**INTRODUCTION**

Parkinson’s disease (PD), the second-most common neurodegenerative disease, is accompanied by dopaminergic neuronal death in the substantia nigra pars compacta. Although most PD cases are sporadic, considerable effort has been devoted to studying the function of PD-associated genes to gain insights into the onset and progression of PD.

PTEN-induced putative kinase 1 (PINK1) is a PD-related gene that, when mutated, causes an autosomal recessive, early-onset PD [1]. PINK1 has pro-survival, anti-apoptotic and cytoprotective functions [2]. PINK1 regulates ATP generation, oxygen consumption [3-5], and reactive oxygen species (ROS) production [6] through regulation of mitochondrial function [7-10]. In addition, PINK1 acts through the AKT-mTOR pathway to regulate survival, proliferation, metabolism, and inflammation, among other cellular functions [11-16].

MicroRNAs (miRNAs) are small, noncoding RNA molecules...
that influence diverse biological functions, including brain development and diseases, through regulation of mRNA and protein expression. Expression of miRNAs is highly dynamic, and miRNAs are crucial for proper neuronal and glial differentiation during brain development [17-21]. Accordingly, deregulation of miRNAs has been reported in several types of neurodegenerative diseases [22-36]. In the brain of Alzheimer’s disease patients and aged individuals, miRNAs that regulate expression of β-site amyloid precursor protein (APP)-cleaving enzyme (BACE), including miR-107, miR-298 and miR-328, are decreased [25, 26]. In the case of Huntington’s disease, expression of miR-9/9*, which targets REST (repressor element 1 silencing transcription factor), is also decreased [27]. In multiple sclerosis, miR-155 and miR-326 are increased, but miR-15a, miR-15b, miR-181c and miR-328 are down-regulated [28]. miRNAs are also linked to PD. In the adult midbrain, specific depletion of Dicer, an endoribonuclease necessary for maturation of miRNAs, causes degeneration of dopaminergic neurons, suggesting that miRNAs maintain dopaminergic neurons in adulthood. Expression of miR-133b, which regulates maturation of dopaminergic neurons, is downregulated in PD patients [29]. miR-9 and miR-326 regulate human dopamine D2 receptor expression [30]. The G2019S mutant of LRRK2 (leucine-rich repeat kinase 2) inhibits the functions of let-7 and miR-184*, which cause neuronal toxicity by forcing cell-cycle progression in post-mitotic neurons [31]. Recently, miR-330 has been identified as a novel gene that is related to PD [32]. In addition, miR-7 and miR-153 have been shown to suppress expression of α-synuclein, a major component of Lewy bodies in PD patients [33-35], and miR-155 has been shown to mediate the anti-inflammatory effects of DJ-1 (PARK1), a PD-related gene [36]. In Drosophila, pathogenic G2019S and I1915T mutants of LRRK2 antagonize let-7 and miR-184*, and induce death of dopaminergic neurons [31].

In this study, we show that expression of miRNAs, particularly miR-326, miR-330 and miR-3099 increases during brain development and neural stem cell (NSC) differentiation. Interestingly, PINK1 deficiency decreases expression of these miRNAs during these processes. Collectively, these findings suggest that defects in the expression of these miRNAs could be linked to the development of PD caused by a PINK1 deficiency.

MATERIALS AND METHODS

Animals

PINK1-deficient mice were a generous gift from Drs. Zhuang (Chicago University) and Kang (Columbia University). PINK1-deficient mice were generated by replacing a 5.6-kb genomic region of the PINK1 locus, including exons 4-7 and the coding portion of exon 8, with a PGK-neo-polyA selection cassette flanked by FRT sequences, as previously described [37]. Heterozygous mice were bred to generate PINK1-null mice and their WT littermate controls for experiments. Mice were genotyped by multiplex PCR of genomic DNA extracted from tail snips. The first primer pair amplified part of intron 6 of PINK1 (present in all mice); the second primer pair amplified part of neo (absent in WT mice); and the third primer pair amplified intron 6 of PINK1 (absent in homozygous mutants). The absence of PINK1 expression was verified by reverse transcription-PCR (RT-PCR) and in situ hybridization (data not shown). All animal procedures were approved by the Ajou University School of Medicine Ethics Review Committee for Animal Research (Amc-119).

