miR-107 Regulates Granulin/Progranulin with Implications for Traumatic Brain Injury and Neurodegenerative Disease

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Granulin (GRN, or progranulin) is a protein involved in wound repair, inflammation, and neoplasia. GRN has also been directly implicated in frontotemporal dementia and may contribute to Alzheimer's disease pathogenesis. However, GRN regulation expression is poorly understood. A high-throughput experimental microRNA assay showed that GRN is the strongest target for miR-107 in human H4 neuroglioma cells. miR-107 has been implicated in Alzheimer's disease pathogenesis, and sequence elements in the open reading frame—rather than the 3' untranslated region—of GRN mRNA are recognized by miR-107 and are highly conserved among vertebrate species. To better understand the mechanism of this interaction, FLAG-tagged Argonaute constructs were used following miR-107 transfection. GRN mRNA interacts preferentially with Argonaute 2. *In vitro* and *in vivo* studies indicate that regulation of GRN by miR-107 may be functionally important. Glucose supplementation in cultured cells that leads to increased miR-107 levels also results in decreased GRN expression, including changes in cell compartmentation and decreased secretion of GRN protein. This effect was eliminated following miR-107 transfection. We also tested a mouse model where miR-107 has been shown to be down-regulated. In brain tissue subjacent to 1.0 mm depth controlled cortical impact, surviving hippocampal neurons show decreased miR-107 with augmentation of neuronal GRN expression. These findings indicate that miR-107 contributes to GRN expression regulation with implica-

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MicroRNAs (miRNAs), ~22 nucleotide noncoding RNAs, play fundamental roles in the human brain.^{1–3} MiRNAs increase the complexity of gene expression regulation and may have helped drive human brain evolution.^{4,5} Endogenous miRNAs perform critical neuroprotective functions^{6–8} with possible direct relevance to many diseases in the brain and elsewhere.^{9–14}

At the molecular level, miRNAs “target” mRNAs through partial hybridization, leading to changes in the rate of polypeptide formation.^{15,16} MiRNAs interact with mRNAs within microribonucleoproteins (miRNPs)¹⁷ using evolutionarily ancient molecular mechanisms. At the core of miRNPs, Argonaute (AGO) proteins bind directly to mature miRNAs. Four paralogous human AGO proteins (AGOs 1–4) help orchestrate miRNA activities.^{17,18} A single miRNA species, in association with AGO proteins, may target hundreds or even thousands of different mRNAs.^{19–21} Complex principles govern how metazoan miRNAs bind to mRNA targets,²² so predicting the physiological interaction of particular mRNA targets is a challenge. Current algorithms for predicting miRNA targets are imperfect and computational predictions tend to differ one from the other. Thus, the use of direct, experimental target identification strategies is important.

Identifying physiological miRNA targets is particularly relevant because the miRNA:mRNA interactions can be pertinent to human illness. Aberrant miRNA expression may contribute to the progression of neurodegenerative

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diseases.^{13,14,23,24} A specific miRNA, miR-107, is down-regulated in Alzheimer's disease (AD) beginning very early in the disease.²⁵

In the present study we sought to identify transcriptome-wide miR-107 targets in human cells. Co-immunoprecipitation (co-IP) experiments that pull down AGO proteins provide a method for characterizing miRNPs along with associated molecules.^{26,27} Using these assays, researchers have isolated multiple proteins, miRNAs, and mRNA targets from miRNPs.^{28–34} We used anti-AGO co-IP and downstream Affymetrix microarray analyses ("RIP-Chip"^{35,36}), to identify miRNA targets. This is a direct method that has been rigorously validated,³⁷ and that can help to guide future computational algorithms.

A notable miR-107 target that we found is *GRN*. This gene has been given multiple names, including granulin(s), progranulin, acrogranin, gp88, proepithelin, PC cell-derived growth factor, epithelial transforming growth factor, and granulin-epithelin precursor.^{38–42} To avoid confusion, we refer to the current consensus identifier "GRN" for the protein and "*GRN*" for the gene or mRNA. *GRN* has been implicated directly in frontotemporal dementias and also may contribute pathogenetically to more prevalent neurodegenerative disease processes including AD.^{43–46} In addition to its involvement in neurodegenerative diseases, *GRN* appears to be relevant in human cancers and a pivotal modulator of cell growth, inflammation, and wound repair.^{42,47}

Materials and Methods

Synthetic miRNA Precursors and Inhibitors

miRNA precursors and inhibitors were purchased from Ambion (Ambion, Austin, TX). When co-transfection to cells, miRNA precursors were used at 25 nmol/L, and miRNA inhibitors were used at 100 nmol/L.

Plasmids and Antibodies

Full-length cDNA (including 5'UTR and 3'UTR) cloned in pCMV6-XL5 plasmid vectors were obtained that express human *GRN* (*GRN*, NM_002087.2), β -site APP-cleaving enzyme 1 (*BACE1*, NM_012104.3), and α -synuclein (*SNCA*, SC119919) (OriGene Technologies, Inc, Rockville, MD). Full-length microtubule-associated protein tau (*MAPT*) was cloned into pCMV6-XL5 by PCR cloning. Plasmids carrying only open reading frame portion of *GRN*, *MAPT*, and *SNCA* were generated by PCR cloning.

Antibodies used in this work are: anti-AGO (2A8)⁴⁸; Goat anti-human ProGRN antibody (AF2420) and BACE1 monoclonal antibody (MAB931) from R&D systems (Minneapolis, MN); anti- β -Actin antibody (600401886) was purchased from Rockland (Gilbertsville, PA). Anti-FLAG M2 affinity gel was from Sigma (St. Louis, MO). Anti-MAP Tau and anti- α -synuclein antibodies were generously provided by Dr. Peter Davies (Albert Einstein College of Medicine, New York, NY) and Dr. Virginia Lee (University of Pennsylvania, Philadelphia, PA).

