

Identification of miRNA Changes in Alzheimer's Disease Brain and CSF Yields Putative Biomarkers and Insights into Disease Pathways

John P. Cogswell^{a,*}, James Ward^b, Ian A. Taylor^c, Michelle Waters^d, Yunling Shi^b, Brian Cannon^e, Kevin Kelnar^e, Jon Kemppainen^e, David Brown^e, Caifu Chen^f, Rab K. Prinjha^g, Jill C. Richardson^g, Ann M. Saunders^a, Allen D. Roses^a and Cynthia A. Richards^h

^a*Department of Pharmacogenetics, GlaxoSmithKline Inc., Research Triangle Park, NC, USA*

^b*Department of Molecular Discovery Research-Informatics, GlaxoSmithKline Inc., Research Triangle Park, NC, USA*

^c*Department of Molecular Discovery Research-Core Technology Discovery Group, GlaxoSmithKline Inc., Research Triangle Park, NC, USA*

^d*Department of Molecular Discovery Research-Metabolic Discovery Technology Group, GlaxoSmithKline Inc., Research Triangle Park, NC, USA*

^e*Asuragen Inc., 2150 Woodard, Austin, TX, USA*

^f*Molecular Cell Biology-RandD, Applied Biosystems, Foster City, CA, USA*

^g*Neurology CEDD, GlaxoSmithKline Inc., Harlow, UK*

^h*Department of Molecular Discovery Research-Infectious Disease Discovery Technology Group, GlaxoSmithKline Inc., Research Triangle Park, NC, USA*

Abstract. MicroRNAs have essential functional roles in brain development and neuronal specification but their roles in neurodegenerative diseases such as Alzheimer's disease (AD) is unknown. Using a sensitive qRT-PCR platform we identified regional and stage-specific deregulation of miRNA expression in AD patient brains. We used experimental validation in addition to literature to reveal how the deregulated brain microRNAs are biomarkers for known and novel pathways in AD pathogenesis related to amyloid processing, neurogenesis, insulin resistance, and innate immunity. We additionally recovered miRNAs from cerebrospinal fluid and discovered AD-specific miRNA changes consistent with their role as potential biomarkers of disease.

Keywords: Alzheimer's disease, inflammation, insulin signaling, microRNA, neurogenesis

Supplemental data available at <http://www.j-alz.com/issues/14/vol14-1.html#supplementarydata>

INTRODUCTION

Alzheimer's disease (AD) is an age-related dementia characterized by memory loss and behavioral changes.

At early stages it is often difficult to conclusively diagnose in the clinic highlighting the need to develop novel biomarkers to inform the development and testing of novel therapeutic agents. AD has a complex progression including early development of neuronal dysplasia, angiogenic changes, release of inflammatory mediators by CNS glial and peripheral immune cells, and development of extracellular amyloid- β plaques and intracellular neurofibrillary tangles [15,

*Corresponding author: John P. Cogswell, GlaxoSmithKline Inc., MA1A.1229, 5 Moore Drive, Research Triangle Park, NC 27709, USA. Tel.: +1 919 483 3449; Fax: +1 919 315 4174; E-mail: John.P.Cogswell@gsk.com.

23,46]. The regional appearance and progression of the tangle pathologies and their impact on the destruction of the memory centers of the brain (i.e., hippocampus and isocortex) provide the basis for the Braak and Braak classification [4]. Genetic alterations that increase the $A\beta_{1-42}$ cleavage product through increased production of the amyloid- β protein precursor ($A\beta$ PP) or through modulation of presenilin function and downstream processing of $A\beta$ PP have been linked to familial forms of AD [23]. Also carriers of the APOE4 allele have been shown to have an earlier age of onset of AD [55]. Recent studies have reported an association between diabetes and the later onset of AD [3,12] supported by observations of decreased glucose utilization and other metabolic defects in AD brain [9,54,61]. Preclinical studies would appear to support a link between AD and dysregulated insulin signaling in that overexpression of GSK3 β in mice results in AD-like pathology and memory deficits while inactivation of the transgene led to improvements in both phenotypes [17].

MicroRNAs (miRNAs) are small regulatory RNAs that bind the 3' UTR's of target genes and inhibit expression post-transcriptionally via inhibition of translation initiation or cleavage of mRNA [19,66]. Each gene with a sizeable 3' UTR is capable of being regulated by multiple miRNAs and furthermore a single miRNA may bind multiple genes [59]. MiRNAs are expressed in a specific spatiotemporal fashion and have been shown to be essential to brain morphogenesis in lower organisms and in neuronal differentiation in cell culture [35–37,56,57,67]. Currently, the few examples of direct links between miRNAs and human diseases of the brain include the intriguing finding of a single nucleotide polymorphism in a family with Tourette's syndrome that increases the binding affinity of miR-189 to the 3' UTR of SLITRK1 [1] and the downregulated expression of miR-133b in midbrain dopaminergic neurons in human brain samples as well as pre-clinical models of Parkinson's disease [32]. To understand whether miRNA expression may be misregulated in AD, the expression of over 300 miRNAs was determined in hippocampus, medial frontal gyrus, and cerebellum from early and late stage AD compared to age-matched controls. These data reveal expression changes in key miRNAs that: 1) are consistent with the regional and time-dependent features of AD pathology; and 2) are linked through their targets to known and novel pathways of disease. We additionally discovered that miRNAs can be detected in cerebrospinal fluid (CSF); and importantly that CSF miRNAs are indeed altered in AD, in particular miRNAs related to multiple disease related pathways such as immune cell differentiation and innate immunity.

MATERIALS AND METHODS

Alzheimer's disease samples and patient data

Age matched hippocampus, medial frontal gyrus, and cerebellum samples from different Braak stages were obtained from the Netherlands Brain Bank (Table 1). Braak stage 5 and Braak stage 1 CSF was obtained from the Kathleen Price Bryan Brain Bank (Table 2).

Brain miRNA isolation and detection

Brain tissue samples were homogenized in Trizol at 10 mg tissue/ml Trizol. After organic extraction the aqueous phase was loaded onto an RNeasy column following standard total RNA protocols (Qiagen). The flow-through from the RNeasy column wash, which contains RNA < 200 bases, was adjusted to a final ethanol concentration of 61% by addition of 0.66 volumes of 100% ethanol and then loaded onto a mirVana filter column (Ambion). The column was washed with 700 μ l of miRNA Wash Solution 1, two times with 500 μ l of miRNA Wash Solution 2/3 (Ambion), and then eluted with 100 μ l 95°C water. Agilent Bioanalyzer analysis showed the RNA eluted from the mirVana column was highly enriched for low molecular weight RNA. RNA concentrations were determined using a ND-1000 spectrophotometer (NanoDrop). MiRNA quantification was performed using a reverse transcription (RT) modification of the TaqManTM miRNA Assays (Applied Biosystems Inc.) [11]. 48-plex RT mixes were obtained from Applied Biosystems, and by concentrating the RT primers to 10 nM each in the final RT reaction [38]. A total of 60 ng RNA was reverse transcribed in multiplexes of up to 48. Six to 7 separate 48-plex RTs were performed on each sample (Supplementary Table 1 – available at <http://www.j-alz.com/issues/14/vol14-1.html#supplementarydata>). After reverse transcription the cDNA was diluted and aliquots were separately amplified using the 48 individual miRNA primer/probe mixes corresponding to the reverse transcription multiplex. PCR reactions were run as triplicates. Each multiplex RT contained 2–3 common snoRNAs that were subsequently used to normalize data from each sample across all pools. The final data set was normalized as described below.

Table 1
Brain sample data

Subject ID	Subject description	Gender	Age (yr)	pH	PMD (hr)	ApoE
BR0-1	Braaks 0, non-demented	female	52	7.16	6:50	3,3
BR0-2	Braaks 0, non-demented	male	80	6.43	6:30	3,3
BR1-1	Braaks 1, non-demented	female	83	6.48	5:30	3,2
BR1-2	Braaks 1, non-demented	female	76	7.20	4:50	3,2
BR1-3	Braaks 1, non-demented	male	82	6.07	7:40	3,3
BR1-4	Braaks 1, non-demented	female	77	6.32	5:40	3,3
BR1-5	Braaks 1, non-demented	male	96	6.65	6:30	3,3
BR3-1	Braaks 3, non-demented	female	84	n/a	9:20	3,3
BR3-2	Braaks 3, non-demented	female	90	6.50	4:45	3,3
BR3-3	Braaks 3, non-demented	female	93	6.25	5:35	3,2
BR3-4	Braaks 3, non-demented	female	91	6.51	5:20	3,2
BR3-5	Braaks 3, non-demented	male	93	6.80	10:25	4,3
BR4-1	Braaks 4, Alzheimer's disease	female	85	6.73	2:45	2,2
BR4-2	Braaks 4, Alzheimer's disease	male	88	6.49	5:00	4,2
BR4-3	Braaks 4, Alzheimer's disease	female	79	6.29	5:20	4,3
BR4-4	Braaks 4, Alzheimer's disease	male	64	6.62	6:00	3,3
BR4-5	Braaks 4, Alzheimer's disease	female	84	6.64	3:50	3,3
BR5-1	Braaks 5, Alzheimer's disease	female	82	6.49	4:35	4,3
BR5-2	Braaks 5, Alzheimer's disease	male	71	6.21	5:20	4,3
BR5-3	Braaks 5, Alzheimer's disease	female	71	6.35	6:45	3,3
BR5-4	Braaks 5, Alzheimer's disease	male	85	6.20	4:25	4,3
BR5-5	Braaks 5, Alzheimer's disease	female	78	6.59	4:00	4,3
BR6-1	Braaks 6, Alzheimer's disease	female	72	6.31	4:55	4,3
BR6-2	Braaks 6, Alzheimer's disease	male	78	6.15	9:55	3,3
BR6-3	Braaks 6, Alzheimer's disease	female	64	6.65	3:40	4,3
BR6-4	Braaks 6, Alzheimer's disease	male	72	6.51	5:30	4,2
BR6-5	Braaks 6, Alzheimer's disease	female	82	6.48	6:00	4,2

