MicroRNA-144 Is Regulated by Activator Protein-1 (AP-1) and Decreases Expression of Alzheimer’s Disease-related A Disintegrin And Metalloprotease 10 (ADAM10)

Cong Cheng¹, Weiguang Li¹, Zheng Zhang¹, Shohei Yoshimura², Qinyu Hao¹, Chi Zhang¹ and Zhao Wang¹

¹ Protein Science Key Laboratory of the Ministry of Education, School of Medicine, Tsinghua University, Beijing 100084, PR China.
² Department of Pharmacology, Okayama University Graduate School of Medicine, Dentistry & Pharmaceutical Sciences, Okayama 700-8558, Japan.

Running title: miR-144 Suppresses ADAM10 Expression

To whom correspondence should be addressed: Zhao Wang, School of Medicine, Tsinghua University, Beijing 100084, China; Tel: 86-10-6277-2240; Fax: 86-10-6277-2675; E-mail: zwang@tsinghua.edu.cn

Keywords: Alzheimer’s disease; MicroRNA; miR-144; ADAM10; Secretase

Background: MicroRNA (miR) dysregulation is found in Alzheimer's disease (AD). A disintegrin and metalloprotease 10 (ADAM10) prevents generation of amyloid β (Aβ) and decrease AD pathology.

Results: miR-144 suppresses ADAM10 expression and is up-regulated by activator protein-1.

Conclusion: miR-144 is negative regulator of ADAM10 and may be involved in AD pathogenesis.

Significance: The first work to demonstrate the function of miRNA-144 and its regulation in the pathogenesis of AD.

SUMMARY

Amyloid β-peptide (Aβ) accumulating in the brain of Alzheimer’s disease (AD) patients is believed to be the main pathophysiological cause of the disease. Proteolytic processing of the amyloid precursor protein by α-secretase ADAM10 (a disintegrin and metalloprotease 10) protects the brain from the production of the Aβ. Meanwhile, Dysregulation or aberrant expression of microRNAs (miRNAs) has been widely documented in AD patients. In this study, we demonstrated that over-expression of miR-144, which was previously reported to be increased in elderly primate brains and in AD patients, significantly decreased activity of the luciferase reporter containing the ADAM10 3'-untranslated region (3'-UTR) and suppressed ADAM10 protein level, whereas miR-144 inhibitor led to an increase of the luciferase activity and the protein. The negative regulation caused by miR-144 was strictly dependent on the binding of the miRNA to its recognition element in the ADAM10 3'-UTR. Moreover, we also showed that Activator protein-1 regulates the transcription of miR-144 and the up-regulation of miR-144 at least partially induces the suppression of the ADAM10 protein in the presence of Aβ. In addition, we found that miR-451, a miRNA processed from a single gene locus with miR-144, is also involved in the regulation of ADAM10 expression. Taken together, our data therefore demonstrate miR-144/451 is negative regulators of ADAM10 protein and suggest a mechanistic role for miR-144/451 in AD pathogenesis.

Alzheimer’s disease (AD) is a neurodegenerative disease that accounts for 50 to 60% of dementia cases in the elderly (1). The amyloid β-peptide (Aβ) is believed to be the essential cause of AD pathology and is produced by sequential proteolytic cleavages of the
amyloid precursor protein (APP) by β- and γ-secretases (2). APP is also processed in a non-amyloidogenic pathway by α-secretase, thereby repressing Aβ formation (3). This alternative processing of APP by α-secretase generates the neuroprotective and neurotrophic soluble APPα ectodomain (4). ADAM10, whose level is decreased in the platelets and neurons of AD patients (5,6), is the most recognized candidate for α-secretase in cell culture and mouse models (7-9). Additionally, APPα is also decreased in the cerebrospinal fluid (CSF) of AD patients (10,11), indicating a reduced amount and/or activity of ADAM10. A previous study demonstrates that neuronal over-expression of ADAM10 in APP transgenic mice significantly decreases Aβ generation, amyloid plaque load, and AD pathology, while over-expression of a dominant negative ADAM10 variant enhances Aβ formation (3). These reports shed light on the important role of ADAM10 in Aβ production and the pathogenesis of AD. Thus, studying the regulation of ADAM10 expression may help to delineate the underlying pathophysiology of AD and offer new therapeutic targets for the disease.

MicroRNAs (miRNAs or miRs) are small (~22 nt) non-coding RNAs (12). In animals, miRNAs regulate gene expression at the post-transcriptional level by leading to target mRNA degradation or repressing protein translation (12,13). miRNAs play very important roles in many aspects of neuronal function and in a variety of physiological processes in the central nervous system (CNS) (14). For instance, miRNAs are implicated in neurite outgrowth (15,16), regulate dendritic spine development (17), promote neurogenesis in the midbrain-hindbrain domain (18), and controls human neuronal differentiation (19). Over-expression of a single miRNA can even shift the overall gene expression profile of a cell from non-neuronal to neuronal (20). Similarly, alterations of miRNA networks cause neurodegenerative disorders, including AD, Parkinson’s disease (PD) (21), and Huntington’s disease (HD) (22). Indeed, recent studies demonstrate that dysregulation of specific miRNAs contributes to amyloidogenesis in the pathogenesis of AD, but all such research has focused on the regulation of APP (23-27) or BACE1 (i.e., β-secretase, β-site APP cleaving enzyme 1) (28-30).

In this study, we used a combination of bioinformatics and experimental techniques to demonstrate that miR-144 is a negative regulator of ADAM10, and Activator protein-1 (AP-1), that can be activated by Aβ and is implicated in AD pathogenesis, is involved in the regulation of miR-144 expression. In the presence of Aβ, miR-144 is up-regulated and contributes to the ADAM10 down-regulation caused by Aβ. Thus, this study presents a mechanism for the ADAM10 protein repression observed in AD patients and provides potential therapeutic targets for the prevention and/or treatment of the disease.

**EXPERIMENTAL PROCEDURES**

_Bioinformatics_ — Prediction of miRNA targets was conducted using miRanda (http://www.microrna.org/). Prediction of the transcriptional start sites (TSSs) of primary microRNAs was performed using miRStart (http://mirstart.mbc.nctu.edu.tw/). The putative promoter sequences of primary microRNAs and the 3’UTR of ADAM10 were retrieved from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Prediction of transcription factors for primary microRNAs was conducted using the Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Sequence conservation was analyzed with the Evolutionary Conserved Region Browser (http://ecrbrowser.dcode.org).

_Cell Culture and Reagents_ — HeLa and human neuroblastoma SH-SY5Y cells were cultured in DMEM (Hyclone) supplemented
with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO₂. 12-O-tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma-Aldrich. Human Aβ42 peptide was obtained from AnaSpec and Sigma. miRNA mimics, inhibitors, and the corresponding negative controls were obtained from RiboBio.