Neurosphere culture

Embryonic neurospheres were cultured from E13.5 mouse brains as previously described [38]. Briefly, forebrains were freed of meninges and gently triturated several times in culture medium using a flame-polished Pasteur pipette. Cells from one brain were plated in a 100-mm Petri dish and cultured in Dulbecco modified Eagle’s medium (DMEM)/F12 media (WelGene, Daegu, Korea) supplemented with N-2, B27 supplement (Gibco-Invitrogen, Carlsbad, CA, USA), 20 ng/ml epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF; BD Biosciences, San Jose, CA, USA). EGF and bFGF were added every 2 days. For serial neurosphere formation, primary neurospheres were collected, incubated with Accumax (Millipore Corp, Bedford, MA, USA), and dissociated. For differentiation, dissociated cells were seeded on plates coated with 0.2 mg/ml poly-L-ornithine and 1 μg/ml fibronectin (Sigma, St. Louis, MO, USA) in the absence of growth factors or in the presence of CNTF (BD Biosciences).

miRNA isolation and quantification

Total RNA, including small RNA, was extracted using an miRNeasy Mini Kit isolation kit (Qiagen, Chatsworth, CA, USA) and reverse transcribed using a miScript Reverse Transcription kit (Qiagen) according to the manufacturers’ instructions. miRNA levels were measured by Q-PCR using miScript SYBR Green PCR kit with miScript universal primer and miRNA-specific primers (Qiagen). RNU_6 was used as an endogenous control miRNA (Qiagen).

Microarray

RNA (1000 ng) was prepared from 1-day-old mouse brains. Biotinylated RNA strands were hybridized at 48°C for 18 hours on an Affymetrix GeneChip miRNA Array 4.0 (Affymetrix, Santa
Clara, CA, US). The GeneChip was washed and stained in the Affymetrix Fluidics Station 450. Fluorescence signals amplified by the branched structure of a 3DNA dendrimer were scanned using an Affymetrix GeneChip Scanner 3000 7G.

**Transfection of miRNA inhibitors and mimics**

For transfection, a mixture of Opti-MEM, 10 nM miRNA inhibitors or mimics, and RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) was added to NSCs on differentiation day 1. Four days later, NSCs were prepared for experiments. Knockdown or overexpression of targets, including miR-326, miR-330, and miR-3099, was confirmed by Q-PCR. Possible cytotoxicity of miRNA inhibitors and mimics was analyzed using an LDH-Cytotoxicity Assay Kit (Biovision, Milpitas, CA, USA).

**Western blot analysis**

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice with modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na₂VO₃, 1 mM NaF) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 2 mM EDTA) and phosphatase inhibitor cocktail (GenDEPOT, Barker, TX, USA). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and identified using antibodies specific for each protein. Antibodies for MAP2 were from Abcam (Cambridge, MA, USA); antibodies for TUJ-1 were from Covance (Berkeley, CA, USA); GFAP antibodies were from Sigma (St. Louis, MO, USA); and GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were incubated with peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA, USA) and visualized using an enhanced chemiluminescence system (Daeil Lab Inc., Seoul, Korea).