Transfections and Dual Luciferase Reporter Assays

For RIP-Chip transfections, H4 cells (American Type Culture Collection, Manassas, VA) were plated in 10-cm culture plates at a density of 2.5×10^6 /plate. Cells were transfected the next day with 25 nmol/L of "Pre-miRNA precursors" and Negative Control #1 (Ambion, Austin, TX) using RNAiMAX (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Cells were cultured for 48 hours after transfection. H4 cell transfection and *Rluc* reporter activity analyses were performed following the protocol described in our previous report.²⁵

Co-IP of miRNPs with Anti-AGO Antibodies

The RIP-Chip co-IP assay with validation has now been described in detail.³⁷ Briefly, protein G agarose beads (Invitrogen, Carlsbad, CA) were incubated with monoclonal anti-AGO,⁴⁸ or nonimmune mouse serum (Pierce Biotechnology, Rockford, IL). After binding, beads were then washed three times in PBS and twice in lysis buffer (25 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 2 mmol/L MgCl₂, 0.5% NP-40, and 5 mmol/L dithiothreitol). H4 cells were harvested 48 hours after transfection. Cell lysates were subjected to preclearance by incubation with pre-blocked protein G beads at 4°C for 60 minutes. An aliquot of lysate after preclearance, but before any co-IP, was removed for total RNA and protein analyses. The remaining lysates proceeded to co-IP with either 2A8-Protein G beads or nonimmune mouse serum-protein G beads at 4°C for 90 minutes. After co-IP, the beads were washed at room temperature as follows: twice with lysis buffer; three times with lysis buffer containing 900 mmol/L NaCl and 1% NP-40; twice more with lysis buffer, and then the beads were transferred to fresh 1.5 ml tube, where there was one final wash with lysis buffer containing 0.05% NP-40. Following the washes, beads and lysates were subjected to DNase treatment by shaking/incubating at 37°C for 20 minutes with 250 μ l of DNA digestion solution containing 40 mmol/L Tris-HCl, pH 8.0, 10 mmol/L MgSO₄, 1 mmol/L CaCl₂, 200 U/ml RNasin, and 40 U/ml DNase I (Promega, Madison, WI). Co-IPed RNA, and also total RNA from lysates, were extracted using Trizol LS (Invitrogen, Carlsbad, CA) as described previously.²⁵

Microarray Analysis and Reverse Transcription Quantitative PCR

Microarray analysis of RNAs isolated from co-IP or from total lysates were performed using Affymetrix Human Gene 1.0 ST chip at University of Kentucky Microarray Core Facility. Eight different biological replicates from three individual experiments were performed for each transfection condition. For quantitative (q)PCR analysis, RNAs were reverse-transcribed with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD), amplified using TaqMan 2 $\times$ PCR Master Mix (Applied Biosystems, Branchburg, NJ) and SYBR as detector. qPCR was

performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Data presented show the average of triplicates from qPCR. Specific qPCR primers were as follows: *GRN* (Forward) 5'-ATGCTGTGTGCTGCGAGGATC-3', *GRN* (Reverse) 5'-GCGCCGATCAGCACAAACAGA-3'; *BACE1* (Forward) 5'-GAC TTCACAAATGTTGGCCTGTCA-3', *BACE1* (Reverse) 5'-CAGTCCGAGAATAACAACATAACCAG-3'; *beta actin* (Forward) 5'-GCGAGCATCCCCAAAGTTCACA-3', *beta actin* (Reverse) 5'-GGCACGAAGGCTCATCATTCA-3'.

Glucose Treatment and GRN Detection in Cell Culture Media

Glucose treatment for H4 cells follow the method previously described.⁴⁹ H4 cells were incubated for 3 hours in Dulbecco's modified Eagle medium (without serum and glucose) containing 2 mmol/L sodium pyruvate. Cells were then incubated in either the same medium, or with added 10 mmol/L glucose. Cell culture media and cells were harvested after 16 hours treatment. Cell culture media were spun briefly to remove residual cells. GRN levels in cell culture media were determined using an enzyme-linked immunosorbent assay (ELISA) as described previously.⁵⁰

Reporter Assays of GRN mRNA Putative miR-107 Binding Sites in Response to Glucose Treatment

To assess the response of a putative miR-107 binding sequence (nucleotides 892–974 on NM_002087.2) in *GRN* to glucose, we first co-transfected H4 cells with pRL-TK reporter constructs bearing miR-107 MRE on *GRN* open reading frame (*GRNwt*) or its mutant (*GRNmut*), six replicates each. Cell culture medium was changed after 24 hours, and the cells were further incubated for 24 more hours. Glucose treatment was performed as described above, followed by conventional Dual Luciferase Reporter Assays. Data shown represents two individual experiments.

Tissue Culture Immunocytochemistry and Semiquantitative Scoring of Staining

30,000 H4 cells were seeded per well onto Lab-Tek II chamber slide system (Nalgene Nunc, Rochester, NY). The following day, transfection of miR-107 and negative control were performed as described above. 48 hours later, the media was aspirated and cells washed and then fixed using 4% paraformaldehyde (10 minutes; Fisher, Pittsburgh PA). Cells were permeabilized with 0.3% Triton-X-100 (Fisher, Pittsburgh, PA) in PBS. Antibody blocking was performed in 5% normal horse serum (Jackson ImmunoResearch, West Grove, PA) in PBS, for 1 hour at room temperature. Blocking solution was aspirated and the cells were incubated overnight at 4°C in a primary antibodies including anti-GRN (1:300; same as de-

scribed above) and anti-human GRP 78/BiP antibody (1:500; Cat. No. G8918, Sigma-Aldrich) diluted in 1% normal horse serum in PBS. The next day, cells were washed (PBS × 3) and incubated in dark for 2 hours, with two secondary antibodies, diluted in 1% normal horse serum: Texas Red conjugated IgG (Code No. 805–075–180, Jackson ImmunoResearch) and DyLight 488 conjugated IgG (Code No. 711-487-003, Jackson ImmunoResearch) were used at 1:800 and 1:500. Following additional PBS washes, slides were mounted in Vectashield (H-1000, Vector Laboratories Inc, Burlingame, CA). Stained slides were viewed by a researcher blinded to the treatment and staining protocols who took photomicrographs using a ×60 objective, using both Texas Red and fluorescein isothiocyanate filters, for a total of 180 different high-power fields (1483 cells total) encompassing a number of controls, only a subset of which (253 cells) were double-labeled for GRN and GRP78. These images were then analyzed blindly by a different researcher who assessed image intensity, granularity of staining, and co-localization of granules on a semiquantitative scale (0 to 10).