Table 2
CSF sample data. Samples 1133, 988 removed by correlation analysis; 795 and 1085 removed by between group analysis

Donor ID	Braak stage	Gender	Age (yr)	PMD (hr)	ApoE	ng RNA/ ml CSF	CSF color
406	Braaks 1, non-demented control	male	77	1:15	2,2	16.3	colorless
707	Braaks 1, non-demented control	male	80	4:15	3,3	12.5	colorless
795	Braaks 1, non-demented control	male	81	7:13	3,4	22	colorless
838	Braaks 1, non-demented control	male	> 90	7:41	3,3	107.4	colorless
855	Braaks 1, non-demented control	female	73	1:37	3,3	33.4	pink
913	Braaks 1, non-demented control	female	67	8:00	3,3	251.1	colorless
922	Braaks 1, non-demented control	male	79	6:54	3,3	24.9	colorless
1037	Braaks 1, non-demented control	female	88	20:30	2,3	32.1	colorless
1076	Braaks 1, non-demented control	male	62	4:50	3,3	37.7	colorless
1169	Braaks 1, non-demented control	male	88	17:20	2,3	145	colorless
988	Braaks 5, Alzheimer's Disease	female	77	6:00	3,4	220.1	pale yellow
990	Braaks 5, Alzheimer's Disease	female	80	5:09	3,3	36.3	colorless
1058	Braaks 5, Alzheimer's Disease	female	> 90	10:52	3,4	29.4	colorless
1085	Braaks 5, Alzheimer's Disease	male	71	6:30	3,4	19.9	colorless
1124	Braaks 5, Alzheimer's Disease	male	56	18:50	3,4	22.1	colorless
1133	Braaks 5, Alzheimer's Disease	female	64	7:20	3,3	24.9	pale pink
1153	Braaks 5, Alzheimer's Disease	female	49	17:31	3,3	18.9	colorless
1259	Braaks 5, Alzheimer's Disease	female	79	8:00	4,4	10.8	colorless
1343	Braaks 5, Alzheimer's Disease	female	77	11:25	2,3	28.8	colorless
1344	Braaks 5, Alzheimer's Disease	male	65	23:30	3,4	14.4	colorless

CSF miRNA isolation and detection

CSF samples for miRNA profiling studies were processed by Asuragen, Inc. according to the company's standard operating procedures. Total RNA was isolat-

ed from 0.75-2ml of cerebrospinal fluid from a total of 20 donors using a glass fiber filter-based biofluid isolation method (Asuragen, Austin, TX). RNA was eluted in 100 μ l of nuclease-free water (Ambion, Austin, TX). The RNA was dried down and resuspended in

14 μ l of Nuclease-Free water. The RNA concentration was determined by measuring 2.0 μ l on a NanoDrop[®] ND-3300 Fluorospectrometer using a modified protocol for the Quant-iT[™] RNA kit (Invitrogen, Carlsbad, CA). CSF miRNA profiling was performed using RT and pre-amplification modifications of the TaqMan[®] miRNA Assays (Applied Biosystems, [64]). 313-plex RT mix and miRNA PreAmp primers were obtained from Applied Biosystems (Struass et al., manuscript in preparation). Four ng total CSF RNA was used in duplicate 313-plex RT reactions. The cDNA was subjected to pre-PCR amplification, 95°C/10min, 55°C/2 min, 72°C/2min followed by 14 cycles of 95°C/15 sec, 60°C/4 min. After pre-PCR amplification the cDNA was diluted and aliquots were separately amplified using 308 individual miRNA primer probe mixes. PCR reactions were run as triplicates.

Normalization of miRNA expression data

TaqMan cycle threshold (Ct) values were converted to expression abundance using the equation $2^{(40-Ct)}$ but log₂-abundance was used for all statistical calculations to ensure data normality. Each multiplex RT contained 2–3 common snoRNAs that were used to account for variation across RT pools. The geometric mean of the snoRNA expression values were used to normalize the RT pools for each sample. Samples were subsequently normalized relative to one another prior to statistical analysis. This cross-sample normalization included all primer sets which were easily detectable, defined as having abundance > 50 in 90% of the samples. The log₂ expression fold changes for each qualifying miRNA versus its median were computed. For each sample, the distribution of these fold changes was compared to the median distribution computed across all samples. The normalizer was defined as the shift from the median distribution within the 40th and 60th percentile range, applied to log₂ abundances. The method has the effect of minimizing the number and extent of reported changes, and as a by-product minimizes expression variance within sample groups. Note “before” and “after” transformed distributions were visually examined for consistency.

Statistical analysis of miRNA expression data

Normalized miRNA log₂ abundances were analyzed using a *t*-test for each pairing of Braak 5,6 versus Braak 0,1 and Braak 3,4 versus Braak 0,1, for each tissue, cerebellum, hippocampus, and medial frontal

gyrus. The *t*-test was used rather than ANOVA because the miRNA multiple pools were changed between the Braak 5,6 and the Braak 3,4 assays. However, the Braak 0,1 was repeated for both assays, and was confirmed to be a stable reference baseline for both *t*-test analyses. The *t*-test results were filtered by P-value, minimum fold change, and minimum median miRNA expression. Supplementary Table 2 (available at <http://www.j-alz.com/issues/14/vol14-1.html#supplementarydata>) has all significant (*t*-test *p*-value > 0.05) data filtered by mean expression > 25. Only data with control sample replicate ≥ 6 and AD sample replicate ≥ 7 were considered in the final analysis. Graph displays fold changes in linear space, with error bars reflecting standard errors converted from the log₂ *t*-test model.

Quality control of CSF miRNA expression data

CSF miRNA expression data was subjected to two quality control analyses, sample correlation analysis, and between groups analysis [13]. Sample correlation analysis was performed using median-centered log₂ miRNA abundances to generate a full correlation matrix. Samples were considered technical outliers when their median correlation was more than 5 times the median absolute deviation (MAD) across all other sample correlation values. Remaining samples were analyzed using Between Groups Analysis from the Bioconductor MADE4 library. Samples whose component score distance was more than 5 times the MAD from the sample group centroid were considered outliers. Remaining samples were subjected to downstream *t*-test analysis and pathway enrichment analysis as described. Table 4 contains all significant (*t*-test *p*-value > 0.05) data filtered by mean expression > 25. Only data with control sample replicate ≥ 5 and AD sample replicate ≥ 5 were considered in the final analysis.

Pathway enrichment analysis of miRNA data

In order to interrogate pathway effects of differential miRNA expression, we used mRNA sequences predicted to be affected by the miRNA's from the MIRANDA and RNAHybrid methods. We applied a 0.01 P-value cutoff to the predictions in order to focus only on the highest quality and most conservative predictions, and required that the predictions also be in agreement between the two methodologies. We generated a pathway library from several available sources and intersected their genes with the miRNA predicted genes. Each pathway thereby contained a set of miRNA's,

Table 3

Effect of miRNA knockdown and overexpression on mRNA abundance. Results of 2 independent transfections. mRNA abundance was measured by quantitative RT-PCR 72 hours after transfection of Pre-miR or Anti-miR and oligo controls using GAPDH levels as a reference. PGAM1 = phosphoglycerate mutase-1; IDH2 = Isocitrate Dehydrogenase 2; RPS6KB2 = p70S6K. * = significant value ($p < 0.05$). Significant results were obtained in the following cell lines: SK-n-AS for miR-32/PGAM1 and miR-423/IDH2; HepG2 for miR-98/IDH2 and miR-193a/RPS6KB2

Transfection	mRNA measured	Experiment 1		Experiment 2	
		% Control	StDev	% Control	StDev
Pre-miR-32	PGAM1	81.12*	4.41	78.18*	3.84
Anti-miR-32	PGAM1	99.36	24.14	105.35	4.5
Pre-miR-98	IDH2	70.03*	1.5	43.98*	5.55
Anti-miR-98	IDH2	84.11	7.25	101.56	6.45
Pre-miR-193a	RPS6KB2	70.36*	5.69	60.03*	5.52
Anti-miR-193a	RPS6KB2	82.06	0.65	109.33	23.28
Pre-miR-423	IDH2	59.33*	7.09	60.19*	4.75
Anti-miR-423	IDH2	82.75	9.03	93.15	0.17

each linked to one or more genes, many of which were therefore represented by multiple miRNA's. The distinguishing feature, that genes are multiply represented, was supported by the understanding that multiple miRNA's may bind and have an additive effect on a single gene. This modification also enabled the analysis to interrogate whether the miRNA changes supported a coordinated effect on specific pathway functions. The full enrichment library for the AD brain data comprised approximately 4,000 total genes despite there being only 202 miRNA's detected above mean expression 20. miRNA's were tested for pathway enrichment, after filtering t -test data for P -value < 0.05 , absolute fold change > 1.5 , and mean expression > 25 . Enrichment was performed using hypergeometric enrichment using the specific universe size defined by detected miRNA's, the predicted mRNA target genes, and the full pathway library. Permutation correction of the P -value was performed to assess random effects from among the detected miRNA's. Details of the methods and steps are in the Bioconductor package "miRNApath" [68].