Luciferase Reporter Gene Constructs—To construct reporter plasmid pmirGLO-ADAM10 3' UTR, the 3'-UTR region of ADAM10 was amplified from human cDNA. The PCR product was digested with SacI and XhoI. Then the fragment, which spans 1267 bps starting from the 35th bp upstream of the stop codon, was cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). To construct the luciferase reporter plasmids pGL3-promoter-2808, promoter-2554, promoter-1895, promoter-1019, promoter-453 and promoter-307, each promoter region of miR-144 was amplified from human genomic DNA and subsequently cloned into KpnI and XhoI site of pGL3-basic plasmid (Promega). The transcription factor over-expression constructs were amplified from human cDNA and then cloned into pcDNA3.1/myc-His A at the KpnI and XhoI sites or BamHI and XbaI sites. The ADAM10 3'-UTR luciferase mutant construct that lacks the putative miR-144 MRE, the ADAM10 3'-UTR luciferase mutant construct that lacks the putative miR-451 MRE and the miR-144 promoter reporter plasmids containing single or double site-specific deletion for each transcription factor AP-1 binding site were generated by using a PCR-mediated deletion mutagenesis protocol (31). The sequences of primers used in aforementioned construction or mutation are listed in Table 1. All constructs and mutants were verified by sequencing at Invitrogen.

Reporter Plasmid Transfection and Luciferase Assay—Transient transfections of HeLa cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For the luciferase assay, cells were plated at a density of 2 × 10⁵ cells/well in 24-well plates and co-transfected after 24 hrs with 0.2 μg pGL3 reporter plasmid and 0.002 μg renilla plasmid (Promega) or 0.1 μg pmirGLO reporter plasmid (Promega), as well as 50 nM miRNA mimics or 100 nM inhibitors per well. Luciferase activities were measured 48 hrs after transfection using the Dual luciferase reporter assay system (Promega) and a GloMax™ 96 Microplate Luminometer (Promega). Transient transfections of SH-SY5Y cells were performed using an Amaxa Cell Line Nucleofector Kit V (Lonza) according to the manufacturer’s instructions.

RNA Extraction and Quantitative RT-PCR—Total RNAs were extracted with TRIzol (Invitrogen), and the first-strand cDNAs were synthesized using the GoScript™ Reverse Transcription System (Promega) according to the manufacturer’s protocol. The quantitative RT-PCR analysis was performed with SYBR® Green Realtime PCR Master Mix (TOYOBO) using the CFX96 Realtime System (BioRad). The primers used for quantitative RT-PCR are as follows: ADAM10 forward, 5'-TCGAACCATCACCCTGCAACCT-3' and reverse, 5'-GCCCAACCAATGAGCCACAATCC-3'; and β-actin forward, 5'-GTCACCAACTGGGACGACATG-3' and reverse, 5'-GATAGCACAAGCCTGGATAGCAAC-3'. β-actin served as an internal control. miRNAs were extracted using an miRcute miRNA isolation kit (TIANGEN). Extracted miRNAs were polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers with an miRcute miRNA first-strand cDNA synthesis kit (TIANGEN) according to the manufacturer’s protocol. Quantitative RT-PCR analysis for mature miR-144 was
performed with an miRcute miRNA qPCR detection kit (TIANGEN) using the primers miR-144 forward, 5′-GGGGGGGGGGGTACAGTATAGTGATGTACTAA-3′; miR-451 forward, 5′-GGGGGGGGCCAAACCGTACCATTAC TGAATTAAA-3′; and U6 forward, 5′-GCAAGGATGACACGAAAATTGTCT-3′. The reverse primer for the quantitative RT-PCR was provided in the miRcute miRNA qPCR detection kit. U6 served as an internal control.

**Protein Extraction and Western Blot Analysis**—Cells were lysed in RIPA buffer (Biosure) supplemented with a protease inhibitor mixture and 1 mM PMSF (Biosure). The protein concentration was measured using a BCA protein assay kit (Pierce). Protein samples were separated by 10-15% SDS-PAGE (Bio-Rad) and electroblotted onto nitrocellulose membranes (Hybond-ECL, GE Healthcare). The membranes were blocked with 5% skim milk in PBST for 2 hrs at room temperature and incubated overnight at 4°C with anti-ADAM10 (1:200, Santa Cruz Biotechnology), anti-APP C-terminal (1:4000, Sigma-Aldrich) or anti-GAPDH (1:2000, CWBIO). Then, they were incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies: goat anti-mouse IgG (1:10,000, ZSGB-BIO) or goat anti-rabbit IgG (1:10,000, ZSGB-BIO). Western blot immunoreactivity was detected using a Super Signal West Pico Kit (Pierce).

**Small RNA Interference (siRNA)**—siRNA duplexes consisting of 21 bp of oligonucleotides were purchased from GenePharma (Shanghai, China). The sequences of siRNA duplexes for c-Jun and negative control (scrambled) were as follows: c-Jun siRNA-1 targeting 5′-GAUGGAACGACCUCUUAUTT-3′, c-Jun siRNA-2 targeting 5′-CCUCAGCAACCUAACCATT-3′, and scrambled siRNA targeting 5′-UUCUCCGAACGUUGACCGUTT-3′. Transfection of siRNA duplexes at concentration of 75 pmol per well for 6-well plate was performed using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instruction.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear proteins from SH-SY5Y cells were extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). The following biotin-labeled probes and unlabeled competitor probes with the same sequences as well as their anti-sense oligos were synthesized at Invitrogen and annealed: 5′-CTCTCTAGGGAAAGGGGCACTGACCCT TGTCATGGACTCAGCGGC-3′, corresponding to AP-1 binding site 3 of miR-144 promoter; 5′-CCATAACCCACCTGGGCTGTGCCTGAC CACAGTTACAGGAGACGCTG-3′, corresponding to AP-1 binding site 5 of miR-144 promoter. EMSA reactions (20 μL) were assembled using LightShift Chemiluminescent EMSA Kit (Pierce), and the reaction contained 50 fmol of biotin-labeled probe, 2 μL 10× binding buffer, 1 μL 1 μg/μL Poly (dI·dC), 0.5 μL 50% glycerol, and/or 2μL NE-PER nuclear extracts, 10 pmol unlabeled probe. Mixtures of protein and DNA were incubated for 20 min at room temperature. Following addition of 5 μL loading buffer, bound and free DNA were resolved by fractionation on 6% native polyacrylamide TBE gel (Bio-Rad) in 0.5× TBE. Gels were pre-run for 40 min at 100 V, and run with samples under the same conditions for 40 min. The DNA/protein complex was transferred onto Biodyne B Nylon Membrane (PALL) at 380 mA for 1 h. The transferred DNA was cross-linked to the membrane at 120 ml/cm² for 60 s using UV-light crosslinking instrument equipped with 254 nm bulbs (DETIANYOU Technology). The biotin-labeled DNA was detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce).