Controlled Cortical Impact Injury and Mouse Brain in Situ Hybridization, Immunohistochemistry, and Quantification of Staining

Male C57BL/6 mice ($n = 7$; 20 to 25 g) were anesthetized using isoflurane and subjected to 1.0 mm depth controlled cortical impact brain injury over the left parietal cortex as previously described in detail.^{51,52} All procedures involving animals were approved by the University of Kentucky Institutional Animal Care and Use Committee. Mouse brain *in situ* hybridization for miR-107 was performed as described in detail^{7,53,54} on perfusion-fixed (4% paraformaldehyde) mice brains that were cut on a freezing microtome. Mice were euthanized 24 hours after injury, and brains were prepared for staining as previously described.⁵² Briefly, 40 μ m-thick sections were stained with 0.5% Cresyl Violet (Nissl stain) or immunolabeled for GRN. After an overnight incubation with anti-GRN antibody (Goat polyclonal, 1:300, R&D systems, MN), sections were incubated in biotin-conjugated IgG (Jackson ImmunoResearch, PA) followed by avidin-biotin-enzyme complex (Vector Laboratories, CA).

For image analyses, one brain section per animal was selected from the epicenter of the cortical impact (−1.80 mm Bregma). A digital image of this coronal section was captured at ×1.25 magnification using an Olympus AX80 microscope. On sections stained for GRN, mir107 and Nissl substance, a researcher blinded to the experiment traced the pyramidal layers of CA1 and CA3 subfields separately within each hippocampus, contralateral and ipsilateral to the impact. The total staining intensity was quantified within each region on GRN or mir107 stained sections using Image-Pro Plus software (version 6.1.0.346, Media Cybernetics, Inc). The total area of Nissl-stained cells in each pyramidal layer region was then quantified. GRN and mir107 staining intensities were normalized to the total

cellular area to correct for potential differences in cell density across animals.

Results

RIP-Chip Assay Procedure

RIP-Chip experiments were performed on H4 cancer cells,⁵⁵ which have a partial glial/neuronal phenotype.⁵⁶ H4 cells were first transfected with miR-107 and various miRNA transfection controls. A Northern blot analysis on co-IPed miRNPs showed that transfected miRNAs were incorporated specifically into the miRNP (Figure 1A). MiRNPs were co-IPed with anti-AGO antibodies bound to protein G-agarose beads. RNA associated with AGO protein complexes were then isolated for microarray profiling, to identify transcriptome-wide miR-107 targets in H4 cells.

After miR-107 transfections were shown to be successful, Affymetrix GeneChip microarrays were used to profile mRNAs that associated with miRNPs following miR-107 transfection. RNAs isolated from co-IPed miRNPs were at very high quality as determined by RIN (RNA Integrity Num-

ber 10.0) in all samples. Eight different biological replicates, in three different batches, were performed for both miR-107 and control miRNAs. RIP-Chip indicated consistently—in every experiment without exception—that *GRN* was the mRNA target most strongly incorporated into the miRNP following miR-107 transfection (Figure 1, B–C). By contrast, *GRN* mRNA was not enriched in miRNPs following transfections with miR-124, miR-128, or miR-320 (data from RIP-Chip with these other miRNAs, and more rigorous assay validation, will be published in a separate manuscript). There was a consistent ~50-fold enrichment in miRNPs after miR-107 transfection, with less *GRN* mRNA in the lysate. This represented an extreme enrichment considering that over 99.9% of the mRNAs were enriched <4-fold, and most not at all, in the assay. Since *GRN* mRNA levels were above average in the miRNP before miR-107 transfection, the fold-change results are not due to an artifact of low

Figure 1. RIP-Chip identified *GRN* as a novel target of miR-107. **A:** Northern blots on RNA from H4 cells harvested two days after transfection and following anti-AGO co-IP show that transfected miR-107 and miR-320 were incorporated specifically into miRNPs. **B:** RIP-Chip identifies *GRN* mRNA as the strongest target recruited to the microribonucleoparticle (miRNP) following transfection with miR-107. The relative enrichment for *GRN* in the miRNP following miR-107 transfection is >50-fold, which held consistently across all experiments. The vast majority of mRNAs were not enriched in the miRNP following miR-107 transfection (see also Supplemental Figure S1 at <http://ajp.amjpatbol.org>). **C:** *GRN* and the other top 10 enriched mRNAs following miR-107 transfections. Levels of these miR-107 targets in miRNPs following miR-320 transfection are shown in parallel. Relative levels of mRNA in the cell lysates before the co-immunoprecipitation are also listed. The right-most column shows whether these mRNAs were predicted as miR-107 targets by computational miRNA target prediction websites MicroCosm (MC), TargetScan (TS), or PicTar (PT).^{62–64}

A Western blots after miRNA transfections

B mRNAs levels in lysate and anti-AGO co-IP: effect of miR-107 transfection

Figure 2. Western blots and mRNA analyses. MiRNA transfections and Western blots were used to evaluate the effects of miR-107 transfections on gene products identified by RIP-Chip. Two days after transfection with miR-107 or negative control miRNAs, H4 cells were harvested and Western blots were performed. **A:** Co-transfection of pCMV6-*GRN* and pCMV6-*BACE1* with miR-107 or negative control miRNA showed that relative to the control miRNA, *GRN* and *BACE1* proteins were knocked down in cells after miR-107 transfection, but β -*Actin* protein levels were not changed. **B:** Results of microarray experiments comparing miR-107 transfection versus negative control miRNA, for “housekeeping” genes *GAPDH* and β -*Actin*, *GRN*, *BACE1*, and two other genes relevant to neurodegenerative disease, *APP* and *MAPT*. Each bar represents three biological replicates; error bars are SD. Only *BACE1* and *GRN* show decreased mRNA in the cell lysates (* P < 0.01) following miR-107 transfection relative to a negative control miRNA transfections, and only *GRN* shows an enrichment in the miRNP following miR-107 transfection (arrow: P < 0.0001). *BACE1* was not enriched in miRNPs.