Cell line selection and transfection of pre-Mir and Anti-miRs

The relative expression levels of 21 miRNAs and 24 mRNAs listed in Supplementary Table 3 (available at <http://www.j-alz.com/issues/14/vol14-1.html#supplementarydata>) were measured by qRT-PCR using TaqMan probes from Applied Biosystems. Expression levels of the miRNAs were determined using TaqMan miRNA Assays (Applied Biosystems) and a proprietary miRNA qRT-PCR technology (Asuragen, patent-pending). For each qRT-PCR assay, 10 ng of the miRNA fractions from the various cell isolates were reverse

transcribed in a 20 μ l reaction with the corresponding miRNA specific RT primer using the RETROscript kit (Ambion, Inc) with the following incubations: 16 °C for 15 min, 42 °C for 15 min, 95 °C for 5 min, then 4 °C while preparing for PCR. Following cDNA synthesis, a 15 μ l PCR was performed using the AB 7900HT (Applied Biosystems), the appropriate PCR primer/probe, 2 μ l cDNA, and TaqMan Universal PCR Master Mix (Applied Biosystems). Cycling conditions were 95 °C for 10 min, then 50 cycles of 95 °C for 5 sec and 60 °C for 30 sec.

mRNA expression levels were determined using TaqMan Gene Expression Assays (Applied Biosystems). mRNA fractions from the ten different cell types were reverse transcribed (10 ng RNA input) with random decamers using the RETROscript kit and the following incubations: 42 °C for 60 min, 95 °C for 5 min, and then 4 °C while preparing for PCR. Following cDNA synthesis, a 10 μ l PCR was performed using the Gene Expression Assays, 2 μ l cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems), and the same cycling conditions as noted above. The appropriate cell line for the analysis of each miRNA was selected based on both the miRNA and mRNA profiling results. Cell lines that expressed relatively low levels of the target miRNA and detectable levels of the target mRNA were selected for the Pre-miR (Ambion) studies for each miRNA. Cell lines that expressed relatively high levels of the target miRNA and detectable levels of the target mRNA were selected for the Anti-miR (Ambion) studies for each miRNA. Using these criteria, we were able to evaluate all of the targeted miRNAs using 4 cell lines; SK-N-AS, HepG2, 22Rv1, and BJ cells. All cell lines were purchased from American Type Culture Collection (ATCC) and cultured using conditions rec-

ommended by ATCC. Transfection conditions for the delivery of small RNA molecules using Lipofectamine 2000 (Invitrogen) were developed. Optimal lipid concentrations for transfection were chosen for each cell line based on a maximal decrease of GAPDH activity in the KDaAlert assay (Ambion) post transfection of a GAPDH siRNA versus its equivalent control as well as minimal toxicity based on equivalent GAPDH protein activity between control oligonucleotide and untransfected cells and minimal visual toxicity.

The optimal transfection conditions for each cell line were used to deliver Pre-miRs or Anti-miRs. The cell lines HepG2, 22Rv1, and SK-N-AS were transfected in triplicate using 0.1 μ l of Lipofectamine-2000 and BJ were transfected with 0.2 μ l of Lipofectamine 2000 using the reverse transfection protocol [50]. In brief, 3 picomoles of pre-miR, anti-miR, or siRNA control (Ambion) were pipetted in triplicate for each cell line into a 96-well tissue culture plate. Lipofectamine 2000 was diluted into 25 μ l volume of OptiMEM (Invitrogen) and incubated for 10 minutes at room temperature. 25 μ l of diluted lipid was added to each well of the tissue culture plate to which the siRNAs had been added. The siRNA/Lipofectamine-2000 mixtures were incubated for 20 minutes at room temperature. 5,000 cells for each cell line listed above in 70 μ l of growth media was added to wells of complexed siRNA. The tissue culture plates were incubated overnight at 37°C and 5% CO₂. The medium was removed and replaced with fresh growth media after 24 hours. GAPDH siRNA, Let7b miRNA, and Pre-miR Negative Control (Ambion) were transfected into each cell line as controls. The samples transfected with GAPDH siRNA were analyzed to confirm the relative experimental transfection efficiency. The samples transfected with let-7b miRNA were analyzed for reduction of nRAS, a gene whose expression has been observed to be regulated by let-7 [28]. The samples were harvested at 72 hours post transfection and analyzed for predicted effects.

Post-transfection RNA isolation and qRT-PCR of mRNA expression

Following transfection of miRNA into the proper cell type, RNA was isolated 24 and 72 hours post-transfection using the MagMAX-96 Total RNA Isolation Kit (Ambion, Inc). 11 μ l of RNA was converted to cDNA using the cDNA synthesis protocol described above. Note that a 1:2 dilution of the cDNA in water was made prior to use. Transfected miRNA effects on endogenous mRNA levels were calculated relative to a

negative control transfected sample. GAPDH mRNA levels were used as an endogenous control to normalize for variances in template input and the presence of contaminants. The comparative Ct method was used to generate fold change in the treated samples relative to the negative control sample. $\Delta\Delta$ Ct values were converted to a percentage wherein the negative control represents 100% expression.

RESULTS

Altered expression of microRNAs in AD hippocampus and medial frontal gyrus

Small RNAs were isolated from age matched hippocampus, medial frontal gyrus, and cerebellum samples from twenty-seven subjects; two Braak stage 0, five Braak stage 1, and five each Braak stage 3,4,5 and 6 (Table 1). MicroRNAs from Sanger release 7.0 and six sno-RNAs were quantified by a customized version of Applied Biosystems' 48-plex RT followed by real-time quantitative PCR assays (assay details in Supplementary Table 1). For statistical analysis data were grouped by tissue region and subject Braak stage. The control group was five Braak 1 and two Braak 0 samples. Two AD groups were obtained by grouping Braak stages 5 with 6 (hereafter called B5,6) and 3 with 4 (called B3,4) (Supplementary Table 2). ANOVA comparison of the B5,6 and B3,4 groups with the control group revealed: 1) a greater number of significant results than would be expected by chance; 2) an even distribution of under- and over-expressed microRNAs; 3) a low standard error overall as expected except at the lowest abundances; and 4) coincident changes in expression for each brain region in isolation, and all combinations thereof. The distribution of significant findings was greatest in hippocampus in B3,4 as expected for the early stages of AD.

As hippocampus and medial frontal gyrus are regions primarily affected by AD pathology, the data were queried for miRNAs whose expression was significantly ($p < 0.05$) altered in both regions as well as either or both regions in the early (B3,4) and late (B5,6) stages of the disease. Expression of twelve miRNAs (miR-200c, -212, -26a, -27a, -30c, -30e-5p, -34a, -381, -422a, -423, -9, -92) was altered in both hippocampus and medial frontal gyrus in either B3,4 or B5,6 groups (Fig. 1A). Expression of nine miRNAs (miR-100, -125b, -132, -145, -146b, -148a, -210, -27b, -425) was shared between B3,4 and B5,6 in either or both hippocampus

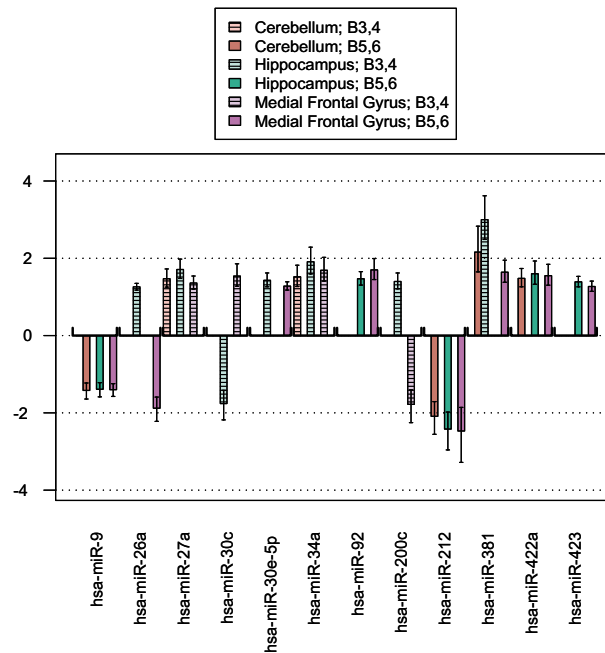


Fig. 1. (A) Directional fold changes for miRNAs altered in both hippocampus and medial frontal gyrus. MiRNA changes in cerebellum shown for reference. All data shown is significant ($p < 0.05$).