**Statistical Analysis**—Results are presented as the mean of three independent experiments ±
RESULTS

MiR-144 Regulates ADAM10 Negatively—The computational program Miranda was used to detect potential miRNAs that could bind to the 3′-UTR of human ADAM10 mRNA (NM_001110). Combined with previous reports, we picked up three miRNAs as potential candidates: miR-9, miR-101 and miR-144, which are implicated in amyloidogenic gene and/or aberrantly express in AD patients (25,29,32). The sequences of the candidate miRNAs and their predicted MREs in the ADAM10 3′-UTR are listed in Table 2. To further investigate the potential function of the candidate miRNAs on ADAM10 expression, luciferase assays were performed in human neuroblastoma SH-SY5Y cells. The result demonstrated that, except for the positive control miR-122 (33,34), only transfection with the miR-144 mimics resulted in a significant luciferase activity down-regulation of the ADAM10 3′-UTR reporter (Fig. 1A). A down-regulation in the luciferase activity after miR-144 transfection was also observed in HeLa cells (data not shown).

Previous genome-wide analysis of miRNA expression demonstrated that compared to global miRNA down-regulation, miR-144 is significantly increased both in elderly primate brains and in AD patients (32). Moreover, bioinformatics analysis revealed that the putative MRE for miR-144 in the ADAM10 3′-UTR is strictly conserved in vertebrates (Table 3). These observations further prompted us to analyze the function of miR-144 on ADAM10 expression. In the miR-144 loss-of-function experiments, we found that miR-144 inhibitor up-regulated the luciferase activity of the ADAM10 3′-UTR reporter (Fig. 1B). Moreover, ADAM10 protein levels are remarkably decreased upon transient over-expression of miR-144 in SH-SY5Y cells (Fig. 1C and D). However, the ADAM10 protein levels were not altered after over-expression of miR-9, which also possesses a putative MRE in the ADAM10 3′-UTR, indicating the specificity of miR-144 in the regulation of ADAM10 levels. Identical data were obtained in HeLa cells (data not shown). In addition, a decrease in the mRNA level of ADAM10 upon miR-144 over-expression was not observed (Fig. 1E).

These results thus identify miR-144 as a negative regulator of ADAM10 and suggest that the repression of the ADAM10 protein induced by miR-144 does not occur via ADAM10 mRNA degradation but may be achieved at the translational level.

Analysis of MiR-144 MRE within ADAM10 3′-UTR—Regulation of target genes by miRNAs is dependent on imperfect base pairing of the miRNAs to the 3′-UTR of their target mRNAs through a 2- to 8-nt seed region located near the 5′-end of the miRNA (35). As shown in Fig. 2A, miR-144 was predicted to possess a putative MRE within the ADAM10 3′-UTR. To investigate whether the miR-144-induced ADAM10 repression occurred via specific binding to the MRE in the ADAM10 mRNA 3′-UTR, a mutant luciferase reporter construct that lacks the potential MRE of miR-144 was generated. The luciferase assay analysis demonstrated that the mutant reporter construct was insensitive to miR-144-mediated inhibition compared to the wild type (Fig. 2B). This result indicates that the miR-144-induced ADAM10 repression depends on the direct binding of miR-144 to its MRE within the ADAM10 mRNA 3′-UTR.

Identification of MiR-144 Promoter—The demonstration that miR-144 is a negative regulator of ADAM10 expression prompted us to examine the transcriptional regulation of miR-144 and its implication(s) in AD. Using the publicly accessible algorithm MiRStart (36), which integrates three datasets including cap
miR-144 Suppresses ADAM10 Expression

analysis of gene expression (CAGE) Tags (37,38), transcriptional start site (TSS) Seq libraries and H3K4me3 chromatin signatures, the site at -371 bp upstream of the miR-144 precursor was predicted as the TSS of the primary miR-144. To experimentally verify this putative TSS, we generated a luciferase reporter construct containing the 2.8-kb region upstream of the precursor miR-144, named pGL3-promoter-2808. According to sequence conservation data, five additional luciferase reporter constructs containing decreasing lengths of the upstream sequence (-2554, -1895, -1019, -453, and -307) were derived. The six luciferase reporter constructs and the pGL3-basic plasmid were then each transfected into SH-SY5Y cells to determine the basal promoter activity. As shown in Fig. 3, the pGL3-promoter-453 construct (i.e., the 453-bp putative promoter region) displayed the highest luciferase activity, which was approximately 10-fold greater than pGL3-basic. The fact that the other four reporter constructs with longer upstream sequences exhibited luciferase activities at comparable levels to that of pGL3-promoter-453 and that pGL3-promoter-307 displayed a dramatic decrease in luciferase activity further support that the TSS is located in the -307 to -453-bp region. The similar result was also observed in HeLa cells (data not shown). These results match our bioinformatics prediction and indicate that the 453-bp region upstream of the precursor miR-144 possesses intact promoter activity triggering miR-144 transcription.

Transcriptional Regulation of MiR-144 — To investigate the key transcription factors involved in miR-144 transcription, the ~1-kb region upstream of the miR-144 TSS was analyzed by transcription factor binding prediction software. SP1, AP-1, CREB, CP2, and TCF4 were predicted to possess putative binding sites in this region. Thus, these five transcription factor were cloned into expression vectors and each co-transfected with the pGL3-promoter-1019 reporter into SH-SY5Y cells. As shown in Fig. 4A, relative to the other four transcription factors, over-expression of c-Jun, a common component of the AP-1 protein complex and can bind to AP-1 recognition sites (39-41), resulted in a strong increase in the luciferase activity. Moreover, the luciferase activity was also increased in a dose-dependent manner after treatment of TPA, which can induce transcription from AP-1-driven promoter (42,43), (Fig. 4B). Meanwhile, the endogenous expression of miR-144 was also significantly increased by c-Jun over expression (Fig. 4C) and TPA treatment (Fig. 4D), but decreased after endogenous c-Jun was knocked down (Fig. 4E).

As shown in Fig. 4F, six potential AP-1 binding sites are predicted to locate in the ~1-kb region upstream of the miR-144 precursor. We used deletion mutagenesis to abolish each site and then performed luciferase assays. The results showed that, compared to the wild type, the mut-3, mut-5 and the double mutation (mutation of binding sites 3 and 5) constructs inhibited the TPA-induced transcriptional activity by 26.1%, 26.4% and 41.6% respectively (Fig. 4G). However, mutation of any other AP-1 binding site did not remarkably affect the transcriptional activity upon TPA treatment. The similar result was observed when c-Jun was over-expressed (Fig. 4H). In addition, the binding properties of the AP-1 at the sites 3 and 5 were further validated by performing EMSA and supershift assay. DNA probes corresponding to AP-1 binding sites 3 and 5, which contained the putative AP-1 binding sequences and their flanking region at both sides in the human miR-144 promoter, were tested. Incubation of either biotin-labeled probe with SH-SY5Y cell nuclear extracts caused a major retarded band (Fig. 4I, lane 3 and 7). Excessive amount of unlabeled probes prevented each labeled probe from binding with AP-1, as indicated by the dim retarded band (Fig. 4I, lane 4 and 8). Additionally, incubation of c-Jun antibody
caused supershift bands (Fig. 4I, lane 2 and 6), indicating the presence of c-Jun in the binding complex. These results fully demonstrate that AP-1 can regulate the transcription of miR-144 by binding the potential sites in its promoter.