mRNA levels at baseline (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

To test whether the changes seen in RIP-Chip are manifest also by altered protein levels in tissue cultured cells, Western blots were performed after miR-107 co-transfections with full-length GRN and BACE1 expressing plasmids. Augmenting miR-107 leads to strong reduction in GRN expression (Figures 2A, and 3). There was also a clear knock-down of previously identified miR-107 target BACE1²⁵ protein levels after miR-107 transfection.

The top ten miR-107 targets identified by RIP-Chip are shown in Figure 1C (see Supplemental Table S1 at <http://ajp.amjpathol.org> for the complete list of miR-107 targets). These are mRNAs that show decreased levels of mRNAs in the lysate and enriched mRNA levels in the miRNP. The correlation was poor between RIP-Chip identified genes and the computerized prediction algorithms for these top targets (Figure 1C). Of the strongest miR-107 targets identified by RIP-Chip, only two were predicted to be miR-107 target according to computational algorithms that assume 3'UTR miRNA binding. Further studies were performed to provide context and validation for the exceptional recruitment of *GRN* mRNA into the miRNP following miR-107 transfection in H4 cells. mRNA from the co-IPs and the lysates (total RNA isolated before co-IP) were evaluated separately via microarrays. Figure 2B shows how the mRNA levels were affected after miR-107

transfection relative to a different control miRNAs according to the microarray data. Included in the analyses were "housekeeping" genes (*GAPDH* and β -actin) and genes related to neurodegenerative diseases (*APP*, *BACE1*, and *MAPT*). We have previously shown that miR-107 targets *BACE1*²⁵. Although miR-107 transfection leads to decreased *BACE1* mRNA in the lysate, there is no increase of *BACE1* mRNA in the miRNP following miR-107 transfection (see Discussion).

Examining miR-107:GRN mRNA Interaction Using Additional Transfection Experiments

To further characterize the interaction between miR-107 and the *GRN* mRNA, we analyzed the results of miRNA transfections on endogenous GRN expression (Figure 3, A and B). In both H4 cells and HeLa epithelial cells, miR-107 transfection but not a negative control miRNA or miR-320 caused endogenous GRN expression to decrease. Higher GRN expression was seen in cells transfected with a miR-107 inhibitor but not miR-320 inhibitor. Next, we tested further the sequence determinants of miR-107 inhibition of GRN expression using seven different miRNAs (Figure 3C). Sequences of transfected miRNAs are shown. miR-103 differs from miR-107 only at a single nucleotide near the 3' end. A more distant paralog also expressed in humans, miR-195, shares the 5' seed region with miR-107 but is otherwise dissimilar. Two additional, nonphysiological miR-107 derived mutant miRNAs were synthesized for transfection experiments: "miR-

Figure 3. miR-107 interactions with *GRN* mRNA appear to be mediated through the 5' seed region of miR-107. Transfections of miRNA confirm that miR-103 and miR-107 target *GRN*. Endogenous GRN protein expression levels are detected in H4 cells and HeLa cells. **A:** Western blot analysis demonstrate that miR-107 transfections, but not a control miRNA in both H4 and HeLa cells, cause knockdown of endogenous GRN without affecting β -actin levels. **B:** Higher GRN expression is seen after miR-107 inhibitor transfection, whereas miR-320 inhibitor does not increase GRN expression. **C:** To better understand the sequence determinants of miR-107:GRN interaction, multiple miRNA sequences were assessed; sequences of transfected miRNAs are shown (upper panel). The 5' seed regions (nucleotides 2 to 7) are indicated. At bottom, Western blots show GRN protein expression after miRNA transfections. Note that all of the transfected miRNAs with the 5' seed region of miR-107 knock down GRN.

Figure 4. miR-107 interacts with the open reading frame of *GRN* mRNA. **A:** *GRN* mRNA cartoon, with the 5'UTR, open reading frame, and 3'UTR roughly to scale. Note the relatively small 5'UTR and 3'UTR. Most computational miRNA target prediction databases with miRNA prediction algorithms do not predict that *GRN* is a target for miR-107. However, sequences complementary to the 5' seed of miR-107 are present in the open reading frame and conserved from zebrafish to humans. **B:** To examine the target location of miR-107, full-length cDNA and only open reading frame expressing pCMV6-XL5 plasmids of *GRN*, *MAPT*, and *SNCA* were co-transfected with miR-107 or a negative control miRNA. Western blot analyses were performed to examine miR-107 effects on these proteins. GRN expressed from both full-length and open reading frame carrying plasmids were clearly knocked down by miR-107, indicating that miR-107 target sites are located in the open reading frame of *GRN*. Expression of MAPT and SNCA from either full length or open reading frame constructs were not affected by miR-107, indicating that miR-107 targeting *GRN* is specific and not related to the plasmid vector.

107 Mut 1" differs from miR-107 in the 5' seed region, whereas "miR-107 Mut 2" differs from miR-107 in the 3' portion. Two other control miRNAs, miR-320 and a negative control miRNA, were also tested. These experiments show that the 5' seed region of miR-107 is necessary to induce strong knockdown of the full-length *GRN* mRNA, whereas the 3' region of miR-107 contributes less.

