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Original Contribution

MicroRNA-7 activates Nrf2 pathway by targeting Keap1 expression

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ABSTRACT

Nuclear factor E2-related factor 2 (Nrf2) is a key transcription factor that regulates the expression of a number of antioxidant and detoxifying genes that provide cellular protection against various stressors including reactive oxygen species (ROS). Nrf2 activity is tightly regulated by a cytoplasmic inhibitory protein called Kelch-like ECH-associated protein 1 (Keap1). The mechanism that controls Keap1 expression, however, remains poorly understood. In the present study, we demonstrate that microRNA-7 (miR-7), which is highly expressed in the brain, represses Keap1 expression by targeting the 3'-untranslated region (UTR) of its mRNA in human neuroblastoma cells, SH-SY5Y. Subsequently, this event results in an increased Nrf2 activity, as evidenced by an increase in the expression of its transcriptional targets, heme oxygenase 1 (HO-1) and glutamate-cysteine ligase modifier subunit (GCLM), and an enhanced nuclear localization of Nrf2. In addition, miR-7 decreases the intracellular hydroperoxides level and increases the level of reduced form of glutathione, indicative of oxidative stress relief. We also demonstrate that targeted repression of Keap1 and activation of Nrf2 pathway, in part, underlies the protective effects of miR-7 against 1-methyl-4-phenylpyridinium (MPP+)-induced toxicity in SH-SY5Y and differentiated human neural progenitor cells, ReNcell VM. These findings point to a new mechanism by which miR-7 exerts cytoprotective effects by regulating the Nrf2 pathway.

1. Introduction

Reactive oxygen species (ROS) are generated from environmental stress as well as normal physiological activities. Increased ROS production, which damages biological macromolecules, such as DNA, proteins, and lipids, has been implicated in several diseases including Parkinson's disease (PD) [1]. Nrf2, a member of the Cap 'n' Collar (CNC) basic leucine zipper transcription factor family, regulates the expression of antioxidant and phase II detoxifying genes to protect against ROS-induced toxicity [2–4]. Nrf2 binds to antioxidant response elements (ARE) in the promoter of genes, such as heme oxygenase 1 (HO-1), glutamate-cysteine ligase modifier subunit (GCLM), and NAD(P)H quinone oxidoreductase-1 (NQO1), and increases their gene expression. In normal physiological conditions, Nrf2 is mainly localized in the cytoplasm complexed with an inhibitory protein, Keap1, where Nrf2 is constantly polyubiquitinated by Cul3-containing E3 ubiquitin ligase complex and targeted for degradation via proteasome pathway. Upon exposure to stressors such as reactive oxygen species (ROS), reactive cysteine residues of Keap1 become oxidized so as to release Nrf2 from the Keap1–Cul3 E3 ligase complex. Consequently, Nrf2 is stabilized and subsequently translocates into the nucleus to function as transcriptional activator of cytoprotective genes [5]. Although Keap1 is regarded as the key regulatory point in Nrf2 pathway, little is known about the regulation of Keap1 expression itself.

MicroRNAs (miRs) are a group of endogenous small non-coding RNAs, which negatively control gene expression by binding to their target sequences in the 3'-UTR of mRNAs [6]. Growing evidence suggested that miR dysfunction has been implicated in various pathological conditions including PD [7–10]. MiR-7 was previously shown to play a protective role in cellular models of PD by directly targeting and downregulating the expression of α-synuclein [11] and RelA [12,13]. In the present study, we show that miR-7 targets Keap1 mRNA and reduces its expression, resulting in

Abbreviations: ARE, antioxidant response elements; CNC, Cap 'n' Collar; CBA, coumarin boronic acid; GCLM, glutamate-cysteine ligase modifier subunit; eGFP, enhanced green fluorescent protein; HO-1, heme oxygenase 1; iTRAQ, Isobaric tags for relative and absolute quantification; MPP+, 1-methyl-4-phenylpyridinium; MTC, S-methyl-l-thiocitrullin; miRs, microRNAs; NQO1, NAD(P)H quinone oxidoreductase-1; NF-κB, nuclear factor κB; PD, Parkinson's disease; ROS, reactive oxygen specie; shRNA, short hairpin RNA; tRFP, turbo red fluorescent protein