**MiR-144 Contributes to the Down-regulation in ADAM10 Protein in the Presence of TPA — In vivo evidence demonstrates that the protein levels of ADAM10 and/or APPsα are decreased in AD patients (5,10,11), whereas expression or activity of AP-1/c-Jun is increased (44-46). Additionally, we have demonstrated that miR-144 acts as a negative regulator of ADAM10 and is regulated by AP-1/c-Jun. Prompted by these observation, we next investigated whether the ADAM10 changes in the presence of TPA, and whether miR-144 might be causally involved in regulating such changes. To test this, we initially determined both ADAM10 mRNA and protein levels in SH-SY5Y cells after TPA treatment. We found that, although the level of ADAM10 mRNA was unchanged in the presence of TPA (Fig. 5A), the level of ADAM10 protein was down-regulated sustainedly in the first 24 hrs and then started to decrease (Fig. 5B and C). The inconsistent changes between the mRNA and protein strongly imply that the down-regulation of ADAM10 protein in the presence of TPA is primarily exerted at the translational level. Meanwhile, using quantitative RT-PCR analysis, we found that the endogenous mature miR-144 was induced after TPA treatment, peaked at 12 hrs, and then started to decrease (Fig. 5D). Notably, the increase in the level of miR-144 exhibited an evident inverse correlation with the change of the ADAM10 protein in at least the first 12 hrs after the initiation of the TPA treatment (Fig. 5E). The incomplete inverse correlation displayed after the 12 h point may be due to involvement of other signal pathways activated by TPA. Furthermore, miR-144 loss-of-function experiments revealed that the repression of ADAM10 protein induced by TPA was restored by miR-144 inhibition (Fig. 5F and G). These data thus further supports the notion that miR-144 is a negative regulator of ADAM10 protein and strongly imply that the AD-associated increase in expression or activity of AP-1/c-Jun as well as elevated expression in miR-144 may contribute to the reduction of ADAM10 protein in AD pathogenesis.

**MiR-144 Contributes to ADAM10 Down-regulation upon Aβ Treatment —**As one of the crucial pathological causes of AD, Aβ phosphorylates and activates c-Jun in vitro (47-49). Thus, we further investigated the levels of miR-144 in SH-SY5Y cells after incubation with Aβ_{42} peptide, which is amyloidogenic and is the major component of neuritic plaques (50,51). Quantitative RT-PCR analysis demonstrated that mature miR-144 levels were strongly increased after incubation with Aβ_{42} peptide (Fig. 6A). Meanwhile, we analyzed ADAM10 protein and mRNA levels after Aβ_{42} treatment. As shown in Fig. 6B and C, the ADAM10 protein level significantly decreased upon treatment with Aβ_{42}. Additionally, APP carboxy-terminal fragment (α-CTF), a membrane-bound APP derivant truncated by ADAM10, was also suppressed, and APP β-CTF was correspondingly increased in the presence of Aβ_{42}, further indicating a decrease in ADAM10 expression or activity. However, ADAM10 mRNA levels were not changed upon Aβ_{42} treatment (Fig. 6D), implying that the Aβ_{42}-induced ADAM10 down-regulation is also mainly achieved at the translational level. To further investigate whether the increase of miR-144 is involved in the down-regulation of ADAM10 protein caused by Aβ_{42}, we performed a miR-144 loss-of-function experiment. As shown in Fig. 6E and F, the repression of ADAM10 protein caused by Aβ_{42} was partially restored after transfection of the miR-144 inhibitor. This result suggests that the Aβ_{42}-induced miR-144 over-expression contributes to the repression of ADAM10
miR-144 Suppresses ADAM10 Expression

protein levels. However, the fact that the miR-144 inhibitor did not completely abolish the decrease in ADAM10 induced by Aβ42 implies that the regulation of ADAM10 expression cannot solely be explained by the action of miR-144 and that additional regulatory pathways must be involved.

MiR-451, A MiRNA Processed from A Single Gene Locus with MiR-144, Is Also Involved in The Regulation of ADAM10 Expression —MiR-451 is co-transcribed with miR-144 from a single locus (52) and is predicted to bind to the ADAM10 3’-UTR (Table 2). In some cases, miR-451 and miR-144 share a same mechanism of transcriptional regulation and display synergistic depressive effects on their target proteins (52,53). Thus, we investigated whether miR-451 is involved in the regulation of ADAM10 protein and whether it is also controlled by c-Jun. We found that, although the putative MRE for miR-451 in the ADAM10 3’-UTR is not conserved in vertebrates (data not shown), over-expression of miR-451 in SH-SY5Y cells also attenuated ADAM10 protein (Fig. 7A and B), and didn’t change ADAM10 mRNA level (Fig. 7C). Meanwhile, miR-451 over-expression significantly decreased the activity of the luciferase reporter containing the ADAM10 3’-UTR, but not that of a mutant luciferase reporter construct that lacks the potential miR-451 MRE (Fig. 7D), indicating that the ADAM10 repression caused by miR-451 also depends on the binding of MRE in ADAM10 3’-UTR. Similar to miR-144, the level of endogenous mature miR-451 was increased by c-Jun over-expression (Fig. 7E) and decreased when c-Jun was knocked down (Fig. 7F). Moreover, in the presence of TPA, the level of miR-451 also changed in a time-dependent manner (Fig. 7G), displayed a nearly identical pattern of miR-144 and also inversely correlated with ADAM10 protein levels in the first 12 hrs after the initiation of the TPA treatment (Fig. 7H). In addition, the level of mature miR-451 was also significantly increased after incubation with the Aβ42 peptide (Fig. 7I). These results suggest that, as another negative regulator of ADAM10 protein, miR-451 may share a same function and mechanism of transcriptional regulation with miR-144 in AD pathogenesis.

