-1-2, Sigma), and β -actin (AC15, Sigma).

2.7 Data Analysis

RT-qPCR experiments were analyzed using qBASEplus demo software [26]. GraphPad Prism was used to perform all statistical analyses and prepare all graphs. Figures were created using Adobe Illustrator. Statistically significant differences were assessed by one-way ANOVA followed by post-hoc Dunnett's correction for multiple comparisons. The α -threshold for significance was set to 0.05 for all experiments.

3. Results

3.1. Multiple miR-101 target sites are predicted in APP 3'-UTR

To identify putative miRNA target sites located within the 1.2 kb 3'-UTR of the human APP transcript, predictions from target site predictor algorithms were compiled and compared. We searched TargetScan [29], PicTar [30], PITA [31], and miRanda-mirSVR [32]. Several putative target sites for miR-101 in APP 3'-UTR were predicted by TargetScan, miRanda-mirSVR, PITA and PicTar (Figure 1A). The first predicted site (seed sequence position 242–248 relative to start of 3'-UTR) is strongly conserved at orthologous positions across multiple vertebrate species. A second predicted site (seed sequence position 531–537) is less strongly conserved across vertebrate species. A third site (position 134–141) predicted by only PicTar and PITA had less favorable thermodynamic stability and therefore was not followed up for further analysis.

3.2. MiR-101 functionally interacts with the APP 3'-UTR via conserved site 1

To test whether miR-101 mediates regulatory effects on gene expression via APP 3'-UTR, we constructed a reporter vector containing the complete APP 3'-UTR inserted downstream of a Renilla luciferase gene. A positive control vector was also constructed containing a miR-1 MRE (perfectly complementary miR-1 site) inserted downstream of Renilla (Figure 1B). Both constructs contained a downstream firefly luciferase gene used as an internal control to normalize for differences in handling and transfection efficiencies. We then co-transfected these constructs into HeLa cells with either miR-101 or miR-1 mimic, respectively, and assessed luciferase reporter expression 48 hours post-transfection. HeLa cells were chosen for their consistency and high levels of transfectability necessary to observe sometimes subtle regulatory effects of miRNA. As expected, positive control (miR-1) transfection completely abolished reporter expression. MiR-101 significantly reduced reporter expression by greater than 40% compared to cells co-transfected with negative control mimic or APP 3'-UTR reporter alone, confirming that miR-101 can downregulate gene expression via elements in the APP 3'-UTR (Figure 1C).

To confirm that this effect was mediated specifically via one of two predicted miR-101 target sites located in the APP 3'-UTR, we introduced mutations in the seed sequences of both target sites in the reporter construct (Figure 1D). We then co-transfected these mutant reporter constructs along with miR-101 mimic into HeLa cells and compared reporter expression to wild-type reporter (Figure 1E). Mutation of site 1 eliminated the inhibitory effect of miR-101 mimic on reporter expression, whereas mutation of site 2 had no effect on this response. Therefore, miR-101 regulates reporter expression specifically via site 1 in APP 3'-UTR. We previously presented parts of these results [33].

3.3. MiR-101 specifically reduces endogenous APP levels without affecting transcript levels

To directly examine whether miR-101 reduces endogenous APP levels, we transfected miR-101 mimic into HeLa cells. In response, APP levels were significantly reduced by > 60% as compared to HeLa cells transfected with negative control mimic (Figure 2A). Therefore, endogenous APP levels are inhibited by miR-101 in human HeLa cells. We confirmed effective delivery of mimic into HeLa by RT-qPCR. MiR-101 levels were enhanced approximately 2000-fold post-transfection (Figure 2D). Notably, APP mRNA levels were not significantly altered following miR-101 transfection (Figure 2E), suggesting a post-transcriptional mechanism that does not involve mRNA destabilization and likely involves direct inhibition of protein translation.

To confirm that the inhibitory effect of miR-101 on APP levels was not limited to HeLa cells, we independently transfected the human astroglial cell line U373 and rat pheochromocytoma cell line PC12 with miR-101 mimic. In both cell lines, APP levels were significantly reduced following miR-101 transfection (Figure 2B,C). Therefore, the inhibitory effect on APP levels is not limited only to human epithelial HeLa cells but is also apparent in human astroglial and rodent neuronal cell lines.

As final confirmation that miR-101 specifically mediates the inhibitory effect on APP expression, we co-transfected miR-101 along with increasing concentrations of an antisense oligonucleotide miR-101 inhibitor into HeLa (Figure 3A). To prevent competition for transfection reagent between miR-101 mimic and inhibitor and anticipated confounding effect this might have on miR-101 mimic delivery and APP expression, a scrambled control inhibitor was included at correspondingly decreasing concentrations, such that total nucleic acid concentration was kept constant across transfection conditions [23]. Even at the lowest concentrations of miR-101 inhibitor, the downregulation of APP levels mediated by miR-101 mimic was abolished. The reversibility of this effect following miR-101 inhibitor co-transfection confirms that it is specifically mediated by miR-101.