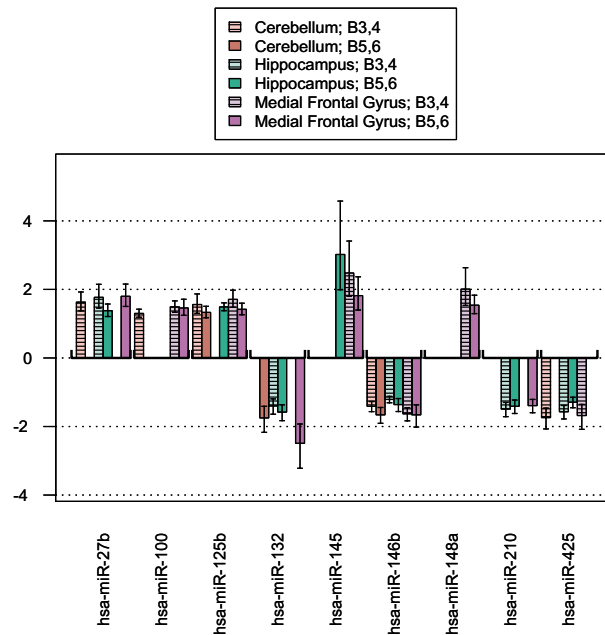


Fig. 1. (B) Directional fold changes for miRNAs altered in both B3,4 and B5,6 groups in either hippocampus, medial frontal gyrus, or both brain regions. MiRNA changes in cerebellum shown for reference. All data shown is significant ($p < 0.05$).

and medial frontal gyrus (Fig. 1B). Of the 21 total miRNAs, nine (miR-145, -148a, -200c, -210, -26a, -30c, -30e-5p, -423, and -92) were significantly changed in hippocampus and/or medial frontal gyrus but not in

cerebellum. Of the 12 miRNAs with additional significant alterations in cerebellum (miR-100, -125b, -132, -146b, -212, -27a, -27b, -34a, -381, -422a, -425, -9) all showed directional concordance between the signif-

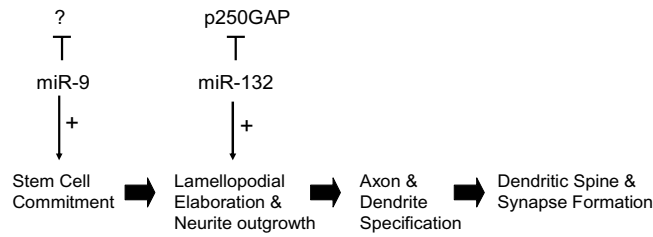


Fig. 2. MiR-9 and miR-132 roles and targets in neuronal life cycle. p250GAP=p250 GTPase activating protein.

icant brain regions. Only miR-146b was consistently altered in both hippocampus and medial frontal gyrus in both the B3,4 and B5,6 stages of the disease. MiR-146b may be a negative regulator of innate immunity and its downregulation in AD brain provides support for an induction of Toll receptor signaling in AD [62].

MiRNAs important in neurogenesis and neuronal differentiation are altered

MiRNA expression can change during the highly staged events that characterize neurogenesis, morphogenesis and differentiation in culture [35]. Expression of two of the miRNAs currently known to regulate these processes, miR-9 and miR-132, were down-regulated in hippocampus and medial frontal gyrus in our filtered data set (Fig. 1A and 1B respectively) [37,57]. Expression of miR-9 is necessary for neurogenesis in cultured stem cells and its downregulated expression in presenilin-1 knockout mice correlates with premature cortical differentiation during embryogenesis [36,37, 57]. MiR-132, an inhibitor of p250GAP translation, is consistently down in hippocampus, a site of neurogenesis in the adult [67]. p250GAP has been characterized as having important roles in the process of neurite extension and neurogenesis. Consistent with these observations miR-132 has been additionally linked to the regulation of MeCP2 and BDNF expression [34]. Figure 2 depicts how the deregulated expression of these miRNAs in AD could impact their key targets in neurogenesis and neuronal differentiation.

MiRNAs mapped to metabolic pathways are altered

In order to assess pathways that may be affected by the deregulated expression of miRNAs in AD, we used mRNA binding predictions at the intersection of the MIRANDA and RNAhybrid methods and developed a pathway enrichment method employing hypergeometric enrichment [29] to assess the probability of a

pathway being coordinately regulated by the AD brain miRNAs [68].

A set of up-regulated miRNAs that suggested coordinate down-regulation of multiple genes and vice versa was suggested by the analysis. Amongst the up-regulated miRNAs included genes linked to amyloid/tau pathway as well as a cluster of metabolic pathways including insulin signaling, glycolysis, and glycogen metabolism. Since the overall impact of the miRNA pathway enrichment was not supportive of an enhancement of amyloidosis and tau hyperphosphorylation we did not pursue validation of these miRNA to target relationships although we note that specific miRNAs predicted to regulate BACE1 and CDK5 could contribute to pathogenesis (data not shown).

Since the pathway enrichment for insulin signaling, glycolysis, and glycogen metabolism were directionally consistent and the recent literature supports a role for altered metabolism in AD pathogenesis we focused instead on establishing whether a subset of the metabolic mRNAs could affect expression of their predicted targets. Since an evaluation of individual protein targets would require antibody validation and the tissue reagents were limited we decided to investigate miRNA modulation of mRNA levels in cells. Some miRNAs have been shown to effect cleavage of their mRNA targets [52] and changes in mRNA expression have been used to elucidate pathways regulated by miRNAs [18, 42]. To evaluate miRNAs that could target mRNA levels, we transfected cultured cells with synthetic (Pre-miR) and antisense (Anti-miR) molecules for a select set of 20 miRNAs, including the metabolic set (Ambion Inc) (Supplementary Table 3). We measured the levels of 20 target mRNAs by qRT-PCR in cells transfected with Pre-miRs and Anti-miRs. We used four different cell lines for the studies and selected the cell lines for each miRNA and target mRNA based upon the endogenous levels of the RNAs in the various cell lines (see Materials and Methods). The introduction of four different synthetic miRNAs (miR-193a, -98, -32, and -423) elicited statistically significant decreases

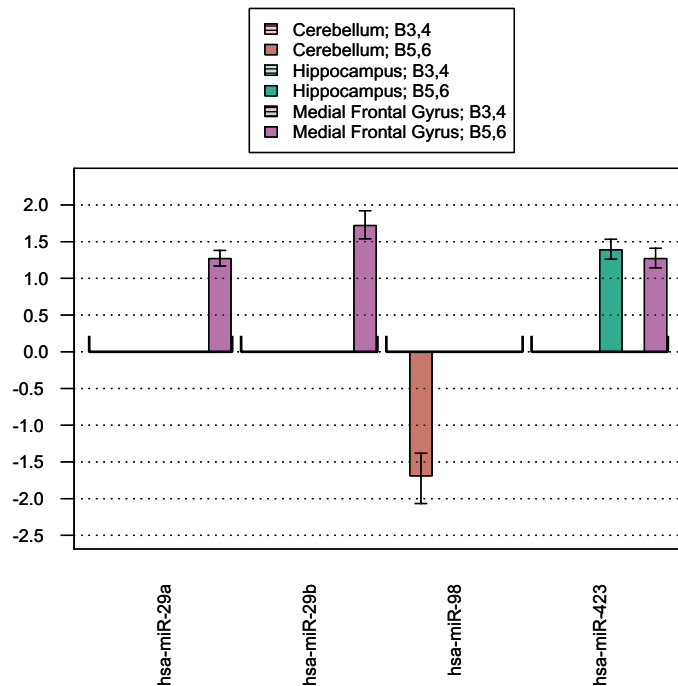


Fig. 3. Directional fold changes for miRNAs relevant to metabolism. All data shown is significant ($p < 0.05$).

es in target mRNA expression in transfected cells (Table 3). Transfection of anti-sense versions of the same miRNAs failed to elicit a corresponding increase in the expression levels of the same mRNA targets. While disappointing, this is typical of in vitro experiments where inhibitors of endogenous miRNAs tend to have less of an effect than transfection of synthetic miRNAs (data not shown). Of the target relationships, isocitrate dehydrogenase regulation is most interesting due to opposing expression and putative impact of its regulatory miRNAs; upregulated miR-423 in hippocampus and medial frontal gyrus suggests downregulation of IDH2 while decreased miR-98 expression in cerebellum, the region least affected by pathology, suggests the converse (Fig. 3).

Additional support that AD altered miRNAs may affect metabolism is seen with the increased expression of miR-29a and miR-29b in B5,6 medial frontal gyrus (Fig. 3) and miR-145 in B5,6 hippocampus and B3-6 medial frontal gyrus (Fig. 1B). MiR-29 was recently shown to be induced in cellular and animals models of insulin resistance and translationally regulates a subunit of the branched chain ketoacid dehydrogenase with potential consequences for glutamate homeostasis [21, 25,30,47,72]. In addition, miR-145 has been shown to translationally inhibit insulin receptor substrate 1 and IRS-1 in turn has been linked to various forms of insulin resistance [58,60,70,71].

