Human microRNA (miR-20b-5p) modulates Alzheimer’s disease pathways and neuronal function, and a specific polymorphism close to the MIR20B gene influences Alzheimer’s biomarkers

Ruizhi Wang1,8, Nipun Chopra1,7,8, Kwangsik Nho2,8, Bryan Maloney1, Alexander G. Obukhov3, Peter T. Nelson4, Scott E. Counts5 and Debomoy K. Lahiri1,✉

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INTRODUCTION
Alzheimer’s disease (AD) is a progressive neurodegenerative disorder with loss of cognitive, executive, and other mental functions, and is the most common form of age-related dementia. Amyloid-β peptide (Aβ) contributes to the etiology and progression of the disease. Aβ is derived from the amyloid-β precursor protein (APP). Multiple microRNA (miRNA) species are also implicated in AD. We report that human hsa-miR20b-5p (miR-20b), produced from the MIR20B gene on Chromosome X, may play complex roles in AD pathogenesis, including Aβ regulation. Specifically, miR-20b-5p miRNA levels were altered in association with disease progression in three regions of the human brain: temporal neocortex, cerebellum, and posterior cingulate cortex. In cultured human neuronal cells, miR-20b-5p treatment interfered with calcium homeostasis, neurite outgrowth, and branchpoints. A single-nucleotide polymorphism (SNP) upstream of the MIR20B gene (rs13897515) associated with differences in levels of cerebrospinal fluid (CSF) Aβ1-42 and thickness of the entorhinal cortex. We located a miR-20b-5p binding site in the APP mRNA 3′-untranslated region (UTR), and treatment with miR-20b-5p reduced APP mRNA and protein levels. Network analysis of protein-protein interactions and gene coexpression revealed other important potential miR-20b-5p targets among AD-related proteins/genes. MiR-20b-5p, a miRNA that downregulated APP, was paradoxically associated with an increased risk for AD. However, miR-20b-5p also reduced, and the blockade of APP by siRNA likewise reduced calcium influx. As APP plays vital roles in neuronal health and does not exist solely to be the source of “pathogenic” Aβ, the molecular etiology of AD is likely to not just be a disease of “excess” but a disruption of delicate homeostasis.

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1Laboratory of Molecular Neurogenetics, Department of Psychiatry, Indiana Alzheimer’s Disease Research Center, Indiana University School of Medicine, Indianapolis, IN 46202, USA. 2Radiology, Indiana University School of Medicine, Indianapolis, IN 46120, USA. 3Anatomy, Cell Biology & Physiology, Indiana University School of Medicine, Indianapolis, IN 46202, USA. 4Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40536, USA. 5Departments of Translational Neuroscience & Family Medicine, Michigan State University, Grand Rapids, and Michigan Alzheimer’s Disease Research Center, Ann Arbor, MI, USA. 6Department of Medical & Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN, USA. 7Present address: DePauw University, Greencastle, IN 46135, USA. 8These authors contributed equally: Ruizhi Wang, Nipun Chopra, Kwangsik Nho ✉email: dlahiri@iupui.edu

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transmembrane amyloid precursor protein (APP) by two enzymes sequentially, \( \beta \)-site APP-cleaving enzyme-1 (BACE1) and the \( \gamma \)-secretase complex \[15, 16\]. In addition, a major contributor to risk of AD is the presence of the APOE\( \varepsilon 4 \) allele on the apolipoprotein E gene (APOE) \[17–25\]. This risk is sufficiently strong and widespread that we considered it necessary to examine potential APOE\( \varepsilon 4 \) effects on outcomes of human tissue/subject based experiments, herein.

APP is a single-pass transmembrane protein found on both neurons and glial cells \[15, 26, 27\]. The protein is hypothesized to play roles in cell adhesion \[28\], neuroproliferation \[29\], neurite outgrowth \[30\], axonal guidance \[31\], and synaptogenesis \[32\]. Hence, regulation of APP levels is important to normal homeostatic function. APP knockout mice are viable but exhibit aberrant long-term potentiation (LTP), impaired locomotor activity, and reduced brain weight \[33–35\], thereby suggesting a necessary role in brain development, learning and behavior.

According to the amyloid cascade hypothesis \[3\], the elimination of amyloid plaque is expected to prevent or arrest AD progression, and, therefore, APP, BACE1 and \( \gamma \)-secretase have been selected as drug targets in the treatment of AD. Unfortunately, conventional drug therapies targeting these proteins have had limited success due to a variety of factors \[3, 16, 36\]. Thus, there is considerable focus on the identification of novel therapies to address the \( \beta \)-\( \varepsilon \) excess problem. MicroRNA (miRNA) manipulation is one such possible therapeutic strategy \[37, 38\].

MiRNAs are short, noncoding RNA that regulate expression of many proteins at the posttranscriptional level, primarily by targeting the mRNA 3'-untranslated region (UTR) \[38\]. Binding of miRNA to its cognate targets usually results in the reduction of protein expression either by inducing mRNA degradation or by inhibiting protein translation via interference with ribosome assembly. In the case of APP, miRNAs can target either the 3'-UTR or the 5'-UTR, subsequently modulating protein levels \[39–43\]. Levels of miRNAs also differ between post-mortem brains obtained from AD patients vs. non-cognitively impaired (NCI) controls. Therefore, miRNA targeting of amyloid metabolism may affect the onset and progression of AD and may constitute an important therapeutic strategy.

The focus of the present study is human hsa-miR20b-5p (miR-20b). Human miR-20b belongs to a cluster of the MIR17 gene family that is highly conserved in vertebrates and negatively regulates APP expression \[44–46\]. The MIR17 gene family consists of three paralogous groups located on chromosomes 7, 13 and X. Another member of the MIR17 family, miR-20a, is a negative regulator of APP expression \[44–46\]. This miRNA is 91% homologous to miR-20b-5p and their seed sequences are identical. On the other hand, their precursor and mature sequences and their chromosomal locations differ \[47\].