DISCUSSION

Brain aging is a major risk factor for the development of AD and other prevalent neurodegenerative disorders (54). This implies that genetic changes occur in the process of brain aging that play coordinating roles with environmental stress to lead to the onset of AD and other neurodegenerative pathologies. However, these genetic changes and their implications in such diseases remain largely unknown. Genome-wide analysis of miRNA expression has revealed that compared to global miRNA down-regulation, miR-144 is the sole miRNA that is consistently elevated in the brains of elderly humans, chimpanzees, and rhesus macaques, and it is also increased in AD patients (32). Because miR-144 could suppress the expression of ADAM10, the elevated miR-144 in aging brains at least partially induces the depletion of ADAM10 protein levels and increases the susceptibility to environmental stress, which may in turn determine the onset of AD. Moreover, growing evidence supports the concept that AD is fundamentally a metabolic disease with derangements in brain glucose utilization and responsiveness to insulin and insulin-like growth factor (IGF) stimulation. The impaired insulin and IGF signaling contributes to AD-associated neuronal loss, synaptic disconnection, tau hyper-phosphorylation, and Aβ accumulation (55). Similarly, miR-144 is also increased and impairs insulin signaling through down-regulation of insulin receptor substrate 1 (IRS1) (56) in impaired fasting glucose (IFG) and Type 2 diabetes mellitus (T2D) patients. This suggests the importance and multiple implications of miR-144 in the
miR-144 Suppresses ADAM10 Expression

Recent reports document that ADAM10 expression is regulated both transcriptionally and post-transcriptionally. Specifically, retinoic acid enhances ADAM10 transcription by promoting the binding of a non-permissive dimer of retinoic acid receptor-α (RARα) and retinoid X receptor-β (RXRβ) to retinoic acid-responsive elements in the promoter of the ADAM10 gene (57,58). ADAM10 transcription is also regulated by PAX2 in renal cancer cells (59) and melanocytes (60). In addition, expression of ADAM10 is suppressed by its 5′-UTR (61), and ADAM10 activity can be blocked by its own predomain (62). Although many reports on ADAM10 regulation have been published in past several years, none present a reasonable mechanism for ADAM10 down-regulation in AD patients. Here, we demonstrated that miR-144 suppresses ADAM10 expression via a classical interaction with the ADAM10 3′-UTR. Thus, the elevated levels of miR-144 in AD patients may be responsible for the down-regulation of ADAM10 protein levels. Additionally, it was observed that the level of ADAM10 mRNA is not affected by miR-144 or TPA (Fig. 1E, 5A), suggesting that the negative regulation of ADAM10 by miR-144 is mainly achieved at the translational level. Same as our basic research finding, the clinical reports showed that ADAM10 proteins levels are decreased in the neurons of AD patients (6), but ADAM10 mRNA levels are increased in hippocampal samples from severe AD cases (63). These two seemingly incompatible observations also imply that translational regulation of ADAM10 exists in the pathogenesis of AD.

The functions of miRNAs in gene regulation have been extensively studied in the last decade. At the same time, research interests have gradually focused on the mechanism(s) by which miRNAs are controlled. In our study, we both identified miR-144 as a negative regulator of ADAM10 and demonstrated that AP-1/c-Jun regulates miR-144 transcription. Consistent with our results, recent studies also demonstrate that c-Jun regulates expression of miRNAs at transcriptional level (64-66). Moreover, there is evidence showing an increase in c-Jun expression in the neurons of AD patients (45,47). In postmortem pathological brain samples, phosphorylated c-Jun staining is found only in the affected regions of the cerebral cortex and hippocampus (67). Furthermore, c-Jun influences the initiation and execution of Aβ-induced neuronal apoptosis (68-70). These findings suggest that the induction of c-Jun is crucial to the pathogenesis of AD. However, previous studies defining the implication of the AP-1/c-Jun pathway in AD mainly concentrate on its involvement in neuronal apoptosis. Here, we demonstrated that AP-1 may directly participate in amyloidogenesis by regulating miR-144.

Notably, AP-1 is not the only transcription factor activated after Aβ treatment in vitro or Aβ deposition in vivo. For instance, there is evidence that Aβ treatment of human brain endothelial cells results in increases in the activities of the AP-1, CREB, GATA, NFATc, and GRE transcription factors (47). Among these, the GATA family was previously reported to regulate transcription of miR-144 and miR-451 (52,53). Thus, a vicious circle in AD pathogenesis composed by Aβ, AP-1/c-Jun, miR-144 and ADAM10 is emerging. In detail, miR-144 elevates in ordinary aging population or in early stage AD patients, and decreases ADAM10 protein levels, which hampers the non-amyloidogenic pathway, and forces metabolism of Aβ to shifts to favor Aβ production. When Aβ is over-produced, brain deterioration begin, then AP-1, GATA, and other transcription factors are recruited or activated, in turn, further promotes miR-144/451 expression and consequently reinforces the process (Fig. 8), which could be accelerate or interrupted by environmental factors. Although, till now, the
first participant who starts the vicious circle is not known yet, we can see that miR-144/451 play a crucial role in it.

In conclusion, this study provides evidence that miR-144 decreases the expression of ADAM10 protein and demonstrates that miR-144 is transcriptionally regulated by AP-1/c-Jun, suggesting miR-144 contributes to AD pathogenesis and pharmacological targeting miR-144 may represent a promising strategy in the management of AD.
REFERENCES

miR-144 Suppresses ADAM10 Expression


Acknowledgments
This work was financially supported by a grant “J&J-Tsinghua Co-managed Fund” and the Chinese National Basic Research Program (973 Project, No. 2007CB507406).