**Luciferase activity**

The effects of miRNAs were examined by measuring luciferase activity using a pGL3 control vector containing the 3'UTR of GFAP, a pRL-Renilla reporter vector, and a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). In detail, the 3'-UTR portion of GFAP (1402-2600 of NM_001131020) was amplified from WT NSCs (at differentiation day 5) using AccuPrime Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and the following primer pair: 5'-GCC GTG TAA TTC TAG (Infusion sequence)-ATA GAT GCG TGC TCC AGC TC-3' (sense) and 5'-CCG CCC CGA CTC TAG (Infusion sequence)-AAA TGA AGA GCA GGG AGC ATA AAG C-3' (antisense). The amplified product, linearized with Xba1 (New England Biolabs, Beverly, MA, USA), was then inserted into the pGL3-control vector using an Infusion cloning kit (Clontech, Palo Alto, CA, USA). Mutagenesis of the 3'-UTR of GFAP (two seed sequences of miR-326 and miR-330) was performed using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Palo Alto, CA, USA). Deletion mutagenesis of the 3'-UTR of GFAP (eight seed sequences of miRNA-3099, Δ1679-2334 of NM_001131020) was performed using AccuPrime Pfx DNA Polymerase (Invitrogen), T4-ligase (Promega), and the primer pair 5'-GCC AAT GTT AAA GGC AGC AAG TCC C-3' (sense) and 5'-CTA TCG GTA TAA CCT AAT TAC ACA GAG CCA G-3' (antisense). For transfections, HEK293T cells (5×10⁴ cells/well) were seeded onto 24-well plates. After 24 hours, 10 nM of each miRNA mimic (miR-326, miR-330, and miR-3099), 250 ng of pGL3-luciferase vectors (control, WT 3'-UTR of GFAP, and mutant 3'-UTR of GFAP), and 20 ng of pRL-Renilla reporter vector were added and cells were transfected using jetPRIME (Polyplus Transfection, New York City, NY, USA). After 48 hours, luciferase activity was measured and normalized to that of Renilla luciferase.

**Statistical analysis**

All data presented in this study are representative of at least three independent experiments. The statistical significance of differences between mean values of two groups was assessed by Student’s t-test. For comparisons of more than two groups, we used a one-way analysis of variance (ANOVA) with Duncan’s post hoc test.

**RESULTS**

**Analysis of the expression of PINK1-regulated miRNAs**

In an effort to identify PINK1 functions in the brain, we analyzed expression of miRNAs in PINK1 wild-type (WT) and knockout (KO) brains. miRNAs were isolated from 1-day-old brains and analyzed using Affymetrix GeneChips. A PINK1 deficiency either increased or decreased several miRNAs; those that exhibited greater than a 2-fold change in expression in the absence of PINK1 are summarized in Table 1. Although the functions of most miRNAs whose expression was altered by PINK1 deficiency are not known, some of these miRNAs are known to be related to tumors and inflammation (Table 1).

Previously, we found that PINK1 deficiency decreases protein but not mRNA levels of glial fibrillary acidic protein (GFAP), a marker of astrocytes, independently of protein degradation during
brain development and differentiation of NSCs [39]. Thus, we examined expression levels of miRNAs that could regulate GFAP expression. We selected miR-326, miR-330, and miR-3099 since these three miRNAs are common candidates that bind to GFAP 3’-UTR and regulate translation based on data bases (TargetScan and miRanda). Using quantitative polymerase chain reaction (Q-PCR),

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Table 1. miRNAs differently expressed in PINK1 KO and WT brain