The next question related to the sequence(s) in *GRN* mRNA that are recognized by miR-107. Full length *GRN* mRNA comprises ~2300 nucleotides (nts). As shown in Figure 4A, *GRN* mRNA has a relatively short 3' UTR—fewer than 300 nts, versus ~750 nts average for human mRNAs.⁵⁷ The 5' seed sequence of miR-107 (GCAGCA) should bind an antiparallel complementary target mRNA sequence (UGCUGC) in *GRN* mRNA. An extraordinary number of these sequence iterations are present in *GRN* mRNA—UGCUGC (20 iterations) and UGCUGU (11 iterations; the latter uridine could allow miRNA hybridization through G:U binding²¹). For perspective, β -Actin mRNA (~1800nts) has only one each of UGCUGC and UGCUGU sequences, and *APP* mRNA (~3600nts) has four UGCUGC and one UGCUGU sequence.

UGCUGC sequences are present in the open reading frame of human *GRN* mRNA (Figure 4A). This suggests a testable hypothesis: miR-107 may target open reading frame sequences. pCMV6-XL5 expression plasmids were constructed to carry only the open reading frames of *GRN*, *MAPT*, and *SNCA*. (*MAPT* and *SNCA* were chosen only because each is associated with neurodegenerative diseases.) As with the full-length *GRN* cDNA clone, *GRN* protein expressed from a plasmid containing only the open reading frame of *GRN* mRNA were also

down-regulated after miR-107 co-transfection. In contrast, *MAPT* and *SNCA* protein expressed from pCMV6-XL5 plasmids carrying either full-length cDNA or only open reading frames were not down-regulated by miR-107 (Figure 4B). This experiment indicates that the effect of miR-107 on *GRN* mRNA is specific, and at least some miR-107 target sequences are present in the mRNA's open reading frame.

We tested directly whether a sequence in the human *GRN* mRNA containing three of the UGCUGC tandems may constitute a target for miR-107. Dual luciferase reporter assays were used (Figure 5). An 82 nts portion of the *GRN* mRNA open reading frame (*GRNwt*), corresponding to nts 892–974 on NM_002087.2, was subcloned into the 3' UTR of pRLTK-*Rluc* plasmid (Figure 5A, top). The same plasmid construct with only nine different nucleotide changes (*GRNmut*), but with identical overall A/G/C/U content, was created as a control (Figure 5A, bottom). *Rluc* plasmids were co-transfected with miR-107, a negative control miRNA, or a miR-107 inhibitor. The *GRNwt* reporter construct, but not *GRNmut*, had luciferase expression knocked down by miR-107 (Figure 5B). These results provide support for the hypothesis that the UGCUGC iterations in the *GRN* mRNA open reading frame are target sites of miR-107.

As they are potential miR-107 targets, *GRN* mRNA's 31 UGCUGC and UGCUGU sequences can be the basis for

Figure 5. Validation of miR-107 potential target sites in *GRN* mRNA open reading frame. Dual luciferase reporter assays were used to validate miR-107 target sites on *GRN* mRNA. **A:** A sequence in the *GRN* mRNA open reading frame (nucleotides 892 to 974 on NM_002087.2) that contains three UGCUGC iterations (top), and a mutated sequence (bottom), were subcloned into the 3'UTR of *Renilla luciferase* (*Rluc*) reporter constructs. UGCUGC iterations are indicated with blue lines. Note that in the *GRNmut* plasmid, there are only three 3-nucleotide sequences changed (green sequences in *GRNwt* were changed to red sequences in *GRNmut*), to alter the predicted seed-binding sequences. **B:** Reporter plasmids were co-transfected with a negative control miRNA, miR-107, or miR-107 inhibitor ($N = 3$ for each condition, with PGL3 transfections to control for transfection efficiency). Luciferase signal, adjusted for transfection efficiency, is in arbitrary units. In these experiments, *Rluc* reporter with the *GRNwt*-containing sequence is knocked down with miR-107 transfection, but not the *GRNmut*. * $P < 0.001$ and ** $P < 0.1$ using Student's *t*-test.

FLAG-AGO co-IPs show enrichment for *GRN* mRNA with AGO2 miRNPs

Figure 6. Differential association of *GRN* with FLAG-tagged human Argonaute (AGO). FLAG-tagged AGO constructs⁵⁸ were co-transfected with miR-107 to test for differential distribution of miR-107 targets *GRN* and *BACE1* in different AGO paralogs. Following co-transfections, lysates were co-IPed using either anti-FLAG (AF) antibody or nonimmunized mouse serum. RNAs that associated with miRNPs were subjected to reverse transcription-qPCR. qPCR results were normalized to total RNA levels. Note that *GRN* mRNA is strongly associated with AGO2, followed by AGO1. By contrast, neither *BACE1* nor β -Actin mRNAs are enriched in the FLAG-AGO co-IPs. These data suggest different pathways for miR-107 targeting *BACE1*. miR-107 regulation of *BACE1* may be through a different mechanism than that of *GRN* because *BACE1* mRNA does not apparently associate stably with AGO 1–3 in H4 cells. Western blots analysis using AF antibody are shown for co-IPed miRNPs.

estimating preliminarily the phylogenetic conservation of the miR-107:GRN mRNA interaction. Sequence alignments were made between human, frog, and zebrafish GRN mRNAs to encompass widely-divergent vertebrate phyla. (miR-103 and miR-107 sequences are identical in humans, frog, and zebrafish.) Remarkably, 14 of the 31 human UGCUGC/UGCUGU sequences were also conserved between these three divergent species (Figure 4A and see Supplemental Figure S2 at <http://ajp.amjpathol.org> for complete alignment data). Each of these potential miR-107 recognition element sequences was in the GRN mRNA open reading frame. Together with our transfection experiments, these data indicate that miR-107 interacts with GRN mRNA at least partly through the 5' miRNA seed and the open reading frame GRN sequences underlying this interaction appear well-conserved among vertebrate species.