In the present report, we identify miR-20b as a negative regulator of APP in human cell lines and a primary human brain (PHB) cell culture. We further show that elevated miR-20b associated with greater risk for AD in post-mortem brains. We also demonstrate that miR-20b's reduction of APP expression is reversed by the addition of an antagoniR to miR-20b. Furthermore, we report that miR-20b can disrupt calcium homeostasis, neurite outgrowth and neuronal branchpoints in a primary human cell culture model. Finally, we identify a SNP approximately 15kb upstream of the MIR20B gene that is associated with AD-related cerebrospinal fluid (CSF) biomarker levels, specifically the 42 amino acid \( \beta \)-\( \varepsilon \) peptide (\( \beta \)-42) and with AD-associated neuroanatomical variation. These data take their place alongside our studies of miRNA regulation of BACE1, membrane metalloendopeptidases (MME), and REI silencing transcription factor (REST) in PHB cultures and donated tissue from subjects who died with mild cognitive impairment (MCI) as well as AD and NCI subjects \[48–53\].

**MATERIALS AND METHODS**

**Cell cultures**

HeLa cells, human glioblastoma/astrocytoma (U373MG/U373), human microglia (HM3C), and human neuroblastoma (SK-N-SH) cells were procured from ATCC (American Type Culture Collection), and grown in EMEM (Corning) supplemented with 10% Fetal Bovine Serum (FBS) until they were ~70% confluent. After a brief trypsin digestion, cells were counted using the trypan-blue exclusion method. About 150,000 cells per well were seeded in a 24-well plate and left undisturbed overnight until transfection was carried out. For neuronal cultures, human neuroblastoma (NB) SK-N-SH cells were differentiated with 10 \( \mu \)M all-trans retinoic acid (ATRA, Sigma) for 7 days in 2% FBS maintenance media and afterwards referred to NBRA.

PHB cultures were grown as described previously \[48\]. Briefly, primary human embryonic brain tissues with no known gene mutations were obtained from the University of Washington Birth Defects Research Laboratory (Seattle), digested with trypsin, and plated in 24-well plates at a density of 150,000 cells per well. Media (neurobasal, Invitrogen) containing B-27 supplement at a ratio of 1:50, Glutamax (Invitrogen) at a ratio of 1:500, and basic fibroblast growth factor (bFGF, Invitrogen) was replenished every 4 days until 17 days in vitro (DIV 17). Transfections were performed from DIV 17 to DIV 20.

**APP 3'-UTR-coupled-reporter assay**

The APP 3'-UTR was inserted downstream within a dual-reporter luciferase plasmid as described previously \[41\]. 50 nM of miR-20b or a commercially supplied negative control mimic (NCM) (ThermoFisher, lyophilized powder) were co-transfected with the full-length, 1.2 kb, APP 3'-UTR dual-reporter luciferase plasmid into HeLa cells and the effect of the miR-20b or NCM on luciferase reporter expression was assessed using the DualGlo luciferase assay (Promega) 48 h post-transfection.

**RNA transfection**

Oligomer mimic of miR-20b (ThermoFisher) or a NCM was resuspended in nuclease free water and used at a concentration of 50 nM for HeLa experiments or 100 nM for the PHB tissue and human glioblastoma cultures. APP siRNA was used at a concentration of 20 nM for HeLa and 50 nM for PHB culture experiments. RNAiMax (ThermoFisher) was used as the transfection reagent at 1.5 \( \mu \)l/well. Mir-20b inhibitor or antagoniR (ThermoFisher) was used at a 100 nM dose in all experiments. Mock-transfected cells were treated with RNAiMax, but no miRNA. The transfection complexes were suspended in Opti-Mem media (ThermoFisher) and distributed 100 \( \mu \)l/well. The volume per well was brought up to 500 \( \mu \)l/well using the appropriate media. Cells were harvested at 72 h unless described otherwise.

**Cell harvesting**

At 72 h post-transfection (or as indicated), conditioned media was collected and stored, and cells were washed with 1x PBS and lysed using 100 \( \mu \)l of Mammalian protein extraction reagent (M-Per, ThermoFisher) supplemented with one tablet of protease inhibitor cocktail (Roche). The cell lysate was centrifuged for 10 min at 15,000 \( \times \)g and the supernatant was collected and used for Western blotting. Toxicity of experimental treatments was measured in cell cultures by lactose dehydrogenase (LDH) assay.

**RNA extraction and quantification from HeLa cells**

For HeLa cultures both total RNA and proteins were extracted via the mirVana Paris kit (ThermoFisher) as per the manufacturer's instructions and stored at ~70 °C. RNA concentration and purity were measured using a Nanodrop spectrophotometer.

**RNA quantification by qRT-PCR**

RNA from cell cultures was reverse transcribed with High Capacity RNA-to- cDNA kit (ThermoFisher). cDNA was subjected to real-time quantitative PCR (qPCR) analysis on QuantStudio 6 Flex instrument (ThermoFisher). Relative quantification was achieved by \( \Delta \Delta C_t \) or "fold change" normalization with the geometric means of housekeeping genes GAPDH and ACTB.

**Western blotting**

An equal amount of protein from treatment samples was denatured by heating for 10 min at 95 °C in the Laemmle sample buffer. The denatured samples were loaded onto a 10% bis-tris, 26-lane gel (BioRad) and run at
200 V for 1.2 h. The gels were transferred onto PVDF membranes using the iBlot dry transfer system (ThermoFisher) and were blocked using 5% Milk in tris-buffered saline with Tween-20 (TBST). Primary antibodies used were as follows: anti-APP (clone 22C11; Millipore, 1:1000), anti-actin (Sigma, 1:500 000), anti-tubulin (Sigma, 1:500 000). After TBST wash, goat anti-mouse (1:3000) secondary antibody conjugated to horseradish-peroxidase was applied for 1 h. After washing, chemiluminescence “Super Signal” reagent (Pierce) was used to visualize protein bands.

Human brain tissue specimens
We obtained well characterized autopsied tissue samples (Table 1) from cognitively normal older adults (non-cognitively impaired/NCI), subjects 0%–100%.

Table 1. Demographics of human brain samples.

| Region | Sex | Age (F, M) | Region | Sex | Age (F, M) |
|--------|-----|------------|--------|-----|------------|
| TL     | 12F, 10M | 82.5 ± 4.8/-2.8, 84.0 ± 2.8/-6.8 | CB     | 12F, 10M | 82.5 ± 4.8/-2.8, 84.0 ± 2.8/-6.8 |
| PCC    | 26F, 13M | 89.6 ± 1.9/-3.8, 85.9 ± 3.5/-3.4 |

*TL: Temporal Lobe, CB: Cerebellum, PCC: Posterior Cingulate Cortex.