Conflict of Interest statement
Conflict of interest: All authors state that they have no conflicts of interest.
Table 1. Sequences of primers used in plasmids construction or mutation.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM10 3'UTR F</td>
<td>ATAGAGCTCAGGAGAGAGTTATCAAATGGG</td>
</tr>
<tr>
<td>ADAM10 3'UTR R</td>
<td>CCCTCGAGAAATGCGAATTITACATCTG</td>
</tr>
<tr>
<td>pGL3-promoter-2808 F</td>
<td>GCGGTACCACTGGTGGCTTCACATCA</td>
</tr>
<tr>
<td>pGL3-promoter-2554 F</td>
<td>GCGGTACCACTGGTGGCTTCACATCA</td>
</tr>
<tr>
<td>pGL3-promoter-1895 F</td>
<td>GCGGTACCACTGGTGGCTTCACATCA</td>
</tr>
<tr>
<td>pGL3-promoter-1019 F</td>
<td>GCGGTACCACTGGTGGCTTCACATCA</td>
</tr>
<tr>
<td>pGL3-promoter-453 F</td>
<td>GCGGTACCACTGGTGGCTTCACATCA</td>
</tr>
<tr>
<td>pGL3-promoter-307 F</td>
<td>GCGGTACCACTGGTGGCTTCACATCA</td>
</tr>
<tr>
<td>ADAM10 3'-UTR 144Mut F</td>
<td>CTTGAATGATCTAATTTTATTTTATTTAAAGAGGCGGAGAGGAGG</td>
</tr>
<tr>
<td>ADAM10 3'-UTR 144Mut R</td>
<td>TTTTCCGCTCTTTAATTAAATAATTGAGATACACTCACAGAGATT</td>
</tr>
<tr>
<td>ADAM10 3'-UTR 451Mut F</td>
<td>AGTGATGATATGCTGAAAAAGACAGCCTTTTTCTTCTTATCATCACAGAGAAAAC</td>
</tr>
<tr>
<td>ADAM10 3'-UTR 451Mut R</td>
<td>GAAAGAAGAAAGAAGAGATCGTCTGTTTTCAGCATATCATCTGATCTGGA</td>
</tr>
<tr>
<td>AP-1 F</td>
<td>ATAGGTACGAGAGAGAGTTATCAAATGGG</td>
</tr>
<tr>
<td>AP-1 R</td>
<td>ATAGGTACGAGAGAGAGTTATCAAATGGG</td>
</tr>
<tr>
<td>CTCF-4 F</td>
<td>ATGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>CTCF-4 R</td>
<td>ATGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>CP2 F</td>
<td>TAATGTAACAGAGATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>CP2 R</td>
<td>TAATGTAACAGAGATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-1 F</td>
<td>AATAGGAATTTGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-1 R</td>
<td>AATAGGAATTTGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-2 F</td>
<td>AAACCTTCCATCGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-2 R</td>
<td>AAACCTTCCATCGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-3 F</td>
<td>TTGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-3 R</td>
<td>TTGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-4 F</td>
<td>TGGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-4 R</td>
<td>TGGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-5 F</td>
<td>TGGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-5 R</td>
<td>TGGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-6 F</td>
<td>TGGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>AP-1 Mut-6 R</td>
<td>TGGGTGAGAATGCGAATTAAATAGCCTGCTGAGGAGGAGGAGGAGG</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; Mut, mutant
Table 2. Candidate miRNAs sequences with their potential MRE in ADAM10 3’-UTR.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>microRNA and ADAM10 3’-UTR alignment</th>
<th>mirSVR score</th>
<th>PhastCon score</th>
</tr>
</thead>
</table>
| miR-9    | 3’ aquaugucgaucauaGGUUUCu 5’ hsa-miR-9  
          | 5’ auggaacaacuacucuCAAAGa 3’ ADAM10 3’UTR | -0.1296       | 0.7766         |
| miR-101  | 3’ aagUCAAUAGUGUCAUGACAu 5’ hsa-miR-101  
          | 5’ cuUAUUAUAAAUUGUGUu 3’ ADAM10 3’UTR | -0.3897       | 0.7596         |
| miR-122  | 3’ guuugugguacaguGUGAGGu 5’ hsa-miR-122  
          | 5’ acauggaaaaacuCAACUCCa 3’ ADAM10 3’UTR | -0.1757       | 0.7766         |
| miR-144  | 3’ ucauguaguagauUUGACAu 5’ hsa-miR-144  
          | 5’ cuuaauuuaaaauUACUGUu 3’ ADAM10 3’UTR | -0.3927       | 0.7596         |
| miR-451  | 3’ uugAGUCAU-UACCAUUGCCAAa 5’ hsa-miR-451  
          | 5’ uuaUCAAAAUAUUAAACGGuu 3’ ADAM10 3’UTR | -1.2235       | 0.6139         |

Table 3. Alignment of the ADAM10 3’-UTR displaying that potential MRE for miR-144 is conserved across species.

Hsa, Homo sapiens; Ptr, Pan troglodytes; Mcc, Macaca mulatta; Mmu, Mus musculus; Gga, Gallus gallus; Cfa, Canis familiaris; Xtr, Xenopus tropicalis.

<table>
<thead>
<tr>
<th>Species</th>
<th>ADAM10 3’-UTR sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsa</td>
<td>GTTTCCTTGTATTATAAAAATTTTTCCGCTCTTAAAA-ATCATGTTAATTGACATACTCAGGATAACAGAGAT</td>
</tr>
<tr>
<td>Ptr</td>
<td>GTTTCCTTGTATTATAAAAATTTTTCCGCTCTTAAAA-ATCATGTTAATTGACATACTCAGGATAACAGAGAT</td>
</tr>
<tr>
<td>Mcc</td>
<td>GTTTCCTTGTATTATAAAAATTTTTCCGCTCTTAAAA-ATCATGTTAATTGACATACTCAGGATAACAGAGAT</td>
</tr>
<tr>
<td>Mmu</td>
<td>GTTTCCTTGTATTATAAAAATTTTTCCGCTCTTAAAA-ATCATGTTAATTGACATACTCAGGATAACAGAGAT</td>
</tr>
<tr>
<td>Xtr</td>
<td>GTTTCCTTGTATTATAGCTTTTTCCGCTCTTAAAA-ATCATGTTAATTGACATACTCAGGATAACAGAGAT</td>
</tr>
<tr>
<td>Cfa</td>
<td>GTTTCCTTGTATTATAAAAATTTTTCCGCTCTTAAAA-ATCATGTTAATTGACATACTCAGGATAACAGAGAT</td>
</tr>
<tr>
<td>Gga</td>
<td>GTTTCCTTGTATTATAAAAATTTTTCCGCTCTTAAAA-ATCATGTTAATTGACATACTCAGGATAACAGAGAT</td>
</tr>
</tbody>
</table>
miR-144 Suppresses ADAM10 Expression

Figure legends

FIGURE 1. Identification of miR-144 as a negative regulator of ADAM10. A. SH-SY5Y cells were transfected with the pmirGLO-ADAM10 3’UTR reporter plasmid (0.05 μg) and miRNA mimics (50 nM) or cel-mir-67 mimics (negative control mimics, NC), which have no sequence identity with any human miRNAs. Luciferase activity was measured 48 hrs after transfection, and the values are shown as fold change of the luciferase activity with respect to the negative control. B. SH-SY5Y cells were transfected with the pmirGLO-ADAM10 3’-UTR reporter plasmid and miR-144 inhibitor (100 nM) or miRNA inhibitor negative control (inhibitor NC). The dual luciferase assay was performed 48 hrs after transfection, and the luciferase activity of the miRNA inhibitor negative control was regarded as 1. C. Western blot analysis of endogenous ADAM10 protein levels in SH-SY5Y cells transfected with the miRNA negative control and miR-144 or miR-9 mimics. GAPDH served as an internal control. D. Intensities of the ADAM10 bands from three independent experiments were quantified and normalized to that of corresponding GAPDH bands. The values were plotted as fold change with respect to the negative control. E. Quantitative RT-PCR analysis of the ADAM10 mRNA levels in SH-SY5Y cells transfected with the miRNA negative control or miR-144 mimics. Values in A, B, D and E are means ± SEM from experiments performed in triplicate. Asterisks indicate significant differences (*p < 0.05).