MiRNA expression is altered in Alzheimer's CSF

Since miRNA expression appears to be more stable than mRNAs and can be detected in human fluids [31] we set out to examine whether miRNAs were: 1) detectable in cerebrospinal fluid; and 2) were differentially expressed between AD and non-affected patients. MiRNAs were isolated from frozen cerebrospinal fluid from 10 Braak 5 and 10 Braak 1 patients, none of whom were used for the AD brain tissue study. In general the RNA yields were low and variable between patients (Table 2). To detect miRNAs in CSF we employed a sensitive pre-amplification procedure that permitted the detection of 201 out of the 242 miRNAs tested. We observed an even distribution of under- and over-expressed microRNAs and a low standard error overall as expected except at the lowest abundances. Two outliers from each set of samples were removed based on Correlation Analysis and Between Group Analysis (BGA). Two of the excluded samples were among the three that were observed to be slightly red in color, suggesting their exclusion due a contribution from contaminating blood cells. *t*-test analysis confirmed significant differences in miRNA populations without substantial change in the significant results before and after outlier removal. Sixty miRNAs were detected as significantly different ($p < 0.05$) between the Braak

stage 5 and Braak stage 1 samples. The distribution of fold changes was evenly distributed in both up and down directions (Table 4). Notably all of the members of the miR-30 family were induced although relevant targets have not been described. Applying the pathway enrichment algorithm to this dataset suggested up-regulation of genes in T cell signaling and inflammation [68]. While the target relationships had not been confirmed for most miRNAs in the enrichment pathways, we did observe that miRNAs linked to immune cell functions including innate immunity (e.g. miR-146b) [62] and T cell activation and differentiation (e.g., miR-181a, miR-142) (Table 4) [10,40,49] were frequently represented. These results show that miRNAs can be recovered from frozen CSF and their expression can distinguish Braak 5 AD from Braak 1 patients.

DISCUSSION

This paper provides the first genome scale description of miRNAs that are differentially expressed in AD brain and importantly further demonstrates their presence and disease-altered expression in cerebrospinal fluid. Through experimental follow up and literature mining of miRNA targets we could establish that the identified miRNAs are useful biomarkers of known and novel pathways contributing to AD pathogenesis. The quantitative changes in expression we have seen in CSF provide initial hope that miRNAs could once published and independently replicated provide accessible biomarkers to aid diagnosis of AD.

We could identify many miRNAs whose expression was altered both early in disease as well as in the regions of the brain most affected by AD pathology. While cause and effect are difficult to establish our observation that many miRNAs were additionally altered in cerebellum suggests expression changes were not merely consequences of pathology (since amyloid plaque pathology is limited in this region). The misregulated expression patterns of miR-9 and miR-132 could explain some features of the alterations in stem cell commitment, neuronal differentiation and actin remodeling seen in AD. While neurogenesis is most important in the developing brain, the process continues in the adult hippocampus [24]. Interestingly, an increased expression of early neuronal markers in the hippocampus of AD patients has been attributed to increased neurogenesis although it is unknown whether the cells expressing these markers go on to become mature neurons [24,

26]. MiR-9, which is downregulated in B5,6 AD brain, is necessary for the differentiation of stem cells to neurons in cell culture [37]. MiR-9's role in neuronal differentiation is mediated in part by phosphorylation of TYR705 on STAT3 although the direct target of miR-9 is unknown. Decreased miR-9 expression is also associated with premature cortical differentiation during the embryonic development of presenilin-1 knock out mice [36]. If miR-9 has a critical role in neurogenesis in vivo our data would suggest that functional deficits may occur in the later stages of the disease.

MiR-132 is an activator of neuronal process outgrowth which translationally inhibits the p250GAP protein and has an emerging role in regulating BDNF and MeCP2 expression [34,67]. MiR-132's decreased expression in B3,4 hippocampus as well as B5,6 hippocampus and medial frontal gyrus suggests deficits in neuronal differentiation even in the earliest stages of the disease. Future exploration of the miRNAs altered in hippocampus and medial frontal gyrus (Fig. 1) should reveal other miRNAs that have roles in neuronal life cycle decisions and progression and pathology of AD.

The miRNA changes observed here are likely to be causes as well as consequences of the complex signal cascades leading to and resulting from formation of $A\beta_{1-42}$ and hyperphosphorylated tau [51]. For example, $A\beta_{1-42}$ is a weak activator of TOLL-receptor (TLR) signalling [43] but its effect on miR-132 and miR-146b needs to be tested since LPS, a strong TLR activator, induces these miRNAs in some non-neuronal cell types but not others [48,62]. In fact, the down-regulation of miR-146b observed here lends support to an innate immunity hypothesis of AD pathogenesis. TLR signaling is implicated in activation of microglia that aid in the phagocytic clearance of $A\beta$ fibrils as well as increased neurodegeneration in animal models of AD [33,39,63]. TLRs are expressed in human AD brain and bacterial infection can exacerbate the time to onset of Alzheimer's disease however there is little direct evidence that TLR pathways are upregulated in AD [7,16]. Mir-146b has an important role in limiting TLR signaling by repressing translation of the downstream effectors IRAK1 and TRAF6 [62]. The down-regulated expression of miR-146b in AD brain should relieve translational repression of TLR signaling and provides support that the exacerbation of innate immunity through TLR signaling could contribute to neurodegeneration [39,62].

The targets of miRNAs shown here as well as in the literature support recent findings of metabolic deficiencies in AD and suggest novel genes and pathways

Table 4

Directional fold changes for miRNAs altered in CSF. Data shown is significant ($p < 0.05$). Expression abundances less than 40 are excluded

miRNA name	Control mean	Group mean	Directional fold change	Control replicates	Group replicates	t-test Pvalue	Std error
hsa-let-7f	7663.96	16264.75	2.12	7	7	4.33E-02	0.48
hsa-miR-105	8553.03	21377.37	2.50	9	7	3.66E-05	0.22
hsa-miR-10a	13108.96	4902.92	-2.67	9	7	0.014398	0.51
hsa-miR-10b	1992.28	960.16	-2.07	9	6	0.033059	0.44
hsa-miR-125a	714011.81	1346274.28	1.89	9	7	0.024531	0.36
hsa-miR-126	156036.85	83587.17	-1.87	9	7	0.02404	0.36
hsa-miR-126*	37753.62	22628.86	-1.67	9	7	0.015319	0.27
hsa-miR-127	214022.02	96778.98	-2.21	9	7	0.047424	0.53
hsa-miR-135a	92375.45	209350.05	2.27	9	7	0.049007	0.55
hsa-miR-138	2711.95	23372.99	8.62	7	2	0.004242	0.75
hsa-miR-141	70.62	182.83	2.59	8	6	0.006058	0.41
hsa-miR-142-5p	18180.09	6426.71	-2.83	9	7	0.044229	0.68
hsa-miR-143	3685.62	1319.53	-2.79	9	6	0.003901	0.42
hsa-miR-146b	102488.11	62347.63	-1.64	9	6	0.027952	0.29
hsa-miR-151	41327.98	73243.95	1.77	9	7	0.00048	0.18
hsa-miR-154	667.10	119.78	-5.57	8	5	0.008535	0.78
hsa-miR-15b	19020.18	5392.72	-3.53	8	7	0.0034	0.51
hsa-miR-181a	31419.34	12271.77	-2.56	9	7	0.042235	0.61
hsa-miR-181c	3975.13	2335.45	-1.70	9	7	0.011449	0.26
hsa-miR-186	66153.66	250975.69	3.79	9	7	0.022184	0.75
hsa-miR-191	452777.91	1366051.74	3.02	9	7	0.004734	0.48
hsa-miR-194	4857.76	2292.67	-2.12	9	7	0.017115	0.40
hsa-miR-195	291373.88	175686.17	-1.66	9	7	0.048921	0.34
hsa-miR-197	89519.01	208382.64	2.33	9	7	0.003468	0.35
hsa-miR-199a*	315824.65	72724.57	-4.34	9	7	0.009408	0.70
hsa-miR-204	52619342.77	300198156.95	5.71	9	7	0.005363	0.76
hsa-miR-205	111.44	287.83	2.58	8	5	0.043015	0.60
hsa-miR-214	18947.27	7759.53	-2.44	9	7	0.046434	0.59
hsa-miR-216	524.91	1652.52	3.15	8	7	0.024177	0.65
hsa-miR-221	26698.22	5359.28	-4.98	9	7	0.001908	0.61
hsa-miR-302b	43.76	158.85	3.63	8	6	0.016914	0.67
hsa-miR-30a-3p	413769.76	879601.20	2.13	9	7	0.036149	0.47
hsa-miR-30a-5p	1629245.30	4992433.01	3.06	9	7	0.016695	0.59
hsa-miR-30b	708987.21	2483172.97	3.50	9	7	0.00047	0.40
hsa-miR-30c	1183823.02	4455795.87	3.76	9	7	0.000281	0.40
hsa-miR-30d	221565.21	771805.04	3.48	9	7	0.013293	0.64
hsa-miR-32	21981.33	49029.27	2.23	9	7	0.029911	0.48
hsa-miR-338	22264.00	10682.01	-2.08	9	7	0.044754	0.48
hsa-miR-345	6310.17	19894.35	3.15	9	7	0.008013	0.54
hsa-miR-362	1121.76	2093.24	1.87	9	7	0.032303	0.38
hsa-miR-371	59.86	328.50	5.49	6	7	0.006945	0.74
hsa-miR-374	18327.35	35675.59	1.95	9	7	0.02724	0.39
hsa-miR-375	14633.72	52779.31	3.61	9	7	0.000816	0.44
hsa-miR-380-3p	61.88	182.84	2.95	9	4	0.039901	0.67
hsa-miR-422b	2870.63	1384.42	-2.07	9	7	0.039726	0.46
hsa-miR-429	231.17	476.89	2.06	9	7	0.049168	0.49
hsa-miR-448	3878.16	11028.75	2.84	9	7	0.015653	0.55
hsa-miR-449	923.81	4920.67	5.33	9	7	0.037863	1.05
hsa-miR-451	104850.04	6869.91	-15.30	9	6	0.010945	1.33
hsa-miR-455	2344.27	1371.68	-1.71	9	7	0.040107	0.34
hsa-miR-494	73.57	433.44	5.89	2	1	0.039138	0.16
hsa-miR-497	23572.28	12554.83	-1.88	9	7	1.55E-02	0.33
hsa-miR-501	803.45	1311.71	1.63	8	7	0.029021	0.29
hsa-miR-517a	430.19	2286.90	5.32	6	6	0.01878	0.86
hsa-miR-517b	480.84	2500.66	5.20	9	6	0.009039	0.78
hsa-miR-518b	178.46	1079.97	6.05	8	7	0.013889	0.91
hsa-miR-518f	66.79	290.21	4.35	7	7	0.032671	0.88
hsa-miR-520a*	45.72	166.05	3.63	8	6	0.03241	0.77
hsa-miR-526a	310.91	1560.38	5.02	9	7	0.009615	0.78
hsa-miR-99a	1967017.19	1216174.16	-1.62	9	7	0.033709	0.29