Table 2. Demographics of ADNI SNP samples.

| Genotype | Number | Sex | Education | Age |
|----------|--------|-----|-----------|-----|
| A0       | 312    | 0 F | 312 M 17 | +2/-2 73.0 ± 5/-4 |
| AA       | 229    | 229 F | 0 M 16 | +2/-3 72.0 ± 6/-6 |
| GA       | 24     | 24 F | 0 M 16 | +2/-0.25 70.5 ± 3/-7.5 |
| G0       | 14     | 0 F | 14 M 18 | +0/-3.5 71.5 ± 5/-2.25 |
| GG       | 1      | 1 F | 0 M 16 | +0/-0 76.0 ± 0/-0 |

*Median years of formal education ± 75th/25th percentiles.

Table 3. ANOVA of NCI/MCI/NCI vs. miR-20b levels (pmol).

| Effect | $\chi^2$ (df) | p     | D   |
|--------|---------------|-------|-----|
| miR-20b | 13.48 (1)     | <0.001| 0.021 |
| ±APOEε4 | 9.77 (1)      | 0.002 | 0.010 |
| Region  | 3.57 (2)      | 0.168 | 0.004 |
| miR-20b × ε4 | 2.08 (1) | 0.149 | <0.001 |

Table 4. ANOVA of NCI/MCI/NCI vs. miR-20b levels (fold change).

| Effect | $\chi^2$ (df) | p     | D   |
|--------|---------------|-------|-----|
| miR-20b | 0.42 (1)      | 0.519 | 0.024 |
| ±APOEε4 | 11.84 (1)     | <0.001| 0.098 |
| Region  | 7.09 (2)      | 0.029 | 0.144 |
| miR-20b × ε4 | 11.98 (1) | <0.001| 0.039 |

Fig. 1 Levels of miR-20b by quantitative real-time PCR (qRT-PCR) in brain autopsy samples alter probability of NCI, MCI, and AD. miR-20b was quantified in temporal lobe (TL), cerebellum (CB), and posterior cingulate cortex (PCC) samples as described in the main text. Effect of miR-20b levels on probability of diagnosis (NCI/MCI/AD) was then modeled. A–F Comparison of absolute quantitation by diagnosis and brain region. Symbols indicate estimated marginal means categories. IQR: Interquartile range for miR-20b within a brain region. $\bar{x}$: Median of miR-20b within a brain region. Significant effects are indicated by line colors and patterns, to wit: NCI, APOE ε4 absent: ; NCI, ε4 present: ; MCI, ε4 absent: ; MCI, ε4 present: . Finally, miR-20b-5p axes for all charts is log scale.
with MCI, or from subjects with AD. Three brain regions were examined, specifically: temporal lobe (TL, superior and middle temporal gyri, Brodmann areas 21/22) and cerebellum (CB, from lateral folia) from the University of Kentucky ADRC (P30 AG0072946), and posterior cingulate cortex (PCC, Brodmann areas 23/32) from the Rush University ADRC (P30 AG010161). We recently described these tissue resources in detail [49]. Briefly, samples were frozen at autopsy and stored at −80 °C. For experimental studies, samples were immersed in liquid nitrogen, lyophilized, and then allowed to incubate for 5 min at room temperature. Then 200 μl of chloroform was added, and the sample was vortexed for 15 s. Following a 3 min incubation at room temperature, the samples were centrifuged at 12,000 × g for 15 min at 4 °C. The upper aqueous layer was transferred to a clean 1.5 ml tube, and an equal volume of 70% ethanol was added. The sample was vortexed and then processed following the manufacturer’s instructions. RNA was eluted in a final volume of 50 μl of nuclease free water, and then quantified to be used as a template for cDNA synthesis.

**RNA extraction from human brain tissue**

RNA was extracted from frozen tissue using a modified Ambion PureLink mini kit protocol (#12183018A). Briefly, between 10 and 25 mg of tissue was placed in a 2 ml round bottom tube. One ml of Trizol (ThermoFisher #15596026) was added. Tissue was sonicated on ice until homogenous and then allowed to incubate for 5 min at room temperature. Then 200 μl of chloroform was added, and the sample was vortexed for 15 s. Following a 3 min incubation at room temperature, the samples were centrifuged at 12,000 × g for 15 min at 4 °C. The upper aqueous layer was transferred to a clean 1.5 ml tube, and an equal volume of 70% ethanol was added. The sample was vortexed and then processed following the manufacturer’s instructions. RNA was eluted in a final volume of 50 μl of nuclease free water, and then quantified to be used as a template for cDNA synthesis.

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**Fig. 2** The rs13897515 SNP alters levels of CSF Aβ and thickness of entorhinal cortex. A Location of rs13897515 relative to MIR20B on X chromosome. B, C CSF Aβ levels were significantly influenced by G allele presence, age, and presence of the APOE ε4 allele. D, E CSF phosphor-tau (pt) levels were significantly influenced by age and APOE ε4 allele presence but not by the SNP allele genotype. F, G Thickness of the entorhinal cortex (EC) was significantly altered by SNP genotype, age and APOE ε4 allele presence. SNP and significant covariate effects are indicated by line/marker colors and patterns, to wit: G allele absent, APOE ε4 absent; – G allele present, ε4 present; – G allele absent, ε4 present; – G allele present, ε4 present; – G allele present, ε4 present; – G allele present, ε4 present. For EC thickness, the most parsimonious model was based on genotype, with the corresponding representations: A0, ε4 absent; – G0, ε4 absent; – G0, ε4 absent; – AA, ε4 absent; – G0, ε4 absent; – AA, ε4 absent; – G0, ε4 present; – A0, ε4 present; – G0, ε4 present; – A0, ε4 present.
**MicroRNA quantification by qRT-PCR**

Quantitation of miR-20b levels was determined using two methods, to wit: miR-20b levels in human tissue were analyzed by qPCR using both relative and absolute quantitative techniques. For relative quantitation, a probe-based assay for miR-20b (TaqMan 001014) was measured and compared to the control small RNA RNU48 (TaqMan 001006 labelled with VIC) [50]. Briefly, template for qPCR was generated using the TaqMan Advanced miRNA reverse transcription kit (ThermoFisher 4366596) following the manufacturer’s recommended protocol with an input of 10 ng of RNA. qPCR was performed on an ABI 7500 instrument in 20 µl reactions, which were incubated for 40 amplification cycles. Each reaction contained 1.3 µl of reverse transcription product as template, 2× master mix minus uracil-N-glycosylase (UNG) (ThermoFisher 444040), and each of the TaqMan assays listed above. Ct values were determined using a constant threshold, and fold change was calculated by the delta-delta Ct method.