FIGURE 2. Analysis of the miR-144 MRE within the ADAM10 3’-UTR. A. Schematic representation of the human ADAM10 3’-UTR indicating the putative MRE for miR-144 and the sequences of the mutant reporter constructs created by PCR-based site-directed mutagenesis. B. The ADAM10 3’-UTR luciferase reporter (3’UTR ) or mutant construct without the predicted miR-144 MRE (mutant 3’UTR) was transfected with either miR-144 mimics or control mimics (NC) into SH-SY5Y cells. The dual luciferase assays were performed 48 hrs after transfection. The fold change in relative luciferase activity was plotted; dark and gray bars indicate luciferase activity of the negative control and miR-144, respectively. Results are presented as means ± SEM from experiments performed in triplicate. Asterisks indicate significant differences (*p < 0.05).

FIGURE 3. Identification of the miR-144 promoter by luciferase assays. Identification of miR-144 promoter activity by luciferase assays in SH-SY5Y cells. The luciferase reporter construct (-2080), containing the ~2-kb region upstream of the precursor miR-144 and its deletion derivate (-2554, -1895, -1019, -453, and -307) was each transfected into SH-SY5Y cells, and dual luciferase assays were performed 48 hrs after transfection. For each reporter construct, the histogram shows the relative luciferase activity levels, evaluated as the ratio between each value versus the pGL3 basic vector; the schematic is on the right. Values are means ± SEM from experiments performed in triplicate.

FIGURE 4. Transcriptional regulation of miR-144. A. The luciferase activity of the pGL3-promoter-1019 reporter after co-transfection into SH-SY5Y cells with an expression plasmid encoding the SP1, c-Jun, CREB, CP2, or TCF4 transcription factor, respectively. Values were calculated as the ratio of the luciferase activities in cells co-transfected with pGL3-promoter-1019 and a transcription factor expression plasmid versus in cells that was co-transfected with pGL3 basic vector and the same expression plasmid. B. Dual luciferase activity assay after treatment with the indicated concentration of TPA in SH-SY5Y cells that transfected with the pGL3-promoter-1019
miR-144 Suppresses ADAM10 Expression

reporter plasmid. Values were calculated as the ratio of the luciferase activities in cells transfected with pGL3-promoter-1019 versus in cells transfected with pGL3 basic vector treated with TPA of same concentration. C, Quantitative RT-PCR analysis of the level of endogenous mature miR-144 in SH-SY5Y cells after 24 hrs transfection of c-Jun expression plasmid. D, Quantitative RT-PCR analysis of the level of endogenous mature miR-144 in SH-SY5Y cells after 12 hrs TPA (50 nM) treatment. E, The levels of miR-144 were analyzed by quantitative RT-PCR 48 hrs after transfection of c-Jun siRNA-1 or siRNA-2 in SH-SY5Y cells. F, Schematic representation of the gene encoding miR-144 and miR-451, as well as the promoter region with six potential AP-1 binding sites with the sequences deleted by PCR-based site-directed mutagenesis in each mutant reporter. G, Each single mutant (mut-1 to mut-6) or double mutant (D.M.) reporter plasmids, or the wild type (wt) plasmid was transfected into SH-SY5Y cells, 24 hrs after the transfection the cells were treated with or without TPA (20 nM), and the dual luciferase activity assay was performed after another 12 hrs. Values were calculated as the ratio of the luciferase activities in cells transfected with wild type or mutant reporter plasmids and treated with TPA versus cells transfected with the same reporter plasmid and treated with DMSO. H, Each mutant reporter plasmids or the wild type plasmid was co-transfected with c-Jun expression vector into SH-SY5Y cells, the dual luciferase activity assay was performed 48 hrs after the transfection. I, 2μL of SH-SY5Y cells nuclear extracts were incubated with 50 fmol biotin-labeled DNA probe corresponding to putative AP-1 binding site 3 in miR-144 promoter (lane 2, 3 and 4) or with the biotin-labeled DNA probe corresponding to binding site 5 (lane 6, 7 and 8). For binding competition, 200-fold excess of unlabeled DNA probe was included in the reaction (lane 4 and 8). For supershift assay, c-Jun antibody was included (lane 2 and 6). Results in A, B, C, D, E, G and H are presented as means ± SEM from experiments performed in triplicate. Asterisks indicate significant differences (*p < 0.05)

FIGURE 5. miR-144 contributes to the down-regulation of ADAM10 protein in response to TPA treatment. A, The mRNA levels of ADAM10 after treatment of TPA (50 nM) for the indicated time intervals were analyzed by quantitative RT-PCR and plotted as the fold change with respect to untreated control. B, Representative results from three independent western blot analyses of ADAM10 protein in SH-SY5Y cells treated with TPA (50 nM) after the indicated time intervals. GAPDH served as an internal control. C, Intensities of the ADAM10 bands for each time interval from three independent experiments were quantified by densitometry. The values were normalized to that of corresponding GAPDH bands and plotted as fold change with respect to the DMSO-treated control. D, Quantitative RT-PCR analysis of the endogenous mature miR-144 after TPA (50 nM) treatment for the indicated time intervals. E, Demonstration of the inverse correlation between ADAM10 protein and mature miR-144 levels in SH-SY5Y cells after TPA treatment for the indicated times intervals. F, SH-SY5Y cells with or without miR-144 loss of function by transfection with the miR-144 inhibitor or mirRNAs inhibitor negative control (miR inhibitor NC) were treated with TPA (20 nM) for 24 hrs, then the levels of ADAM10 protein in the cells were analyzed by western blot. G, Intensities of the ADAM10 bands from three independent experiments were quantified and normalized to that of corresponding GAPDH bands. The values were plotted as the fold change with respect to the negative control. Values in A, C, D, E and G are means ± SEM from experiments performed in triplicate. Asterisks indicate significant differences (*p < 0.05).

FIGURE 6. miR-144 is regulated by Aβ and contributes to the repression of ADAM10 induced
miR-144 Suppresses ADAM10 Expression

by Aβ. A, The level of endogenous mature miR-144 24 hrs after treatment with Aβ_{42} peptide (5 µM) was measured by quantitative RT-PCR and plotted as fold change with respect to PBS-treated control. B, ADAM10 protein, α-CTF, and β-CTF in SH-SY5Y cells 24 hrs after treatment with Aβ_{42} peptide at the indicated concentrations were analyzed by western blot. C, Intensities of the ADAM10 bands from three independent experiments were quantified and normalized to that of corresponding GAPDH bands. The values were plotted as the fold change with respect to the PBS-treated control. D, Quantitative RT-PCR analysis of ADAM10 mRNA in SH-SY5Y cells 24 hrs after treatment with Aβ_{42} peptide (5 µM). E, SH-SY5Y cells with or without miR-144 loss of function by transfection with the miR-144 inhibitor or miRNAs inhibitor negative control (miR inhibitor NC) were treated with Aβ_{42} (5 µM) peptide for 24 hrs, then the levels of ADAM10 protein in the cells were analyzed by western blot. F, Intensities of the ADAM10 bands from three independent experiments were quantified and normalized to that of corresponding GAPDH bands. The relative expression of ADAM10 was plotted as the fold change with respect to the negative control. Results in A, C, D, and F are presented as means ± SEM from experiments performed in triplicate. Asterisks indicate significant differences (*p < 0.05).