| Transcript ID (increase in KO) | Function (ref) | log ratio (KO/WT) |
|-------------------------------|----------------|-------------------|
| mmu-miR-6926-5p               | Unknown        | 1.66              |
| mmu-miR-6921-5p               | Unknown        | 1.37              |
| mmu-miR-7068-3p               | Unknown        | 0.99              |
| mmu-miR-467c-5p               | Unknown        | 0.97              |
| mmu-miR-361-3p                | Down-regulated in prostate and lung cancers (Guzel et al., 2015; Roth et al., 2012) | 0.93 |
| mmu-miR-451a                  | Inhibits cell proliferation (Liu et al., 2015) | 0.91 |
|                               | Tumor suppressor (Babapoor et al., 2014; Fukumoto et al., 2014) | |
| mmu-miR-199b-5p               | Oncogene (Zeng et al., 2015/ Liu et al., 2014) | 0.77 |
| mmu-miR-6915-5p               | Unknown        | 0.74              |
| mmu-miR-1a-3p                 | Unknown        | 0.72              |
| mmu-miR-448-3p                | Unknown        | 0.72              |
| mmu-miR-694                   | Tumor suppressor (Lu et al., 2014) | 0.71 |
| mmu-miR-297b-3p               | Unknown        | 0.70              |
| mmu-miR-297a-3p               | Unknown        | 0.70              |
| mmu-miR-297c-3p               | Unknown        | 0.70              |
| mmu-miR-5616-3p               | Unknown        | 0.68              |
| mmu-miR-29b-3p                | Down-regulated in Alzheimer’s brain (Satoh et al., 2015) | 0.63 |
|                               | Related to inflammation and nephropathy (Xing et al., 2014) | |
| mmu-miR-18b-5p                | Related to gastric cancer (Xia et al., 2014) | 0.62 |
|                               | Down-regulated in stroke (Sorensen et al., 2014) | |
| mmu-miR-1298-5p               | Unknown        | 0.59              |

| Transcript ID (decrease in KO) | Function (ref) | log ratio (KO/WT) |
|-------------------------------|----------------|-------------------|
| mmu-miR-3470b                 | Related to hypersensitivity (Segall et al., 2015) | -2.30 |
| mmu-miR-5620-5p               | Unknown        | -1.26             |
| mmu-miR-7027-5p               | Unknown        | -1.07             |
| mmu-miR-6404                  | Unknown        | -1.03             |
| mmu-miR-3098-5p               | Unknown        | -0.95             |
| mmu-miR-184-5p                | Related to neuropathic pain/inflammation (Gong et al., 2015) | -0.89 |
| mmu-miR-7b-3p                 | Unknown        | -0.81             |
| mmu-miR-7116-3p               | Unknown        | -0.80             |
| mmu-miR-3473c                 | Unknown        | -0.80             |
| mmu-miR-224-3p                | Related to immune imbalance (Wang et al., 2015) | -0.77 |
| mmu-miR-3473b                 | Related to macrophage activation (Wu et al., 2014) | -0.73 |
| mmu-miR-6912-5p               | Unknown        | -0.73             |
| mmu-miR-7071-5p               | Unknown        | -0.69             |
| mmu-miR-6969-3p               | Unknown        | -0.65             |
| mmu-miR-7040-5p               | Unknown        | -0.61             |
| mmu-miR-7026-5p               | Unknown        | -0.60             |
| mmu-miR-7007-3p               | Unknown        | -0.60             |
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we found that PINK1 deficiency decreased expression of all three miR-326, miR-330, and miR-3099. In the normal brain, expression levels of miR-326, miR-330, and miR-3099 gradually increased from embryonic day 12.5 (E12.5) to postnatal day 1~7 (P1~7), and slightly decreased (miR-3099) or further increased (miR-326 and 330) at 8 weeks (Fig. 1A). Interestingly, at P1, expression levels of all three miRNAs were lower in the PNIK1-KO brain than in the WT brain (Fig. 1B; miR-330 and miR-3099, p<0.05; miR-326, p=0.053). Because expression of these miRNAs changed during development of the brain, we next examined expression of these miRNAs during differentiation of NSCs. After the induction of differentiation, the expression of markers of neurons (MAP2 and TUJ-1), astrocytes (GFAP), and oligodendrocytes (CNPase) increased at 2 days, and was further increased at 5 days (Fig. 2A). Interestingly, levels of all miR-326, miR-330, and miR-3099 expression are increased during development of mouse brains and are reduced by a PINK1 deficiency. (A) Whole brains were collected at the indicated embryonic ages and from adult mice, and expression patterns of miR-326, miR-330, and miR-3099 were analyzed by Q-PCR. (B) Expression of miR-326, miR-330, and miR-3099 were measured in WT and PINK1-KO mice brains at postnatal day 1. Data are means±SEMs of three samples (∗p<0.05). Data shown are representative of at least three independent experiments (∗p<0.05).