**Table 5.** ANOVA of SNP rs138397515 status effects.

| Outcome | Effect | F (df, df) | p | ω² |
|---------|--------|------------|---|-----|
| CSF Aβ | G allele | 1.38 (1, 577) | 0.040 | 0.005 |
| Age    |        | 4.42 (1, 577) | <0.001 | 0.019 |
| APOE e4 | G allele | 32.11 (1, 577) | <0.001 | 0.148 |
| CSF pr | G allele | 0.23 (1, 577) | 0.633 | <0.001 |
| Age    |        | 14.69 (1, 577) | <0.001 | 0.021 |
| APOE e4 | G allele | 84.79 (1, 577) | <0.001 | 0.130 |
| EC Thickness | Genotypeb | 14.04 (3) | 0.003 | 0.012 |
| Age    |        | 36.39 (1) | <0.001 | 0.062 |
| APOE e4 | G allele | 4.48 (1) | 0.003 | 0.015 |

*aStatistic for EC thickness is χ² (df). bGenotypes in sample consist of A0, G0, AA, GA, and GG.

**A. APP mRNA (NM_000484.4)**

| Species/miRNA | miR-20b-5p |
|---------------|-------------|
| Human         | miR-20b-5p  |
| Rat           | miR-20b-5p  |
| Mouse         | miR-20b-5p  |
| Chicken       | miR-20b-5p  |
| Pig           | miR-20b-5p  |
| Cow           | miR-20b-5p  |
| Horse         | miR-20b-5p  |
| Cat           | miR-20b-5p  |
| Hedgehog      | miR-20b-5p  |
| Squirrel      | miR-20b-5p  |
| Aardvark      | miR-20b-5p  |
| Armadillo     | miR-20b-5p  |

**B. miR-20b-5p**

**C. Luciferase Reporter Assay**

Fig. 3  **MiR-20b target conservation on APP 3′-UTR and site confirmation.** A The hsa-miR-20b sequence was used to probe the APP 3′-UTR sequence with the STarMir utility. [57] A single putative site was predicted, with 100% homology to the 8-base seed sequence. Additional miRNA species previously confirmed to regulate APP translation are also shown [39–42]. B Multiple sequence alignment of homologous regions from 12 additional mammalian species. Seed sequence (outlined) was conserved across multiple genera and families. Homology was compared between each animal target sequence and hsa-miR-20b-5p sequence and vs. the human 3′-UTR target sequence. All sequences maintained at least 63% homology to complete miR-20b-5p sequence and at least 91.3% homology to the human target sequence. C APP 3′-UTR-coupled-reporter activity. Reporter clone with full APP 3′-UTR sequence was co-transfected with NCM and miR-101-3p and miR-20b mimics. miR-20b significantly (p ≤ 0.05) reduced levels of luciferase reporter expression. Symbols indicate estimated marginal means categories. Samples sharing a symbol did not significantly differ from each other. Error bars represent standard errors of means (SEM).
unbound dye. Coverslips with Fura-2 loaded cells were kept in dark for 60 min to allow Fura-2AM to Fura-2 conversion before imaging was performed. Coverslips were placed on an inverted Zeiss microscope equipped with a back-illuminated Andor charge-coupled device camera. The coverslip was mounted into a perfusion system with continuous supply of either HEPES Buffer or HEPES buffer-containing 70 mM KCl. The coverslip was flushed with HEPES buffer for 10 min. During this time, regions of interest (ROI) were selected based on cell morphology; an attempt was made to include mostly non-aggregated cells. This was followed by a 5 min application of HEPES buffer containing 70 mM KCl to depolarize the neurons. Fura-2 was excited at 350 nm and 380 nm while the emitted light was collected using a 510 nm filter. The ratio of fluorescence intensities acquired at 350 nm (F350) and 380 nm (F380) excitation wavelengths was calculated for analysis. Only cells responding to KCl were selected for analysis, as they were likely neurons.

Data for characterization of SNP in human tissues (ADNI)

Raw data used for analysis of MIR20B gene associated SNP effects were obtained from the Alzheimer’s Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). These data included serial magnetic resonance imaging (MRI), positron emission tomography (PET), biological markers, and clinical and neuropsychological assessments (Table 2).

Data analysis

Data were modeled for all experiments except brain level miR-20b analysis and entorhinal cortex thickness by generalized linear models (glm) followed by analysis of variance/deviance (ANOVA) and estimated marginal means using false discovery rate (fdr) to correct for multiple comparisons. A distinct advantage of the glm over ordinary least squares analysis is that the assumptions of normal distribution and similar variances (homoscedasticity) are greatly relaxed and do not need to be satisfied.

Fig. 4 Treatment with miR-20b and related oligomers produces parallel outcomes for APP mRNA, intracellular APP, and secreted APP. HeLa cells were treated with oligomers: Specifically, miR-20b mimic, antagoniR to miR-20b, mimic + antagoniR, siRNA to APP, and a commercially obtained negative control mimic (NCM). Cultures and media were harvested and prepared for qRT-PCR, Western blotting, and LDH, as described in the text. Data were checked for multivariate normality then analyzed with MANOVA followed by estimated marginal means with the false discovery rate adjustment. Pillai’s trace (df, df): 2.17 (5, 1); p < 0.001, $\eta^2 = 0.542$. A Western blot of intracellular and secreted APP (sAPP), along with β-actin. B qRT-PCR relative outcomes, mock = 1. C Relative densitometry of intracellular APP, mock = 1. D Relative densitometry of sAPP, mock = 1. E Relative LDH signal, mock = 1. Symbols indicate statistical categories. Samples sharing a symbol do not differ at $p \leq 0.05$. Error bars represent standard errors of means (SEM).
demonstrated for a valid analysis. Brain miRNA level data were analyzed by ordinal logistic regression (olr) followed by ANOVA. The olr presumes that the response variable is ordered, such as NCI, MCI, and AD representing progressive severity of condition. Since EC data were collected using two distinct MagFields, specifically 1.5T and 3T, we used a generalized mixed-level model with random intercepts for MagField to account for likely differences in measurements caused by the two settings. When MANOVA was used, assumptions of multivariate normality and sphericity were tested by Marria’s and Bartlett’s tests, respectively. Data were found to be multivariate normal and to have homogeneous covariate matrices and was thus appropriate for MANOVA.