We caution that both UGC and UGU are codons for the amino acid cysteine, and GRN is a highly cysteine-rich protein. However, most other top mRNA targets for miR-107 are not cysteine-rich proteins, and of 16 mRNAs on the Affymetrix GeneChip Human Gene 1.0 ST microarray with "cysteine-rich" in their gene name, none was enriched in the miRNP after miR-107 transfection relative to controls (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

Testing Different AGO miRNPs for miR-107: GRN mRNA

Four different paralogous AGO proteins (AGOs 1–4) are expressed in humans. We examined whether our anti-AGO antibody, which was raised against AGO2,⁴⁸ was

Figure 7. Glucose treatment is associated with decreased GRN secretion at least partly mediated through miR-107. H4 cells were transfected with 25 nmol/L miR-107 or a negative control miRNA precursor for 48 hours. Control cells were incubated in Dulbecco's modified Eagle medium cell media without glucose (NG) or supplemented with 10 mmol/L glucose. Glucose treatment has previously been shown to increase miR-107 expression.⁴⁹ GRN in media was measured by ELISA, and the miR-107 levels in the cells were determined by qPCR with values normalized to 5S RNA. miR-107 levels (yellow bars) were up-regulated in response to glucose supplementation in cells transfected with control miRNA. Inversely, GRN (blue bars) was reduced in cell media following glucose supplementation. In cells transfected with miR-107, GRN levels in media are lower than in control NG, but GRN levels did not differ between NG and Gluc treated cells ($P > 0.05$). Data are average (relative to control) of two representative experiments with four replicates each. Statistical data were analyzed using Student's *t*-test. Comparing ELISA values, glucose versus NG: * $P < 0.01$; *** $P < 0.0001$. Comparing miR-107 values, glucose versus NG: ** $P < 0.006$; Error bars are standard errors of the means.

causing bias in terms of detecting AGO-bound GRN and BACE1 mRNAs. miR-107 was co-transfected with plasmids that contain FLAG-tagged AGO constructs that were described previously.⁵⁸ AGO4 is only lowly-expressed at the mRNA level in H4 cells and FLAG-tagged AGO4 did not express well in our hands despite multiple attempts (data not shown). GRN, BACE1, and β -Actin mRNA were not significantly bound to FLAG-AGO4 in those experiments (data not shown). Thus, only results of AGO1-AGO3 experiments are shown (Figure 6).⁵⁸ FLAG-AGO3 expresses at lower level than FLAG-AGO1 and FLAG-AGO2. However, downstream reverse transcription-qPCR was performed normalizing the overall RNA levels following anti-FLAG co-IPs. Following miR-107 transfections, the highest concentration of GRN mRNA was seen in FLAG-AGO2 co-IP, followed by FLAG-AGO1. mRNA for β -Actin and BACE1 were not detected in significant amounts in any FLAG-AGO co-IP. These data indicate that GRN mRNA appears to associate mainly with AGO2 in miRNPs.

The Effect of miR-107 on GRN Expression in Tissue Culture

Tissue culture experiments were used to determine whether stimuli known to affect miR-107 also cause altered GRN secretion in cultured cells (Figure 7).⁴⁹ The amount of GRN protein in the cell culture media was measured using an ELISA assay.⁵⁰ Transfection of miR-107 in H4 cells resulted in decreased GRN levels ($P <$

Figure 8. Glucose treatment in H4 cells resulted in reduced luciferase activity of reporter construct bearing GRN miR-107 recognition element. H4 cells were first transfected with reporter plasmids (nucleotides 892–974 on NM_002087.2, GRNwt and GRNmut, $N = 6$ for each condition) along with transfection control plasmid pGL3. Cells were allowed to recover before 16-hour glucose treatment (10 mmol/L or none). Dual luciferase reporter assays were performed. Relative GRNwt was normalized to GRNmut and taken as 100% under "No Glucose" conditions. Data represents two individual experiments. Corrected luciferase signal is in arbitrary units. *Rluc* reporter with the GRNwt-containing sequence is knocked down relative to GRNmut in cells with 10 mmol/L glucose. Error bars are SD. * $P < 0.01$ using one-tailed Student's *t*-test.

10^{-5}) compared with GRN levels in negative control miRNA transfections. A prior study showed that changing glucose levels in tissue cultured cells leads to a selective increase in miR-107 expression.⁴⁹ We used this paradigm for H4 cells and hypothesized that miR-107 increase would cause decreased GRN expression. As expected, glucose treatment led to increased miR-107 expression relative to cells with no glucose supplementation ($P < 0.003$). The ELISA assay confirmed that GRN levels were decreased following glucose supplementation ($P < 10^{-5}$). We compared the effects of glucose supplementation on GRN expression, in cells transfected with miR-107 relative to cells transfected with a control miRNA. Glucose supplementation did not further reduce GRN levels in miR-107 transfected cells, probably because miR-107 levels are very high and GRN is low in both of these conditions. These data suggest that the effect of glucose supplementation on GRN level is mediated at partly through miR-107. Finally, we hypothesized that glucose supplementation would lead to increased miR-107 which would knock down the luciferase reporter construct with the subcloned sequence corresponding to the open reading frame of GRN (nucleotides 892 to 974 on NM_002087.2). These experiments (Figure 8) show that the *GRNwt* mRNA derived sequence (versus the subtly-mutated *GRNmut*) indeed leads to specific attenuation of the reporter protein following glucose supplementation.

Since miR-107 transfection was associated with decreased GRN secretion, we hypothesized that miR-107 transfection might lead to altered cellular processing of GRN. Immunocytochemistry was performed on H4 cells (Supplemental Figure S3 at <http://ajp.amjpathol.org>). These studies showed that in cells transfected with a control miRNA (or no miRNA), the GRN immunocytochemistry showed a granular staining pattern that could be present throughout the cells' cytoplasm. Following miR-107 transfection, the staining became less granular throughout the cytoplasm and tended to be found in a "huff"-type pattern near to the cell nuclei (Supplemental Figure S3 at <http://ajp.amjpathol.org>).