to explore. Recent studies have reported an association between diabetes and the later onset of AD [3, 12]. Regulation of brain insulin levels and the loss of insulin receptor signaling molecules have been linked to amyloid-mediated pathogenesis as well as memory [60,70]. IRS-1 is a key insulin receptor signaling molecule whose loss of expression is linked to insulin resistance [71]. IRS-1 expression is decreased in AD and is regulated during memory tasks in rats [70]. MIR-145 has recently been shown to translationally inhibit IRS-1 and miR-145's increased expression in the memory forming regions of AD patients could be an additional mechanism leading to decreased IRS-1 expression in AD [58,71]. Of further relevance to brain insulin signaling is our finding that miR-29a and -29b are increased in B5,6 medial frontal gyrus since miR-29 is induced in models of insulin resistance and overexpression of miR-29 decreases uptake of glucose and insulin [25]. MiR-29b has also been convincingly shown to inhibit translation of the dihydrolipoyl branched chain acyltransferase (DBT) [47]. DBT is a subunit of the branched chain alpha-ketoacid dehydrogenase (BCKD) that is the last committed step in degradation of branched chain amino acids. Branched chain amino acids are essential nitrogen donors for glutamate synthesis. Despite the accumulation of branched chain amino acids and ketoacids, patients with BCKD deficiencies become glutamate deficient and experience neurologic deterioration [72]. Branched chain alpha keto-acids prevent the uptake of glutamate in rat cerebral cortex slices and cause neurotoxicity in cell culture suggesting a possible role in glutamate excitotoxicity [21,30]. Perhaps BCKD becomes deficient in AD brain due to the induction of miR-29 and plays a contributory role in pathways leading to excitotoxic neuronal cell death. In addition, our finding that IDH2 can be regulated by miR-423 and miR-98 agrees with reports that isocitrate dehydrogenase enzyme activity is decreased in AD prefrontal cortex and suggests a mechanism for the relative sparing of the cerebellum [8]. Isocitrate dehydrogenase 2 is an important enzyme in the control of mitochondrial redox balance and its decreased expression could be an important contributor to the oxidative stress that is observed in AD [27, 41]. Dehydrogenase mRNAs in general well as other mitochondrial enzymes are decreased in AD although IDH2 and BCKD have not been directly examined [2, 6]. MiRNAs-29, 423, 98, and 145 appear to be useful biomarkers for insulin resistance, glutamate and redox homeostasis and their targets, IDH2 and BCKD, provide testable hypotheses with respect to their expression in Alzheimer's disease brain.

While this paper was being prepared the altered expression of a limited set of miRNAs in AD hippocampus was reported [44]. This paper only measured 13 miRNAs of relevance to brain development and some individual results differ from our study. Possible explanations for these differences may be in the samples themselves, the differences in the sensitivity and specificities of the technologies employed, or the normalization methods used. In our experience using column purified small RNA samples, putative small RNA housekeeping genes demonstrate significant expression variation between samples that could influence data normalization (see snoRNAs in Supplementary Table 2; 5S and U6 data not shown). Our method for normalization relies on identifying the least variant miRNAs for a given set of samples and using a normalization adjustment to scale expression to the least variant set. Also, because of high sequence similarity, microRNAs within the same family are more difficult to distinguish by oligonucleotide hybridization than by the stem-loop RT primer used in our detection system.

This paper provides the first indication that miRNA levels in CSF are affected by AD. Whether miRNAs are present in CSF as a result of cell destruction, production of exosomes [65], or even transport mechanisms, as seen in plants, is unknown. While some miRNAs are expressed at high levels in the brain (e.g., miR-181) or are highly enriched in the choroid plexus at the interface between blood and CSF (e.g., miR-204) [14], there was no obvious relationship between the miRNAs altered in CSF and the absolute levels in sites of AD mediated destruction or the directional changes in those regions. We propose that the miRNAs detected in these frozen CSF samples are derived from immune cells in the CSF. In normal CSF, ~ 80% of resident leukocytes are blood-derived CD4+ memory T cells with the remainder comprised largely of monocytes and dendritic cells [5,53]. The trafficking of these immune cells from blood and local lymph nodes into CSF and ultimately brain is important in immunosurveillance. In acute inflammatory diseases such as multiple sclerosis, an influx of B cells or monocytes into CSF can be measured although total cell counts are not altered in AD. The pathway enrichment algorithm predicts a downregulation of miRNAs in AD CSF involved in T lymphocyte signaling and inflammatory pathways [68]. The mere fact that many of these immune-related miRNAs are coordinately decreased in AD supports a deregulation in inflammatory signaling rather than an influx of inflammatory cells. For example, miR-146b was down-regulated in CSF which, similar to brain, sup-

ports an upregulation of innate immunity. Although toll receptor signaling is normally associated with monocytes, T lymphocytes have these pathways as well [45]. MiR-181a and miR-142-5p, which were downregulated in CSF, have roles in lymphocyte lineage determination [10,40,49] while miR-181 has been shown to also affect T cell sensitivity [40] consistent with alterations of these pathways in AD CSF. Further support comes from miR's-191, 221, 142, and 15b which are expressed at specific stages of T lymphocyte development and show either up or down-regulated expression in AD CSF [49]. Interestingly, miR-221 can inhibit c-Kit expression while miR-10a inhibits the Homeobox gene Hox-A1 and both c-Kit and Hox-A1 have roles in hematopoiesis and T cell development [20,22,69].

Cell-specific responses in AD brain and CSF could be both causes and consequences of the disease. Further work is required to characterize those miRNAs that contribute versus those that respond to hyperphosphorylation of Tau or $A\beta_{1-42}$. Certainly miR-29 and miR-145 appear to be biomarkers of insulin resistance while miR-146b helps establish a role for innate immunity in AD pathogenesis. Furthermore IDH2 and BCKD are interesting miRNA targets to interrogate in the future with respect to the brain pathogenesis. Further research will focus on whether the miRNA changes in CSF can be linked to AD specific signaling molecules, and, if any of these changes are specific to AD, can be used to follow the course of AD or overlap with other neurodegenerative diseases involving inflammation. Collectively these data illuminate the potential of miRNAs to provide insights into AD pathogenesis and initial hope that microRNAs could provide accessible biomarkers to aid clinical diagnosis.

ACKNOWLEDGMENTS

This work was supported by GlaxoSmithKline, Inc., with whom many of the authors are employed and/or own equity. The authors would like to thank Dr. R. Ravid, Netherlands Brain Bank, The Netherlands, for arrangement/donation of brain tissue and Christine Hulette of the Kathleen Price Bryan Brain Bank funded by NIA AG05128 for arrangement/donation of cerebrospinal fluid. Thanks also for support from Mark Hurler and Craig Meyer, GlaxoSmithKline, Inc. for training in the scale factor normalization method and Christine Debouck whose vision got us started on miRNA evaluations.