Sample sizes were determined by comparison to our earlier work, which has generated significant and repeatable results [40–42]. We calculated coefficients of determination ($D$) for ordinal logistic models via $D = \text{mean}(p, c) - (\text{mean}(p, c) - 1)$, where mean($p, c$) is the mean of each probability that corresponds to the specific level (in this case NCI/ MCI/AD) reported in the data, and the second term is the remaining predicted probability sum for that point for input levels of the response variable is calculated. This method is an extension of Tjur’s $D$ for binomial logistic models [55] and is analogous to the $R^2$ coefficient of determination. To obtain values for each effect in the model, models were generated without an effect and $D$ was calculated. Since $D$ is an analogue for $R^2$, the Fisher transformed $Z$ of the full model and each sub-model were calculated from $\sqrt{D}$ subtracted from transformed $\sqrt{D}$ for the overall model. The result was back-transformed and squared to produce a partial $D$ for each effect.

**Construction of interaction networks based on miR-20b and AD**

We probed the STarMirDB utility with several AD-related mRNA sequences. Positive outcomes of this search were then used as inputs to NetworkAnalyst to map minimum networks for human hippocampus protein-protein interactions (PPI), frontal cortex PPI, hippocampus co-expression, frontal cortex co-expression, and signaling pathways. Proteins/genes found in these networks were then used as inputs to STarMirDB to investigate further miR-20b targeting. Networks were visualized by the R igraph package.

**RESULTS**

miR-20b alters probability of AD, MCI, and NCI in elderly adult human brain samples

Quantitation of miR-20b in post-mortem samples of NCI, MCI, and AD brains (Table 1) determined a complex relationship. Raw qRT-PCR signals were quantified by both absolute (Fig. 1A–C, Table 3) and relative levels (Fig. 2A–C, Table 3).

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**Table 6.** ANOVA of APP levels vs. oligomer treatments in multiple cell lines.

| Effect          | F (df, df) | p     | D   |
|-----------------|------------|-------|-----|
| Treatment       | 6.39 (6, 90) | <0.001 | 0.188 |
| Cell Line       | 0.32 (7, 90) | 0.926 | <0.001 |
| Treatment x Line| 3.15 (20, 90) | <0.001 | 0.214 |

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**Fig. 5** Treatment with miR-20b reduced levels of APP in U373 astroglial and HeLa epithelial cells, while miR-20b reduced levels of APP in HMC3 microglial cells and in human primary brain cultures (PHB) and was reversed by co-treatment with antagomiRs. U373, HMC3, PHB, and NBRA cells were cultured and treated with miR-20b mimic or antagomiR as specified in the main text. A Western blot and densitometry of APP and β-actin in U373 cells. miR-20b significantly reduced levels of APP (relative to β-actin). B Western blot and densitometry of APP and β-actin in HMC3 cells. Like U373, miR-20b significantly reduced levels of APP. C Western blot and densitometry of APP and β-actin in NBRA cells. miR-20b did not reduce levels of APP measured. Co-treatment with miR-20b antagomiR eliminated miR-20b reduction of APP in U373, HMC3, and PHB. Error bars represent standard errors of means (SEM).
Hoperation, no independent association with miR-20b levels (Fig. 1D). Quantitation of miR-20b produced a model where diagnosis had probability of AD increased in all three brain regions. Relative probability of AD overall, as expected. As miR-20b levels increased, (Fig. 1A) APOE miR-20b levels and ε4 allele interacted with regard to miR-20b levels. When the model tested effects of miR-20b on diagnosis (AD/MCI/NCI) in different brain regions (TL, CB, PCC). We tested potential covariates of age, sex, and presence/absence of at least one APOE ε4 allele. For both absolute and relative quantitation, a model that included APOE ε4 presence/absence as a covariate was selected. When examining absolute quantities of miR-20b, differences by miR-20b levels and APOE ε4 presence had significant effects (Fig. 1A–C). APOE ε4 presence was associated with increased probability of AD overall, as expected. As miR-20b levels increased, probability of AD increased in all three brain regions. Relative quantitation of miR-20b produced a model where diagnosis had no independent association with miR-20b levels (Fig. 1D–F). However, APOE ε4 allele presence and diagnosis significantly interacted with regard to miR-20b levels. When the APOE ε4 allele was absent, miR20b levels associated with reduced probability of AD and increased probability of NCI. Effects were pronounced in TL and CB samples but were very weak in PCC. Comparing fit of models by D (Supplementary Table 1) showed that the relative miR-20b model had greater accuracy than the absolute quantified miR-20b model. Low levels of prediction for MCI may be an artifact of the sample, which consisted primarily of NCI and AD subjects.

A single-nucleotide polymorphism (SNP) near miR-20b gene was associated with altered levels of CSF Aβ and entorhinal cortex thickness

We queried the ADNI database for SNPs in or near the MIR20B gene on ChrXq26.2. One biomarker-linked SNP was identified (rs138397515). This SNP was 14.7 kb upstream of MIR20B and consisted of an A→G transition (Fig. 2A). Out of 580 subjects (Table 2), distribution among A0/AA/G0/GA/GG genotypes was 312/229/14/24/1, where “A0” and “G0” indicate male subjects. Hardy–Weinberg χ² tests indicated that the alleles were within H-W equilibrium (p = 0.775). The “GG” genotype had only one representative and was excluded from further analysis. CSF biomarkers (CSF Aβ1-42 and CSF p-tau) from the dataset were compared to SNP variation (Fig. 2). Analysis was carried out on the 580 subjects as a single sample group. However, SNP effects were also tested vs. potential covariates of subject age, subject education (years of attainment), subject sex, and presence of at least one APOE4 allele. Higher CSF Aβ levels were associated with the presence of at least one G allele (Fig. 2B, C, p = 0.040, Table 5). CSF Aβ also was associated with subject age and presence of at least one APOE4 allele (p < 0.001). When we examined hippocampus volume and entorhinal cortex thickness, we found no association between rs138397515 status and hippocampus volume. However, the SNP genotype did associate with differences in entorhinal cortex thickness (Fig. 2F, G; p = 0.003, Table 5), specifically EC thickness increased along with genotype in the order G0 < A0 < AA < GA.