Studies of miR-107:GRN in the Mammalian Brain

We sought to evaluate preliminarily whether miR-107 may contribute to mammalian brain *GRN* regulation. A basic consideration relates to the level of miRNA expression in the human brain. It has been shown previously that miR-659 may also regulate *GRN* in the human brain.⁴⁶ miRNA profiling experiments were performed to determine the expression levels of miR-107 relative to miR-659 and to other miRNAs in human cerebral cortex. RNA was isolated from gray matter from the superior and middle temporal cerebral cortical gyri (Brodmann Areas 21 and 22) of ten different nondemented human brains as described.^{25,56} cDNA miRNA array experiments were then performed as described previously in detail.^{59,60} The arrays demonstrate that miR-107 is expressed at far higher levels than miR-659 in this brain area (array results

and patient characteristics are shown in see Supplemental Figure S4^{59,60,93} at <http://ajp.amjpathol.org>).

An *in vivo* model was used to test whether a stimulus that affects miR-107 levels would also affect GRN protein expression (Figure 9, A–J).⁶¹ A prior study showed that miR-107 is specifically decreased in hippocampal Cornu Ammonis (CA) fields subjacent to cortical traumatic brain injury (TBI) at 24 hours after the injury.⁶¹ We hypothesized that this could be a model to test preliminarily if changes in miR-107 are associated with changes in GRN expression. We performed depth controlled cortical impact brain injury (1.0 mm) in the parietal cortex of mice ($N = 7$). A day after the injury, the mice were sacrificed and stained for miR-107 using *in situ* hybridization. As expected, neuronal miR-107 expression was decreased in the hippocampal CA1 and CA3 in the side ipsilateral to the injury, relative to the contralateral side and out of proportion to the change in neuronal staining using Nissl stain (Figure 9). Immunohistochemical stains were performed with a GRN antibody on near-serial sections. In inverse correlation to the decrease in neuronal miR-107 staining, the GRN protein expression showed an augmentation on the side ipsilateral to the injury. Note that the GRN expression is augmented in CA3 hippocampal neurons ipsilateral to the injury and is not only seen in leukocytes and microglial cells.

Discussion

The current study provides evidence that miR-107 helps regulate GRN expression in human cells. We were initially interested in assessing miR-107 targets due to prior experiments showing miR-107 is down-regulated in AD cerebral cortical gray matter.²⁵ Surprisingly, RIP-Chip assays revealed that *GRN* mRNA is the strongest miR-107 target transcribed in cultured H4 cells. Further experiments support the hypothesis that the miR-107:GRN interactions are physiologically important (see Figure 10). miR-107 regulation of *GRN* appears to be relevant to glucose metabolism in cultured H4 cells, and we also found a correlative relationship between miR-107 and GRN expression in a mouse model of TBI. These experiments have implications about neurodegenerative diseases, cancer, and tissue-level responses to injury.

In addition to describing a novel mechanism of GRN regulation, our data also provide new information about how miRNAs interact with mRNAs. Since we had previously formulated hypotheses of miRNA targeting based on computational predictions, the observation that miR-107 targets *GRN* was unexpected. Moreover, only two of the top fifteen strongest miR-107 targets detected in the RIP-Chip assay are predicted according to *MicroCosm*, *TargetScan*, or *PicTar* computational algorithms^{62–64} (other computational algorithms, *ma22*²¹ and *RNAhybrid*⁶⁵, were also tested, and these did predict miR-107/*GRN* binding although the predicted binding specificities were unclear). Furthermore, miR-107 appears to target *GRN* through sequences located in the open reading frame of *GRN* mRNA. The tendency for miRNAs to target

Figure 9. Assessing miR-107 and GRN expression in response to mouse traumatic brain injury (TBI). In 1.0-mm depth-controlled cortical impact experiments, miR-107 expression is decreased and GRN protein is increased in hippocampal neurons. A prior study had shown that hippocampal fields in TBI show decreased miR-107 expression 24 hours after injury.⁶¹ We hypothesized that diminished miR-107 expression would correlate with increased GRN protein levels. Note that in a representative case (A–I) miR-107 levels (A–C) are attenuated in ipsilateral hippocampal CA1 and CA3 fields, and GRN levels (D–F) are increased in near-serial sections, relative to the side contralateral to the injury. Higher-power photomicrographs (B, C, E, F, H, I) were taken from CA3 in an area indicated (G). Scale bars = 500 μ m (A, D, G) and 50 μ m (B, C, E, F, H, I). These changes were measured using unbiased methods in seven different mice. Only the hippocampal pyramidal layers (CA1 and CA3) were evaluated to prevent bias from the GRN-immunopositive microglial cells. Results of the quantitation are shown (J). In this TBI model, miR-107 expression decreased relative to the contralateral side and GRN increased (* P < 0.05 by Student's t -test) in near-serial section hippocampal fields, compatible with the hypothesis that GRN expression changes are associated with altered miR-107. The intensity of staining per unit area is expressed as mean $\pm$ SEM.

regions outside the 3'UTR has been described but is not known as a widespread phenomenon.^{66,67}

There has been prior evidence of different AGO proteins associated with distinct miRNA targets.^{68,69} The strong association between AGO2 to *GRN* mRNA (especially after miR-107 transfection) provides greater detail about the molecular pathway of miR-107 targeting on GRN. This is the first example we know of a strong miRNA target that is associated preferentially with AGO2 in human neural-derived cells.

After determining that *GRN* gene expression is regulated in part by miR-107, we sought evidence as to

Figure 10. A schematic diagram about miR-107 and GRN. miR-107 and GRN may be important in linking glucose metabolism and brain disease, including neurodegeneration and brain trauma.

whether the interaction was physiologically relevant. Prior studies point to roles of miR-107 in cellular metabolism,^{49,70} with the caveat that naming a single discrete cellular function for a miRNA can be overly simplistic. Levels of miR-107 expression in cultured cells are relatively high in high-glucose or low-folate culture media conditions.^{49,71} In all known vertebrates, the genes for miR-107 and paralogous miR-103 reside within introns of pantothenate kinase paralog genes. Pantothenate kinase polypeptides catalyze the enzymatic rate-determining step in acetyl-CoA formation.⁷⁰ Since most of the detection and functional assays would show extensive overlap with regard to miR-107 and miR-103 (see Figure 3), further discussion will refer to “miR-103/107” to include these genes together. When we perturb levels of glucose in the media of H4 cells, we found evidence that glucose metabolic pathways may recruit miR-103/107 to regulate *GRN* expression. Significantly, transfection of miR-107 into H4 cells led to lower GRN expression to such a degree that further regulation of GRN by glucose was no longer detectable.