3.4 Endogenous miR-101 regulates expression of APP specifically via 3'-UTR site 1

To determine whether endogenous miR-101 regulates APP levels, we tested commercially available “Target Protectors”. These are modified RNA oligonucleotides complementary to specific target sites within a transcript and no other sequences in the transcriptome. They act to blockade the interaction between miRNA-RISC complexes and specific miRNA target sites. We transfected a custom-designed target protector against APP 3'-UTR site 1 into HeLa and assessed APP levels. Indeed, APP levels were elevated 1.7-fold above mock-transfected cells after β -actin normalization (Figure 3B). Thus, endogenous miR-101 in human HeLa cells regulates APP expression specifically via site 1 in 3'-UTR.

3.5. MiR-101 displays differential expression levels in different cell types

To characterize miR-101 expression levels across multiple cell types of different origins, we used qPCR to assay miR-101 levels in HeLa, HEK293T, NT2, naïve SK-N-SH, differentiated SK-N-SH, and differentiated NT2N. MiR-101 levels were comparable in different cell lines tested. However, as compared to post-mitotic differentiated NT2N neuronal cultures, miR-101 levels were low in HeLa and HEK293T cells. CNS-like [24] differentiated NT2N model neuron cultures demonstrated highest miR-101 levels. Therefore, miR-101 appears to be preferentially expressed in model CNS neurons.

4. Discussion

The network of cis-acting elements and trans-acting factors that govern APP expression is complex and still not fully elucidated. Studies of APP promoter have revealed a complex structure with many proximal and distal regulatory elements that mediate constitutive and stimulated regulatory activities [3–6,34–35]. Regulatory elements in the 5'UTR can drive promoter activity through a CAGA box [36], among others. Post-transcriptional regulation is also mediated via regulatory elements in the 5'UTR, including an iron responsive element [9] involved in regulating translation of the APP transcript. The APP 3'-UTR contains several stability control elements that regulate APP mRNA stability, including a 29-nt control element that destabilizes the transcript [10]. Polyadenylation of APP 3'-UTR also regulates transcript stability through inclusion or exclusion of a specific GG dinucleotide sequence in various polyadenylated transcripts [37]. Thus, regulation of APP expression involves a complex, intertwined network of trans-acting factors working on a dense landscape of cis-acting elements located throughout the APP gene and transcript.

MiRNAs are another class of trans-acting regulatory molecules that post-transcriptionally regulate protein production via cis-acting recognition elements located within 3'-UTRs. Here, we have identified miR-101 as a regulator of APP expression via a specific recognition element located at position 242–248 in the APP 3'-UTR. This is the first study, to our knowledge, that demonstrates this relationship in human cells, which is actually quite meaningful because AD is a specifically human disease. A recent study [38] showing a similar interaction in rodent neurons suggests that this regulatory relationship is well conserved across mammalian species – an observation further supported by strong conservation of the miR-101 target site in APP 3'-UTR. We have also for the first time compared miR-101 expression levels across a series of human cultured cell types, demonstrating highest expression in model CNS postmitotic neurons in culture. This finding suggests that miR-101 is likely to be a relevant regulator of APP levels in human CNS neurons.

The location of the miR-101 target site is of interest because it is in close proximity to the known 29-nt cis-acting element (position 200–229) important in control of APP transcript stability [10]. Given that hnRNP C and nucleolin are known to bind to this element [39], it is tempting to speculate that there might be interactions between these two factors and the RISC complex proteins that miR-101 is expected to deliver to APP 3'-UTR. Such interactions could have synergistic or inverse influences on APP expression. This possibility warrants further follow-up in future studies.

MiR-101 has ascribed functions apart from regulating APP expression. MiR-101 expression is significantly reduced in multiple types of cancers, including prostate [40], colon [41] and gastric [42], among others. Consistent with these findings, functional studies have clearly demonstrated that miR-101 acts as a tumor suppressor by inhibiting expression of the oncogenes EZH2, COX2, FOS, and MCL-1 [40–41,43–44]. These findings might explain the somewhat low expression levels of miR-101 observed in some of the transformed cell lines assessed in our study (e.g. HeLa, HEK293T). MiR-101 also has connections to another neurodegenerative disorder, as it has been shown to regulate expression of ATXN1, implicated in spinocerebellar ataxia type I [45].