miR-20b targets the APP mRNA through its 3′-UTR

Query of the hsa-miR-20b sequence against the APP 3′-UTR revealed putative sites at base pairs 3158–3180 (Fig. 3A), predicted ΔG = −24.9. The site is highly conserved across multiple lineages of placental mammals, as shown by Multiz (58) alignment (Fig. 3B). We assessed the effect of miR-20b on the activity of the 3′-UTR in HeLa cells. We tested an APP 3′-UTR luciferase reporter clone (41) by treatment with mimic for miR-20b (Fig. 3C). Treatment reduced luciferase signal vs. mock and fold change (Fig. 1D–F, Table 4) methods, then analyzed with ordered logistic regression to predict probabilities of each member of an ordered set of outcomes [56]. In this case, the order was NCI (no disease), MCI (mild/moderate disease), and AD (severe/worse disease).

Our model tested effects of miR-20b on diagnosis (AD/MCI/NCI) in different brain regions (TL, CB, PCC). We tested potential covariates of age, sex, and presence/absence of at least one APOE ε4 allele. For both absolute and relative quantitation, a model that included APOE4 presence/absence as a covariate was selected. When examining absolute quantities of miR-20b, differences by miR-20b levels and APOE4 presence had significant effects (Fig. 1A–C). APOE ε4 presence was associated with increased probability of AD overall, as expected. As miR-20b levels increased, probability of AD increased in all three brain regions. Relative quantitation of miR-20b produced a model where diagnosis had no independent association with miR-20b levels (Fig. 1D–F). However, APOE ε4 allele presence and diagnosis significantly interacted with regard to miR-20b levels. When the APOE ε4 allele was absent, miR20b levels associated with reduced probability of AD and increased probability of NCI. Effects were pronounced in TL and CB samples but were very weak in PCC. Comparing fit of models by D (Supplementary Table 1) showed that the relative miR-20b model had greater accuracy than the absolute quantified miR-20b model. Low levels of prediction for MCI may be an artifact of the sample, which consisted primarily of NCI and AD subjects.
and NCM treatment ($F$ (df, df) = 24.136 (3, 16); $p < 0.001; \omega^2 = 0.776). In addition, we transfected cells with miR-101-3p as a positive control. This oligo also reduced 3'-UTR reporter activity, confirming our previous results [41].

**Treatment by miR-20b alters levels of APP mRNA and protein**

Treatment of HeLa cells by miR-20b mimic significantly reduced levels of APP mRNA (Fig. 4B), intracellular APP (Fig. 4A, C), and secreted APP (sAPP) in conditioned media (Fig. 4D). Treatment by siRNA against APP resulted in even greater reduction of these outcome measures. Co-treatment with miR-20b and an antagomiR significantly reduced sAPP levels. However, since this was not accompanied by reduction in APP mRNA or intracellular APP protein, this outcome may be a result of an interaction between the NCM oligomer and APP cleavage or secretion protein mRNAs.

As a multivariate outcome experiment, this was analyzed by MANOVA after confirming multivariate normality by Mardia’s test and sphericity by Bartlett’s test. (Pillai’s trace (df, df): 2.17 (5, 1); $p < 0.001, \eta^2 = 0.542$).

**miR-20b reduced levels of APP in other human cell cultures**

We further transfected miR-20b in several additional human cell cultures, including astrocytic (U373), microglial (HMC3), differentiated neuroblastoma (NBRA), and PHB cultures. To explicitly test whether apparent differences in response among cell lines could be detected, we tested a model of APP ~ Treatment + Line + Treatment x Line, each blot normalized to “Mock = 1”. We discovered that cell line, treatment, and the interaction of treatment and cell line/culture were significant (Table 6), indicating that apparent response differences among cell lines/cultures were also significant (Fig. 5). Treatment of miR-20b mimic reduced APP levels in U373 cells (Fig. 5A). MiR-20b treatment also significantly reduced levels of APP in HMC3 (Fig. 5B) and PHB cultures (Fig. 5C). When ATRA-differentiated neuroblastoma cultures (NBRA) were treated with miR-20b mimic, APP reduction was not significant (Fig. 5D).

**miR-20b and APP siRNA treatments each reduce calcium influx in a developmental human brain model culture**

We tested the effect of miR-20b and APP siRNA on calcium influx of PHB using Fura-2 imaging (Fig. 6, Table 7). An “idealized” Fura curve (Supplementary Fig. S1A), would progress through “pre-flow”, from first cycle “0” to “I”, which is the beginning of the influx. Influx would then progress to “P”, the peak. It would then be followed by active discharge to “II”, after which it would be passive tailing off. The distance from I to P, “b”, is the “time to peak”. The distance “a” from P to II is the “discharge time”. The height “a” is the “peak amplitude”, and the height “a” is the “ratio decay magnitude”. Six comparisons were made based on different normalizations of the Fura-2 curves. Length of “pre-flow” and

![Fig. 7 Treatment with miR-20b reduced neurite branchpoints in PHB cultures.](image-url)
heights of 350/380 signal for this segment were based on raw data (Supplementary Fig. S1B). The length of “pre flow” was significantly longer for mock than for miR-20b or siRNA treated cells (Fig. 6A). However, 350/380 ratios did not significantly differ among treatments (Fig. 6B). To compare distances b, b', and a (peak height), positions of I for each treatment were normalized to one baseline. Neither (peak height), positions of I for each treatment were normalized among treatments (Fig. 6B). To compare distances b, b' and trace heights were normalized to one baseline. Neither (peak height), positions of I for each treatment were normalized among treatments. However, mean peak amplitude (Fig. 6E) was significantly higher for mock-treated cells than for either miR-20b or siRNA treated cells. However, ω² (less-biased analog of η²) was quite small (0.093). To compare distance a' (discharge magnitude), traces were scaled to minimum and maximum of each trace (min = 0, max = 1). No significant differences were found by treatment (Fig. 6F).