References

- [1] J.F. Abelson, K.Y. Kwan, B.J. O'Roak, D.Y. Baek, A.A. Stillman, T.M. Morgan, C.A. Mathews, D.L. Pauls, M.-R. Rasin, M. Gunel, J.A. Spertus, J.F. Leckman, L.S. Dure, R. Kurlan, H.S. Singer, D.L. Gilbert, A. Farhi, A. Louvi, R.P. Lifton, N. Sestan and M.W. State. Sequence variants in *SLITRK1* are associated with Tourette's syndrome, *Science* **310** (2005), 317–320.
- [2] M.Y. Aksenov, H.M. Tucker, P. Nair, M.V. Aksenova, D.A. Butterfield, E. Estus and W.R. Markesbury. The expression of several mitochondrial and nuclear genes encoding the subunit of electron transport chain complexes, cytochrome c oxidase, and NADH dehydrogenase, in different brain regions in alzheimer's disease, *Neurochem Res* **24** (1999), 767–774.
- [3] J.P. Blass, G.E. Gibson and S. Hoyer, The role of the metabolic lesion in Alzheimer's disease, *J Alzheimers Dis* **4** (2002), 225–232.
- [4] H. Braak and E. Braak, Neuropathological staging of Alzheimer's related changes, *Acta Neuropathol* **82** (2004), 239–259.
- [5] M. Britschgi and T. Wyss-Coray, Immune cells may fend off Alzheimer disease, *Nat Med* **13** (2007), 408–409.
- [6] W.M. Brooks, P.J. Lynch, C.C. Ingle, A. Hatton, P.C. Emson, R.L.M. Faull and M.P. Starkey, Gene expression profiles of metabolic enzyme transcripts in Alzheimer's disease, *Brain Res* **1127** (2006), 127–135.
- [7] M. Bsibsi, R. Ravid, D. Gveric and J.M. van Noort, Broad expression of Toll-like receptors in the human central nervous system, *J Neuropathol Exp Neurol* **61** (2002), 1013–1021.
- [8] P. Bubber, V. Haroutunian, G. Fisch, J.P. Blass and G.E. Gibson, Mitochondrial abnormalities in Alzheimer brain: Mechanistic implications, *Ann Neurol* **57** (2005), 695–703.
- [9] S. Chang, T.R. Ma, D. Miranda, M.E. Balestra, R.W. Mahley and Y. Huang, Lipid- and receptor-binding regions of apolipoprotein E4 fragments act in concert to cause mitochondrial dysfunction and neurotoxicity, *Proc Natl Acad Sci USA* **102** (2005), 18694–18699.
- [10] C.-Z. Chen, L. Li, H.F. Lodish and D.P. Bartel. MicroRNAs modulate hematopoietic lineage differentiation, *Science* **303** (2004), 83–86.
- [11] C. Chen, D.A. Ridzon, A.J. Broomer, Z. Zhou, D.H. Lee, J.T. Nguyen, M. Baribisin, N.L. Xu, V.R. Mahuvakar, M.R. Andersen, K.Q. Lao, K.J. Livak and K.J. Guegler, Real-time quantification of microRNAs by stem-loop RT-PCR, *Nucleic Acids Res* **33** (2005), e179.
- [12] S. Craft, Insulin resistance syndrome and Alzheimer disease: pathophysiologic mechanisms and therapeutic implications, *Alzheimer Dis Assoc Disord* **20** (2006), 298–301.
- [13] A. Culhane, Multivariate analyses of microarray data using Ade4, Bioconductor v2.1, <http://bioconductor.org/packages/bioc/html/made4.html>, Posted 8 October 2007, Accessed January 30, 2008.
- [14] M. Deo, J.-Y. Yu, K.-H., Chung, M. Tippens and D.L. Turner, Detection of mammalian microRNA expression by in situ hybridization with RNA oligonucleotides, *Dev Dynamics* **235** (2006), 2538–2548.
- [15] B. De Strooper, Loss-of-function presenilin mutations in Alzheimer disease. Talking point on the role of presenilin mutations in Alzheimer disease, *EMBO Reports* **8** (2007), 141–146.
- [16] M.D.M. Dunn, M. Mullee, V.H. Perry and C. Holmes, Association between dementia and infectious disease: Evidence from

- a case-control study, *Alzheimer Dis Assoc Disord* **19** (2005), 91–94.
- [17] T. Engel, F. Hernandez, J. Avila and J.J. Lucas, Full reversal of Alzheimer's disease-like phenotype in a mouse model with conditional overexpression of glycogen synthase kinase-3, *J Neurosci* **26** (2006), 5083–5090.
- [18] C. Esau, S. Davis, S.F. Murray, X.X. Yu, S.K. Pandey, M. Pear, L. Watts, S.L. Booten, M. Graham, R. McKay, A. Subramaniam, S. Propp, B.A. Lollo, S. Freier, C.F. Bennett, S. Bhanot and B.P. Monia, miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting, *Cell Metab* **3** (2006), 87–98.
- [19] A. Esquela-Kerscher and F.J. Slack, Oncomirs – microRNAs with a role in cancer, *Nat Rev Cancer* **6** (2006), 259–269.
- [20] N. Felli, L. Fontana, E. Pelosi, R. Botta, D. Bonci, F. Facchiano, F. Liuzzi, V. Lulli, O. Morsilli, S. Santoro, M. Valtieri, G.A. Calin, C.-G. Liu, A. Sorrentino, C.M. Croce and C. Peschle, MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor downmodulation, *Proc Natl Acad Sci USA* **102** (2005), 18081–18086.
- [21] C. Funchal, A.M. Rosa, M. Wajner, S. Wofchuk and R.P. Pureur, Reduction of glutamate uptake into cerebral cortex of developing rats by the branched-chain alpha-keto acids accumulating in maple syrup urine disease, *Neurochem Res* **29** (2004), 747–753.
- [22] R. Garzon, F. Pichiorri, T. Palumbo, R. Iuliano, A. Cimmino, R. Aqeilan, S. Volnia, D. Bhatt, H. Alder, G. Marcucci, G.A. Calin, C.-G. Liu, C.D. Bloomfield, M. Andreeff and C.M. Croce, MicroRNA fingerprints during human megakaryocytopoiesis, *Proc Natl Acad Sci USA* **103** (2006), 5078–5083.
- [23] M. Goedert and G. Spillantani, A century of Alzheimer's disease, *Science* **314** (2006), 777–781.
- [24] A.F. Hallbergson, C. Gnatenco and D.A. Peterson, Neurogenesis and brain injury: managing a renewable resource for repair, *J Clin Invest* **112** (2003), 1128–1133.
- [25] A. He, L. Zhu, N. Gupta, Y. Chang and F. Fang, Overexpression of miR-29, highly upregulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes, *Mol Endocrinol* **21** (2007), 2785–2794, doi:10.1210/me.2007.0167.
- [26] K. Jin, A.L. Peel, X.O. Mao, L. Xie, B.A. Cottrell, D.C. Henshall and D.A. Greenberg, Increased hippocampal neurogenesis in Alzheimer's disease. *Proc Natl Acad Sci USA* **101** (2004), 343–347.
- [27] S.-H. Jo, M.-K. Son H.-J. Koh, S.-M. Lee, I.-H. Song, Y.-O. Kim, Y.-S. Lee, K.-S., Jeong, W.B. Kim, J.-W. Park, B.-J. Song and T.-L. Huh, Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial NADP⁺-dependent isocitrate dehydrogenase (IDPm), *J Biol Chem* **276** (2001), 16168–16176.
- [28] S.M. Johnson, H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K.L. Reinert, D. Brown and F.J. Slack, RAS is regulated by the let-7 microRNA family, *Cell* **120** (2005), 635–647.
- [29] N. Johnson, A. Kemp, A. and S. Kotz, *Univariate Discrete Distributions*, Second Edition, John Wiley and Sons, New York, 1992.
- [30] P. Jouvet, P. Rustin, D.L. Taylor, J.M. Pocock, U. Felderhoof-Mueser, N.D. Mazarakis, C. Sarrat, U. Joashi, M. Kozma, K. Greenwood, A.D. Edwards and H. Mehmet, 2000, Branched chain amino acids induce apoptosis in neural cells without mitochondrial membrane depolarization or cytochrome c release: Implications for neurological impairment associated with maple syrup urine disease, *Mol Biol Cell* **11** (2005), 1919–1932.
- [31] J. Kemppainen, J. Shelton, K. Kelnar, S. Volz, H. Peltier, A. Szafranska, D. Ovcharenko, T. Illmer, M. Labourier, G. Latham and D. Brown, microRNAs as biomarkers in blood and other biofluids, <http://www.asuragen.com/pdfs/posters/biomarkers.pdf>, Accessed January 30, 2008.
- [32] J. Kim, K. Inoue, J. Ishii, W.B. Vanti, S.V. Voronov, E. Murchison, G. Hannon and A. Abeliovich, A microRNA feedback circuit in midbrain dopamine neurons, *Science* **317** (2007), 1220–1224.
- [33] M. Kitazawa, S. Oddo, T.R. Yamasaki, K.M. Green and F.M. LaFerla, Lipopolysaccharide-induced inflammation exacerbates Tau Pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease, *J Neurosci* **25** (2005), 8843–8853.
- [34] M.E. Klein, D.T. Liou, L. Ma, S. Impey, G. Mandel and R.H. Goodman, Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA, *Nat Neurosci* (2007), doi:10.1038/nn2010.
- [35] K.S. Kosik and A.M. Krichevsky, The Elegance of the MicroRNAs: A Neuronal Perspective, *Neuron* **47** (2005), 779–782.
- [36] A.M. Krichevsky, K.S. King, C.P. Donahue, K. Khrapko and K.S. Kosik, A microRNA array reveals extensive regulation of microRNAs during brain development, *RNA-A Publication of the Rna Society* **9** (2003), 1274–1281.
- [37] A.M. Krichevsky, K.-C. Sonntag, O. Isacson and K. Kosik, Specific microRNAs modulate embryonic stem cell-derived neurogenesis, *Stem Cells* **24** (2006), 857–864.
- [38] K. Lao, K.L. Xu, V. Yeung, C. Chen, K.J. Livak and N.A. Strauss, Multiplexing RT-PCR for the detection of multiple miRNA species in small samples, *Biochem Biophys Res Commun* **343** (2006), 85–89.
- [39] S. Lenhardt, L. Massilon, P. Follett, F.E. Jensen, R. Ratan, P.A. Rosenberg, J.J. Volpe and T. Vartanian, Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway, *Proc Natl Acad Sci USA* **100** (2003), 8514–8519.
- [40] Q.-J. Li, J. Chau, P.J.R. Ebert, G. Sylvester, H. Min, G. Liu, R. Braich, J. Manoharan, J. Soutschek, P. Skare, L.O. Klein, M.M. Davis and C.-Z. Chen, miR-181a is an intrinsic modulator of T cell sensitivity and selection, *Cell* **129** (2007), 147–161.
- [41] M.T. Lin and M.F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, *Nature* **443** (2006), 787–795.
- [42] P. Linsley, J. Schelter, J. Burchard, M. Kibukawa, M.M. Martin, S.R. Bartz, J.M. Johnson, J.M. Cummins, C.K. Raymond, H. Dai, N. Chau, M. Cleary, A. Jackson, M. Carleton and L. Lim, Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression, *Mol Cell Biol* **27** (2007), 2240–2252.
- [43] M. Lotz, S. Ebert, H. Esselmann, A.I. Iliev, M. Prinz, N. Wiazewicz, J. Wiltfang, J. Gerber and R. Nau, Amyloid beta peptide 1-40 enhances the action of Toll-like receptor-2 and -4 agonists but antagonizes Toll-like receptor-9-induced inflammation in primary mouse microglial cell cultures, *J Neurochem* **94** (2005), 289–298.
- [44] W. Lukiw, Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus, *Mol Neurosci* **18** (2007), 297–300.