MiR-101 may be directly implicated in AD pathogenesis beyond physiological regulation of APP expression. Two independent studies have demonstrated reduced expression of miR-101 in AD brain cortical samples relative to controls [15,17] while another study has demonstrated downregulation primarily in AD cortical white matter [14]. It has not been shown whether dysregulated miR-101 in AD leads to increased APP expression, as would be expected. Studies correlating expression of miR-101 with APP levels in AD and control samples would be valuable in confirming this expected functional sequelae of miR-101 dysregulation.

Beyond miR-101, other miRNA are capable of regulating APP expression. Members of the miR-20a family (miR-20a, -17-5p, and -106b) have been shown to endogenously regulate APP expression [18,20], as have miR-106a, miR-520c, and miR-16 [19,21]. One recent study has also identified miR-124 as a regulator of APP splicing [22]. Clearly we are just beginning to uncover an extensive repertoire of miRNA utilized by cells to regulate APP expression and processing.

In conclusion, this study provides strong evidence that miR-101 regulates levels of APP in human cell cultures of different types and origins. Since APP and A β are central players in the pathways implicated in AD, reducing APP expression has been suggested as a potential strategy for mitigating the pathological processes underlying AD. These results suggest that miR-101 represents a novel target for the therapeutic modulation of APP levels. Either direct

delivery of miR-101 to the CNS or upregulation of endogenous expression would be expected to reduce APP levels in the brain. MiR-101 is expressed from two independent genomic loci on chromosome 1 and 9 that are both located in intergenic regions. The promoter elements that govern transcription of miR-101 are ill-defined and only beginning to be characterized. Understanding the regulatory elements that control miR-101 expression may reveal novel ways to modulate its expression and as a consequence, APP expression. Future studies will need to dissect this regulatory network and determine if it can be manipulated in ways that might prove salutary as a potential AD therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank grants from Alzheimer's Associations (Zenith award) and the NIH (AG18379 and AG18884) to DKL. We also thank J.T Rogers, A.B. Niculescu, J.A. Bailey and B. Ray for their assistance.

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Research Highlights

- MiR-101 inhibits expression of reporter under control of APP 3'UTR in human cells
- MiR-101 mediates this effect through one of two predicted target sites
- MiR-101 reduces expression of endogenous APP in human cultured cells
- APP expression is regulated by endogenous miR-101 in human cells

Figure 1.

MiR-101 targets human APP 3'-UTR via a conserved site. (A) Schematic of the APP transcript, including two putative miR-101 target sites predicted by the TargetScan, PicTar, miRanda-mirSVR, and PITA algorithms. (B) Schematic of APP 3'-UTR and positive control reporter constructs. (C) APP 3'-UTR reporter was transfected into HeLa cells alone and co-transfected with either negative control mimic or miR-101 mimic. Positive control reporter was co-transfected along with miR-1. Dual luciferase assays were performed 48 hours post-transfection. Reporter expression presented as scaled Renilla/firefly luciferase ratios. (D) Two predicted target sites in the APP 3'-UTR were mutated by site-directed mutagenesis. (E) Wild-type and target site mutant reporter constructs were transfected into HeLa cells either alone or along with negative control and miR-101 mimic. Luciferase expression was assessed 48 hours post-transfection. luc=luciferase; prom=promoter; WT=wild-type; error bars represent SEM. * $p < 0.05$, $n = 6$.

Figure 2.

MiR-101 downregulates endogenous APP expression in multiple human cell types. HeLa cells (A), human astroglial U373 cells (B), and rodent pheochromocytoma PC12 cells (C) were transfected with miR-101 mimic or negative control and APP levels assayed 72 hours post-transfection by Western blot. siRNA transfection included as delivery control (n=4). (D) Efficient delivery of miR-101 into HeLa cells confirmed by RT-qPCR 72 hours post-transfection (two technical replicates). (E) APP mRNA levels in HeLa cells were assayed by RT-qPCR 72 hours post-transfection (two technical replicates). RQ=relative quantity; error bars represent SEM; * p<0.05 relative to mock-transfected cells.

Figure 3.

Endogenous miR-101 regulates APP levels in human cells. (A) MiR-101 mimic (50 nM) was co-transfected with increasing concentrations of miR-101 antisense LNA oligonucleotide inhibitor. APP levels were assayed by Western blot. (B) Target protectors directed against miR-101 site 1 in APP 3'-UTR and negative controls were transfected into HeLa cells. APP levels were assayed by Western blot 72 hours post-transfection. Error bars represent SEM. * $p < 0.05$, $n = 4$.

Figure 4. Differential expression of miR-101 in various cell types. RT-qPCR quantification of miR-101 levels in a series of cell lines. Diff=differentiated; error bars represent SEM of two technical replicates.