Table 9. Major AD-related proteins used as network building seeds.

| Protein | Uniport | Full name | Category a | miR-20b b |
|---------|---------|-----------|------------|-----------|
| ADAM10  | O14672  | Disintegrin and metalloproteinase domain-containing protein 10 | Amyloid | + |
| ADAM17  | P78536  | Disintegrin and metalloproteinase domain-containing protein 17 | Amyloid | + |
| ADAM9   | Q13443  | Disintegrin and metalloproteinase domain-containing protein 9 | Amyloid | + |
| APOE    | P02649  | Apolipoprotein E | Regulator | + |
| APP     | P05067  | Amyloid precursor protein | Amyloid | + |
| BACE1   | P56817  | Beta-secretase 1 | Amyloid | + |
| ECE1    | P42892  | Endothelin-converting enzyme-1 | Clearance | |
| GSK3A   | P49840  | Glycogen synthase kinase-3 alpha | Tau | |
| GSK3B   | P49841  | Glycogen synthase kinase-3 beta | Tau | |
| IDE     | P14735  | Insulin-degrading enzyme | Clearance | + |
| IL1A    | P01583  | Interleukin-1 alpha | Amyloid | + |
| IRP1    | P21399  | Iron-responsive element-binding protein 1 | Amyloid | + |
| IRP2    | P48200  | Iron-responsive element-binding protein 2 | Amyloid | + |
| MAPK13  | O15264  | Mitogen-activated protein kinase 13 | Tau | |
| MAPT    | P10636  | Microtubule-associated protein tau | Tau | + |
| MME     | P08473  | Membrane metallo-endopeptidase | Clearance | + |
| PSD95   | P78352  | Postsynaptic density protein 95 | Synaptic | |
| PSEN1   | P49768  | Presenilin-1 | Amyloid | |
| PSEN2   | P49810  | Presenilin-2 | Amyloid | |
| REST    | Q13127  | RE1-silencing transcription factor | Regulator | |
| SNAP25  | P60880  | Synaptosomal-associated protein 25 | Synaptic | + |
| SNCA    | P37840  | Alpha-synuclein | Regulator | + |
| SYPH    | P08247  | Synaptophysin | Synaptic | |

aClassification vs. AD relationship, specifically, Amyloid; APP, APP processing enzyme, or APP translation factor; Clearance: Aβ clearing enzyme; Regulator: Protein with functions/effects on both Aβ and hyperphosphorylated tau protein; Tau: either MAPT or one of its major kinases.
bPredicted or confirmed to interact with miR-20b-Sp.
discovered network members, even within the same brain region. On the other hand, within network type, there was extensive overlap for PPI between hippocampus and frontal cortex but no overlap between hippocampus and frontal cortex for coexpression. Literature search reveals that the majority of these proteins may have some function in AD, and expanding analysis of miR-20b to include these targets in AD research may be fruitful.

**DISCUSSION**

Our integrated studies used different approaches, such as bioinformatics, biochemical, cellular, genetics, physiology and neuropathology, to reveal potential novel roles for miR-20b in AD. We showed that miR-20b represses levels of APP by targeting a predicted 7-mer m8 on the APP 3′-UTR. This repression was seen in epithelial, astrocytic, microglial, and a primary human mixed-brain cell culture model. This work adds to other reports of miRNA regulating APP levels [39–42, 44, 54, 59, 60]. Our cell culture work also showed that miR-20b reduces APP 3′-UTR activity and protein. Furthermore, we demonstrated that miR-20b and APP siRNA reduce intracellular Ca2+ transients in response to neuron membrane depolarization. Since our work also shows that miR-20b-5p reduces APP in microglial cells, future work should address whether the miRNA can also affect intracellular calcium levels in microglial cells. Treatment by miR-20b also resulted in reduction in neurite branchpoints and neurite length in PHB cell cultures. A SNP near MIR20B gene was associated with significant differences in a CSF biomarker (CSF Aβ42) levels and thickness of the entorhinal cortex detected via MRI. Finally, AD progression (NCI/MCI/AD) corresponded to increases in brain miR-20b levels when miRNA was measured by pMol, and we found evidence of interaction of APOE ε4 allele presence and miR-20b levels.

Our work adds knowledge about miR-20b’s important role in vital cellular- and organ-level pathways. For instance, miR-20b negatively modulates many different targets including endothelial PAS domain 1 [61], vascular endothelial growth factor in the lung [62], ephrin B2 and B4 in human placental tissue [63], proteinase-activated receptor-1 [64], phosphatase and tensin homolog [65, 66], signal transducer and activator of transcription 3 [67], induced myeloid leukemia cell differentiation protein Mcl-1 [68], B-cell translocation gene [69], IL-1 receptor-associated kinase 4 [70], hypoxia-inducible factor 1-alpha [71], and protein kinase B [72].

We report that transfection of miR-20b reduced both synaptic branch lengths and branchpoints in human neuronal culture. APP is expressed in the presynaptic membrane and is trafficked along with vesicles in the pre-synapse [73, 74]. Similarly, Aβ fragments are generated at both the pre-synaptic and post-synaptic neurons [75]. Previous research suggests that dimerization of APP is important to neurite growth [76], and, therefore, it is possible that our observed miR-20b effect on neurite outgrowth influences APP dimerization. It may be noteworthy that, in addition to APP and Aβ synaptic activity, BACE1 also exerts control on synaptic function [77]. The neurite-based measures of cells treated with miR-20b experienced a noteworthy increase in variability on days 3 and 4. This was most pronounced when branchpoints and length were normalized to cell body area. Such variation could be technical or biological. Technically, culture cell confluence was high, which was necessary to maintain health of primary human brain cultures. However, such density may have interfered with...
Fig. 9 Coexpression networks of miR-20b, AD-related targets. Networks were generated by NetworkAnalyst as described in the text. Network nodes are color coded by protein function related to AD. A Hippocampus PPI network. B Frontal cortex PPI network. C Summary of network members in addition to "seed" members (or "novel" members).