**Fig. 1.** miR-326, miR-330, and miR-3099 expression are increased during development of mouse brains and are reduced by a PINK1 deficiency. (A) Whole brains were collected at the indicated embryonic ages and from adult mice, and expression patterns of miR-326, miR-330, and miR-3099 were analyzed by Q-PCR. (B) Expression of miR-326, miR-330, and miR-3099 were measured in WT and PINK1-KO mice brains at postnatal day 1. Data are means±SEMs of three samples (∗p<0.05). Data shown are representative of at least three independent experiments (∗p<0.05).

**Fig. 2.** miR-326, miR-330, and miR-3099 expression are increased during differentiation of NSCs, and are reduced by PINK1 deficiency. NSCs were differentiated by growth factor withdrawal (A–C) or treatment with 1 ng/ml CNTF (D–F). (A, D) Expression of markers of neurons (MAP2 and TUJ-1), astrocytes (GFAP), and oligodendrocytes (CNPase) was analyzed 2 and 5 days after NSC differentiation (A) and 4 days after treatment with the indicated amount of CNTF (D). (B, E) miR-326, miR-330, and miR-3099 expression were measured by Q-PCR during WT NSC differentiation. (C, F) miR-326, miR-330, and miR-3099 expression were measured in WT and PINK1-KO NSC at differentiation day 3.
three miRNAs increased during differentiation of NSCs at 1 and 3 days (Fig. 2B). Furthermore, levels of these three miRNAs were significantly lower in PINK1-KO NSCs than in WT NSCs (Fig. 2C).

**GFAP expression is regulated by miR-326, miR-330, and miR-3099**

Because these miRNAs, particularly miR-326 and miR-330, are known to be related to astroglioma [29, 40], we wondered whether these miRNAs regulated differentiation of NSCs into astrocytes. We first examined the expression levels of these miRNAs in the presence of CNTF, which drives differentiation of NSCs into astrocytes [41]. As expected, CNTF caused a concentration-dependent increase in GFAP expression (Fig. 2D). Under these differentiation-inducing conditions, expression of miR-326, miR-330 and miR-3099 increased (Fig. 2E); the levels of these miRNAs were also lower in KO NSCs than in WT NSCs (Fig. 2F).

In serial experiments, we found that miR-326, miR-330, and miR-3099 all positively regulated GFAP expression. Specifically, inhibitors of these miRNAs significantly reduced GFAP levels in WT NSCs at differentiation day 5, without affecting TUJ-1 levels (Fig. 3A). Conversely, we found that mimics of miR-326, miR-330, and miR-3099 increased GFAP expression at this same time.

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**Fig. 3.** Effects of inhibitors and mimics of miR-326, miR-330, and miR-3099 on GFAP expression. At differentiation day 1, WT NSCs (A, C) or PINK1-KO NSCs (B, D) were transfected with miRNA inhibitors (10 nM) targeting miR-326, miR-330, and miR-3099 (A, C) or miRNA mimics (10 nM) of miR-326, miR-330, and miR-3099 (B, D). WT NSCs were also treated with a mixture of the three miRNA inhibitors (10 nM each) or 30 nM control miRNA. RNU_6 was used as a control. Protein levels of GFAP and TUJ-1 were analyzed by Western blot at differentiation day 5 (A and B, upper panels). GAPDH was used as a loading control. Band intensities of GFAP were quantified (A and B, lower panel). Cell death was measured by monitoring LDH release (C, D). Neither miRNA inhibitors nor mimics induced cell death (C, D). Data are means±SEMs of three samples (*p<0.05; **p<0.01). Data shown are representative of at least three independent experiments.
point in PINK1-KO NSCs, without changing expression of TUJ-1 (Fig. 3B). Neither inhibitors nor mimics of miRNAs affected cell viability (Fig. 3C, D).