FIGURE 7. miR-451 is also involved in the regulation of ADAM10 expression. A, Western blot analysis of ADAM10 protein in SH-SY5Y cells after transfection with miR-451 or miR-144 mimics. The miR-144 mimics were used as a positive control. B, Intensities of the ADAM10 bands from three independent experiments were quantified and normalized to that of corresponding GAPDH bands. The values were plotted as fold change with respect to the negative control. C, Quantitative RT-PCR analysis of ADAM10 mRNA in SH-SY5Y cells after transfection with miR-451. D, The ADAM10 3’-UTR luciferase reporter (3’UTR ) or mutant construct without the predicted miR-451 MRE (mutant 3’UTR) was transfected with either miR-451 mimics or control mimics (NC) into SH-SY5Y cells. The dual luciferase assays were performed 48 hrs after transfection. The fold change in relative luciferase activity was plotted; dark and gray bars indicate luciferase activity of the negative control and miR-451 respectively. E, Quantitative RT-PCR analysis of the level of endogenous mature miR-451 in SH-SY5Y cells 24 hrs after transfection of c-Jun plasmid. F, 48 hrs after transfection of c-Jun siRNA-1 or siRNA-2 in SH-SY5Y cells, levels of endogenous mature miR-451 were analyzed by quantitative RT-PCR. G, Quantitative RT-PCR analysis of the endogenous mature miR-451 after TPA (50 nM) treatment for indicated time intervals. H, Demonstration of the inverse correlation between the levels of ADAM10 protein and endogenous mature miR-451 in SH-SY5Y cells after TPA (50 nM) treatment for the indicated time intervals. I, The level of endogenous mature miR-451 24 hrs after the treatment with Aβ_{42} peptide (5 µM) was measured by quantitative RT-PCR and plotted as the fold change with respect to the PBS-treated control. Results in B, C, D, E, F, G, H, and I are means ± SEM from experiments performed in triplicate. Asterisks indicate significant differences (*p < 0.05).

FIGURE 8. Regulatory feed-back loop encompassing AP-1, miR-144, miR-451 and ADAM10. miR-144 and miR-451 binds to each MRE with the 3’-UTR of ADAM10 mRNA and decrease ADAM10 expression at the transcriptional level. The decrease in ADAM10 protein promotes amyloidogenesis. In turn, the increase in Aβ may activate AP-1 and other signaling pathways that may be involved in the regulation of miR-144 and miR-451.
Figure 1

A

B

C

D

E

miR-144 Suppresses ADAM10 Expression

20
miR-144 Suppresses ADAM10 Expression

Figure 2

A

human ADAM10 mRNA

5'UTR

ORF

3'UTR

5' cuaaauuuuuuuuuUACUGUu 3' ADAM10 3'UTR

3' ucauguaguagauAUGACAu 5' miR-144

5' cuaaauuuuuuuUACUGUu 3' ADAM10 3'UTR mut

B

Relative Luciferase Activity

0.0 0.2 0.4 0.6 0.8 1.0 1.2

mutant 3'UTR 3'UTR

NC

miR-144

* Figure 3

Relative Luciferase Activity


Luc Luc Luc Luc Luc Luc

0 5 10 15
miR-144 Suppresses ADAM10 Expression

Figure 4

A

Relative Luciferase Activity

vector c-Jun CREB Sp1 CP2 TCF4

B

Relative Luciferase Activity

DMSO 20 50 100 200 TPA (nM)

C

Mature miR-144 Fold Change

vector c-Jun

D

Mature miR-144 Fold Change

control TPA

E

Mature miR-144 Fold Change

scramble siRNA 1 siRNA 2

F

AP1 binding site

1 2

TTTAGCC****GTAAGG
TAGGAC****CAGGAA
CAGGTTGCTCTTCTG

G

Relative Luciferase Activity

DMSO TPA

H

Relative Luciferase Activity

vector c-Jun

I

unlabeled probe c-Jun antibody nuclear extract bind-probe

super shift shift
miR-144 Suppresses ADAM10 Expression

Figure 5

A

ADAM10 mRNA Fold Change

B

ADAM10

GAPDH

0 2 6 12 24 48 Time (h)

D

Mature miR-144 Fold Change

F

ADAM10

GAPDH

DMSO + ++

TPA − + +

milR inhibitor NC + + −

milR-144 inhibitor − − +

C

ADAM10 Protein Fold Change

E

Mature miR-144 Fold Change

G

Relative Expression Level

0 2 6 12 24 48 Time (h)
miR-144 Suppresses ADAM10 Expression

Figure 6

A

miR-144 Fold Change

B

ADAM10
β-CTF
α-CTF
GAPDH

0 1 5 Aβ (μM)

C

Relative Expression Level of ADAM10

D

Fold Change of ADAM10 mRNA

E

ADAM10
GAPDH

PBS + − −
Aβ + + −
mir inhibitor NC + − −
mir-144 inhibitor − + +

F

Relative Expression Level of ADAM10

http://www.jbc.org/ Downloaded from by guest on September 1, 2017
miR-144 Suppresses ADAM10 Expression

Figure 7

A) ADAM10 and GAPDH expression levels in NC, miR-451, and miR-144 conditions.

B) Relative expression level of ADAM10 in NC, miR-451, and miR-144 conditions.

C) Fold change of ADAM10 mRNA in NC and miR-451 conditions.

D) Relative luciferase activity of 3'UTR and mutant 3'UTR with NC and miR-451 conditions.

E) Mature miR-451 fold change with vector and c-Jun conditions.

F) Mature miR-451 fold change with scramble, siRNA 1, and siRNA 2 conditions.

G) Mature miR-451 fold change over time (0, 2, 6, 12, 24, 48 hours).

H) ADAM10 and mature miR-451 protein fold change over time (0, 2, 6, 12, 24, 48 hours).

I) Mature miR-451 fold change with control and Apo-5 μM conditions.
miR-144 Suppresses ADAM10 Expression

Figure 8

TPA → Aβ → AP-1 → ADAM10 → ADAM10 mRNA → miR-144 → miR-451
MicroRNA-144 Is Regulated by Activator Protein-1 (AP-1) and Decreases Expression of Alzheimer's Disease-related A Disintegrin And Metalloprotease 10 (ADAM10)

Cong Cheng, Weiguang Li, Zheng Zhang, Shohei Yoshimura, Qinyu Hao, Chi Zhang and Zhao Wang

J. Biol. Chem. published online April 1, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.381392

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2013/04/01/jbc.M112.381392.full.html#ref-list-1