An APP overexpression experiment could conclusively demonstrate that miR-20b alters calcium influx and neurite outgrowth via an APP-mediated pathway. However, multiple studies have already reported that APP promotes neurite outgrowth both in vitro and in vivo. For example, APP increases axonal arborization in Drosophila brains [79]. APP knockdown by shRNA inhibits neurite outgrowth in neuron cultures, while APP knockout in vivo reduces neurite numbers and lengths [80]. Similarly, APP regulates intracellular calcium levels. Expression of human APP in rat cortical neurons increased calcium influx [81]. Based on our data, APP sRNA and miR-20b treatment both reduce calcium influx. We can infer that miR-20b reduces calcium influx at least in part due to APP downregulation. Nevertheless, we still must admit that we do not know whether miR-20b alters expression of other proteins independent of APP, which in turn regulate neurite outgrowth and ion channels. Future work should involve a comprehensive “omic”-type experiment.

Small changes in the membrane expression of calcium-permeable channels can result in large-scale cellular events. These can be beneficial, for example, in the insertion of NMDAR receptors during the events of LTP. Aβ is involved in LTP and normal memory processes [82–85], suggesting that excess removal of Aβ may impact learning. APP may itself modulate trafficking of NMDAR to the membrane [86]. Therefore, identifying miRNA that may influence Aβ levels would be beneficial to our understanding of Aβ’s role in the synapse.
A. Signaling Network

Fig. 10  Signaling network of miR-20b, AD-related targets. Network was generated by NetworkAnalyst as described in the text. Network nodes are color coded by protein function related to AD. A Signaling network. B Summary of network members in addition to “seed” members (or “novel” members).

Taken together, our data suggest that increasing miR-20b levels results in loss of neuronal function, as measured by neurite length and branching, as well as a reduction in KCl-evoked intracellular calcium influx. We posit two potential mechanisms (Fig. 11). First, miR-20b reduces voltage-gated Ca²⁺ channels (VGCC) expression directly. A search of several neuronal subunits of VGCC revealed multiple potential miR-20b binding sites (Supplementary Tables 2–3). Nevertheless, we speculate that the reduction observed may also be at least in part due to miR-20b reversing calcium influx by reducing APP. This may induce a subsequent reduction of available Aβ to insert into the membrane and potentially to regulate VGCC function, possibly by lowering the channel’s activation threshold [87], specifically because our APP siRNA transfection induced a response that was similar to that induced by miR-20b.

We successfully measured miR-20b in post-mortem brains of NCI, MCI, and AD patients. This adds to previous work that measured miR-20b in circulating CNS-derived exosomes in the blood [88–90] as well as in peripheral tissue [91, 92]. Our work suggests levels of miR-20b differ in post-mortem brains of AD vs. NCI patients, when miR-20b is absolutely quantified. By contrast, using the ΔΔCT (fold change) method resulted in no detected differences. In addition, our analysis suggests that miR-20b may increase in older individuals suffering from AD, thereby suggesting a novel interaction of changing miRNA levels with age. This observation should be investigated further. It is also important to note that our brain samples were limited to three brain regions: the PCC, the cerebellar cortex, and the TL. Therefore, future studies should measure levels of miR-20b in other brain regions, such as hippocampus and entorhinal cortex.

Our data suggest that the rs138397515 (A/G) SNP, located upstream of the MIR20B gene on Chromosome X, is associated with increase in CSF Aβ1–42. In addition, the SNP status was associated with differences in entorhinal cortex thickness detected by MRI. According to the ALFA project [93], studying the genomes of Alzheimer’s patients and their families, frequency of the minority “G” allele varies little among many populations (0.02–0.03) except among Asian populations, where the allele is not recorded and African-origin populations, where frequency is ~0.003 [93]. How miR-20b expression is affected by this upstream SNP will require further investigation. This SNP has the potential to participate on the complexity of effects influencing AD onset in disparate racial and ethnic groups. While apparent racial differences in AD have been reported [94], it remains unknown to what extent these differences have genetic, environmental, iatrogenic, or other unknown basis [95].

An apparent paradox underlies our present work. We demonstrated by cell culture work that application of miR-20b reduces levels of APP. However, miR-20b appears to be increased in one model relating miR-20b levels to probability of AD. Likewise, elevated levels of miR-20b appear to hamper neuronal communication (measured by neurite growth) and calcium response. Notably, the model that represented miR-20b in brain by relative quantification (~ΔΔCT/fold change) showed a definite decrease in probability of AD as miR-20b increased, and this model has a higher R² (R² analogue) than did the model based on absolute quantification of miR-20b. In addition, while APP and Aβ aggregation are associated with AD pathogenesis and pathology, APP serves necessary functions, these functions may differ in developmental vs. late-life stages. For example, APP plays a vital role in neuronal pruning [96], ensuring normal development of the brain. Insufficient pruning is widely accepted as a potential cause of autism spectrum disorder (ASD) [97, 98]. Here we used PHB culture as a proxy of a “developmental model”.

Taking the different outcomes and models used into account, we propose that one function of miR-20b in APP regulation is to...
Different domains of APP (right panel) are as shown: Intercellular, Transmembrane and Cytoplasmic. 

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not be a practical treatment. In addition, since the familial AD types that underlie most mouse models only represent minority of human AD cases, we would also investigate inducible mouse AD models [108].

Furthermore, three clinical outcome instruments (Alzheimer’s Disease Assessment Scale–Cognitive Subscale (ADAS-COG), specifically ADAS-COG 13-item [109], Mini-Mental State Examination [110], and Rey Auditory Verbal Learning Test (RAVLT)) [111] were evaluated to measure cognitive decline and MRI changes. Acta Neuropathol. 1991;84:562. https://doi.org/10.1007/s00401-013-1236-0

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AUTHOR CONTRIBUTIONS
RW and NC carried out cell culture experiments, RNA transfections, UTR-derived reporter clone and western blotting assays. RW did RNA extraction and quantification. NC performed Fura experiments. KN supplied and extracted ADNI data. AGO contributed to analysis of Fura experiments. PTN contributed brain tissue specimens. SEC contributed brain material and qRT-PCR of same. BM performed data analysis, and figure design. DKL performed study design and provided overall direction and facilities as well as manuscript writing and checking. All authors contributed to paper drafting, editing, and writing.

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The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Debomoy K. Lahiri.

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