Next, we examined whether these three miRNAs increase the translational efficacy of GFAP through direct binding to the 3'-UTR of GFAP mRNA using pGL3-luciferase constructs containing WT or GFAP 3'-UTR mutants because miRNAs regulate gene expression through binding to the 3'-UTR. GFAP 3'-UTR mutants were prepared by mutation of two common binding sites of miR-326 and miR-330 complementary sequences (CCCAGAG), or deletion of all eight miR-3099 binding sites (CTAGCCTA) (Fig. 4A). Consistent with expectations, application of miR-326, miR-330 or miR-3099 increased luciferase activity of normal GFAP 3'-UTR-containing constructs, but not that of GFAP constructs containing mutated 3'-UTRs (Fig. 4B). These results suggest that expression of miR-326, miR-330, and miR-3099 regulate GFAP expression during NSC differentiation. Taken together, our findings indicate that levels of the miRNAs, miR-326, miR-330 and miR-3099, may be regulated by PINK1 and contribute to the expression of GFAP during differentiation of NSCs.

**DISCUSSION**

In this study, we found that PINK1 deficiency changes expression of several miRNAs, including miR-326, miR-330 and miR-3099 that may be related to cell proliferation/cancer and inflammation (Table 1). We further found that miR-326, miR-330, and miR-3099 control GFAP expression during NSC differentiation, suggesting that a PINK1 deficiency could cause abnormal development of GFAP-expressing astrocytes.

Generally, miRNAs are thought to suppress mRNA translation. However, there are evidences to show that miRNAs can increase mRNA translation. For example, miR-369-3 levels are increased in HEK293T and Hela cells, and increase translation of the miR-369-3 target gene, tumor necrosis factor-α (TNF-α) [42]. Likewise, in *X. laevis* oocytes, xlmiR-16 increases the translation efficiency of Myt1 (myelin transcription factor 1) mRNA, which is important for maintaining the immaturity of oocytes [43, 44]. In addition, depending on tumor progression, miR-206 differentially regulates KLF4 (Kruppel-like factor 4) translation [45]. Several other miRNAs, including miR-122, have also been shown to increase translation [46-48]. In our study, we found that miR-326, miR-330, and miR-3099 increased GFAP expression, showing that inhibitors...
of these miRNAs decreased GFAP protein levels in WT NSCs, and mimics of these miRNAs increased GFAP expression in PINK1-KO NSCs (Fig. 3). Finally, using luciferase reporter constructs in HEK293T cells, we confirmed that miR-326, miR-330, and miR-3099 have a translation-activating effect on the 3’-UTR of GFAP (Fig. 4), suggesting that these miRNAs target the 3’-UTR of GFAP. miRNAs are known to be linked to brain development and neurodegenerative diseases, including Alzheimer’s disease, Huntington’s disease, multiple sclerosis, and PD [25–35]. Notably, previous studies have reported possible link between neurodegenerative diseases and miR-326, miR-330, and miR-3099 [30, 32, 40, 49, 50]. miR-326 overexpression leads to severe multiple sclerosis [49] and regulates dopamine D2 receptor expression [30]. miR-330 enhances cell proliferation, promotes cell migration and invasion, and suppresses apoptosis by activating extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways [40]. miR-330 is also associated with PD [32], and miR-3099 affects embryogenesis and neuronal differentiation [50]. In the current study, we discovered that miR-326, miR-330, and miR-3099 influence GFAP expression and/or astrogliogenesis. Furthermore, PINK1 was found to regulate expression of these miRNAs, suggesting a possible link between PINK1 and PD through these miRNAs.

For several decades, studies on PD and other neurodegenerative diseases have focused on neurons. However, genes related to neurodegenerative diseases are expressed in glia and NSCs, which means that mutations of these genes could cause defects in these cells. Therefore, it is also necessary to study neurodegenerative diseases in the context of these cells, which support neuronal survival and function in diverse ways.

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