Unlike miR-103/107, GRN has been studied extensively for decades. GRN has been implicated in numerous human illnesses including neoplastic and neurodegenerative disease. In multiple human cancers, including some brain cancers, GRN is a vital growth factor and/or demonstrates high expression.^{72–76} miR-107 has been shown to be repressed epigenetically in some cancer cells,⁷⁷ which could cause substantially increased GRN protein levels—thereby potentiating tumor growth. Inhibition of miR-107 has been shown to cause increased growth of human lung adenocarcinoma cells,⁷⁸ perhaps through this mechanism.

Genetic and pathological roles for *GRN* in neurodegeneration have also been suggested. We found miR-107 decreased in AD brains.²⁵ It would be interesting to know if this change correlates with altered neuronal *GRN* regulation that may contribute to AD pathogenesis.^{79,80} However, GRN protein is expressed highly in glial and inflammatory cells, which are up-regulated in AD brain.⁸¹ Tissue-level analyses (eg, immunoblots of total brain ho-

mogenates) would thus be in appropriate to study the connection between miR-107 and GRN expression because the question of cell type-specific GRN expression cannot be distinguished. *GRN* germline mutations are responsible for a subset of frontotemporal dementia (FTLD),^{43,44,82} which is a devastating neurological disease. In FTLD cases with *GRN* mutations, inheritance follows an autosomal dominant pattern and pathogenesis is thought to be due to *GRN* haploinsufficiency rather than a toxic gain of function mechanism.^{83,84} It is unknown whether miR-107 factors into that regulation. Also unknown is which of the pathways involving *GRN* are specifically responsible for the neuronal loss that occurs in FTLD. The RNA processing protein TDP-43 is aberrantly deposited in FTLD brains with *GRN* mutations so these cases are termed FTLD-TDP (although many FTLD-TDP cases are not due to *GRN* mutations).^{85–87} In the current study, *TDP-43* mRNA is transcribed at moderate-to-high levels in H4 cell lysates (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Using the RIP-Chip assay, we did not find *TDP-43* mRNA incorporated into the AGO-miRNP, either after miR-107 or other miRNA transfections. A prior study provided support for the hypothesis that *GRN* is regulated by a human brain miRNA; a single nucleotide polymorphism in the *GRN* 3'UTR can raise FTLD risk because that polymorphism alters miR-659 regulation of *GRN*.⁴⁶ In the present study we show that expression levels of miR-103/107 is far higher than miR-659 in the temporal cortex of ten elderly nondemented persons. Nevertheless, it is still possible that both miR-103/107 and miR-659 act on *GRN*.

In contrast to the roles played in human diseases, GRN may contribute to wound healing and other normal brain functions.^{42,47,81} Here we found correlative support in a mouse model for the hypothesis that miR-107 expression decrease may contribute to an increased expression of GRN after TBI. We performed these experiments because it was shown previously that miR-107 is down-regulated in rodent TBI.⁶¹ GRN protein expression is altered in ventral horn neurons in a mouse sciatic nerve axotomy model.⁸⁸ Further, there is abundant evidence that GRN normally functions in wound repair outside the brain.^{47,74,89} We note that RIP-Chip identifies strong miR-107 targets—for example, meteorin and ninjurin—that are involved in axonal regeneration and extension.^{90–92} For these reasons, it is tempting to speculate that miR-107 plays a role in modulating neuronal repair and regeneration in the mammalian brain through molecular regulation of *GRN* and other mRNA targets.

Although strong regulation of *GRN* by miR-103/107 was demonstrated in the current study, there are some limitations to our approach. We used RIP-Chip assays on H4 cells. Derived from a brain tumor diagnosed as “neuroglioma” (a tumor classification no longer in use), H4 cells express a miRNA profile that more closely resembles cultured astrocytes than cultured neurons.^{55,56} As with any particular cell type, there are unavoidably many genes that are untranscribed that we could not evaluate for miRNA binding in the current study.

Aside from the inherent limitations of the cells used, RIP-Chip assays do not provide all of the information

about miRNA:mRNA interactions. AGO-miRNPs have been shown to exert gene expression regulation via multiple mechanisms.^{17,93} For the mRNA to be “read out” on a microarray downstream of the anti-Ago co-IP, the target mRNA must be stably bound to the miRNP. When AGO2 works as a “slicer” to enzymatically cleave target mRNAs,⁹⁴ one would expect a less stable miRNA:mRNA association and hence lower yield of mRNA targets. We note that *BACE1* mRNA, which we have shown is targeted by miR-103/107 (including a description of a specific miRNA recognition element in *BACE1* mRNA),²⁵ is not specifically enriched in the RIP-Chip assay. However, we showed that *BACE1* mRNA is specifically reduced in the lysates of miR-107 transfected cells, and miR-107 transfections indeed knock down *BACE1* at the protein level. These observations are compatible with the hypothesis that a distinct mechanism other than stable AGO association is involved in miR-107:*BACE1* regulation. Given the known multiple avenues of miRNA-based gene expression regulation,^{17,93,95} these results suggest that there are alternative pathways of miR-107 gene expression regulation. These pathways are a focus of ongoing research in our laboratory and may involve “slicing” or destabilization of *BACE1* mRNA, a more tenuous association between *BACE1* mRNA and AGO, or some other mechanism(s). Our results underscore once again that small noncoding RNAs are relevant to human disease, and that there is much yet to be learned about this complex mode of gene expression regulation.

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