- [45] H. MacLeod and L.M. Wetzler, T cell activation by TLRs: A role to TLRs in the adaptive immune response, *Science STKE* (2007), **402**, pe48.
- [46] J. Marx, Alzheimer's disease: A new take on tau, *Science* **316** (2007), 1416–1417.
- [47] B.D. Mersey, P. Jin and D.J. Danner, Human microRNA (miR29b) expression controls the amount of branched chain a-ketoacid dehydrogenase complex in a cell, *Hum Mol Genet* **14** (2005), 3371–3377.
- [48] S.A. Moschos, A.E. Williams, M.M. Perry, M.A. Birrell, M.G. Belvisi and M.A. Lindsay, Expression profiling *in vivo* demonstrates rapid changes in lung microRNA levels following lipopolysaccharide-induced inflammation but not in the anti-inflammatory action of glucocorticoids, *BMC Genomics* **8** (2007), 240.
- [49] J.R. Neilson, G.X. Zheng, C.B. Burge and P.A. Sharp, Dynamic regulation of miRNA expression in ordered stages of cellular development, *Genes Dev* **21** (2007), 578–589.
- [50] D. Ovcharenko, S. Hunicke-Smith, K. Kelnar, D. Brown and R. Jarvis, High-throughput RNAi screening *in vitro*: From cell lines to primary cells, *RNA* **11** (2005), 985–993.
- [51] G. Pigino, A. Pelsman, H. Mori and J. Busciglio, Presenilin-1 mutations reduce cytoskeletal association, deregulate neurite growth, and potentiate neuronal dystrophy and tau phosphorylation, *J Neurosci* **21** (2001), 834–842.
- [52] R.S. Pillai, MicroRNA function: multiple mechanisms for a tiny RNA? *RNA-A Publication of the Rna Society* **11** (2005), 1753–1761.
- [53] N. Rebenko-Moll, L. Liu, A. Cardon and R. Ransohoff, Chemokines, mononuclear cells and the nervous system: heaven (or hell) is in the details, *Curr Opin Immunol* **18** (2006), 683–689.
- [54] E.M. Reiman, K. Chen, G.E. Alexander, R.J. Caselli, D. Bandy, D. Osborne, A.M. Saunders and J. Hardy, Correlations between apolipoprotein E epsilon4 gene dose and brain-imaging measurements of regional hypometabolism, *Proc Natl Acad Sci USA* **102** (2005), 8299–8302.
- [55] A.M. Saunders, W.J. Strittmatter, D. Schmechel, P.H. George-Hyslop, M.A. Pericak-Vance, S.H. Joo, B.L. Rosi, J.F. Gusella, D.R. Crapper-MacLachlan and M.J. Alberts, Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease, *Neurology* **43** (1993), 1467–1472.
- [56] G.M. Schratt, F. Tuebing, E.A. Nigh, C.G. Kane, M.E. Sabatini, M. Kiebler and M.E. Greenberg, A brain-specific microRNA regulates dendritic spine development, *Nature* **439** (2006), 283–289.
- [57] L.F. Sempere, S. Freemantle, I. Pitha-Rowe, E. Moss, E. Dmitrovsky and V. Ambros, Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation, *Genome Biol* **5** (2004), R13.1–R13.11.
- [58] B. Shi, L. Sepp-Lorenzino, M. Prisco, P. Linsley, T. deAngelis and R. Baserga, MicroRNA 145 targets the insulin receptor substrate-1 and inhibits the growth of colon cancer cells, *J Biol Chem* **282** (2007), 32582–32590.
- [59] P. Sood, A. Kreck, M. Zavolan, G. Macino and N. Rajewsky, Cell-type-specific signatures of microRNAs on target mRNA expression, *Proc Natl Acad Sci USA* **103** (2006), 2746–2751.
- [60] E. Steen, B.M. Terry, E.J. Rivera, J.L. Cannon, T.R. Neely, R. Tavares, X.J. Xu, J.R. Wands and S.M. de la Monte, Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease – is this type 3 diabetes? *J Alzheimers Dis* **7** (2005), 63–80.
- [61] J. Strum, R. Shehee, D. Virley, J. Richardson, M. Mattie, P. Selley, S. Ghosh, C. Nock, A. Saunders and A. Roses, Rosiglitazone induces mitochondrial biogenesis in mouse brain, *J Alzheimers Dis* **11** (2007), 45–51.
- [62] K.D. Taganov, M.P. Boldin, K.-J. Chang and D. Baltimore, NF- κ B-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses, *Proc Natl Acad Sci USA* **103** (2006), 12481–12486.
- [63] K. Tahara, H.-D. Kim, J.-J. Jin, J.A. Maxwell, L. Li and F. Ken-ichiro, Role of toll-like receptor signaling in A β uptake and clearance, *Brain* **129**(2006), 3006–3019.
- [64] F. Tang, P. Hajkova, S. Barton, K. Lao and M. Surani, MicroRNA expression profiling of single embryonic stem cells, *Nucleic Acids Res* **34** (2006), e9.
- [65] H. Valadi, K. Ekstrom, A. Bossios, J. Sjostrand, J.J. Lee and J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, *Nat Cell Biol* **9** (2007), 1–6.
- [66] M.A. Valencia-Sanchez, J. Liu, G.J. Hannon and R. Parker, Control of translation and mRNA degradation by miRNAs and siRNAs, *Genes Dev* **20** (2006), 515–524.
- [67] N. Vo, M.E. Klein, O. Varlamova, D.M. Keller, T. Yamamoto, R.H. Goodman and S. Impey, A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis, *Proc Natl Acad Sci USA* **102** (2005), 16426–16431.
- [68] J. Ward, miRNAPath: Analysis of miRNA pathway enrichment, Bioconductor v2.1, <http://bioconductor.org/packages/bioc/html/miRNAPath.html>, Accessed January 30, 2008.
- [69] C. Waskow and H.R. Rodewald, Lymphocyte development in neonatal and adult c-Kit-deficient (c-Kit^{W/W}) mice, *Adv Exp Med Biol* **512** (2002), 1–10.
- [70] G.S. Watson and S. Craft, Insulin resistance, inflammation and cognition in Alzheimer's disease: Lessons for multiple sclerosis, *J Neurol Sci* **245** (2006), 21–33.
- [71] M.F. White, IRS proteins and the common path to diabetes, *Am J Physiol Endocrinol Metab* **283** (2001), E413–E422.
- [72] M. Yudkoff, Y. Daikhin, I. Nissim, O. Horyn, B. Luhovyy, A. Lazarow and I. Nissim, Brain amino acid requirements and toxicity: The example of leucine, *J Nutr* **135** (2005), 1531S–1538S.