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the activation of the Nrf2 pathway. In addition, cytoprotective effect of miR-7 against MPP+, a mitochondrial toxin, was significantly diminished when Nrf2 pathway was inhibited either by silencing the expression of Nrf2 or overexpression of Keap1 cDNA lacking 3'-UTR. These results imply that targeted repression of Keap1 and subsequent activation of Nrf2 pathway underlies the protective effect of miR-7. Our current studies suggest that miR-7 could help to activate Nrf2 pathway and to promote cell survival under stress.

2. Methods

2.1. Materials

MPP+, hydrogen peroxide and S-methyl-L-thiocitrulline (MTC) were purchased from Sigma. Caumarin boronic acid was purchased from Cayman Chemical.

2.2. Plasmids

Flag-Keap1 and HO-1 promoter luciferase construct were gifts from Dr. Ken Itoh (Hirosaki Univ. Japan) [14]. 3′-UTR of human Keap1 mRNA was amplified using forward primer 5′-TTGATCTC-TAGAGGCACTTTTGTTTCTTGGGC-3′ and reverse primer 5′-CGGGTTTCTAGACAGGGTGAAAGACACTAGTT-3′ and inserted into pGL4.51 plasmid (Promega) using Xba1 restriction enzyme in the correct orientation. The mutations in the Keap1 3′UTR at the predicted miR-7 binding sites were generated by site-directed mutagenesis using the Pfu polymerase (Stratagene) with following primers: Mutant 1, 5′- A T A A C C C A T C A A C C G G G A A G G G -3′, 5′- C C C T T C C C G G T T G A T G G G T T A T -3′; Mutant 2, 5′-C C T C T C T C C T G C T A G C C T G C T C T T T -3′, 5′- A A A G A G C A G G C T A G C A G G A G A G A G G -3′; Mutant 3, 5′- T A A C T A G T G T C A A T C A C C C T G A A -3′, 5′- T T C A G G G T G A T T G A C A C T A G T T A -3′;. All the mutations were confirmed by DNA sequencing.

2.3. Cell Cultures and Transfection

Human neuroblastoma cell line, SH-SY5Y was purchased form American Type Culture Collection. Cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen). Cells were transfected with pre-miR-SC (scrambled; Ambion), pre-miR-7 (Ambion), siRNA-NT (nontargeting; Ambion), siRNA-Nrf2 (Ambion), anti-miR-SC (Ambion) and anti-miR-7 (Ambion) by using Lipofectamine RNAiMax according to manufacturer's instructions. Co-transfection of Flag-Keap1, pGL4.51-Keap1 3′UTR and pGL4.51-mutant Keap1 3′UTR with pre-miRs was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Human neural progenitor cells, ReNcell VM (EMD Millipore) were maintained in DMEM with F12 (1:1; Hyclone, Thermo Scientific) supplemented with 1 × B27 (Life Technologies), glutamax (Invitrogen), 10 U/ml heparin (Sigma), 50 ug/ml Genticin (Invitrogen), 20 ng/ml epidermal growth factor (EGF; Sigma), and 20 ng/ml basic fibroblast growth factor 2 (bFGF-2; Sigma). These cells were differentiated into tyrosine hydroxylase (TH)-positive neurons by using the protocol previously described [15].

2.4. Lentivirus production and infection

HEK293T cells were used for the production of lentivirus expressing miR-SC (Open Biosystems), miR-7 (Open Biosystems), shRNA-NT (Open Biosystems) and shRNA-Nrf2 (Open Biosystems). Lentivirus isolation and infection into target cells were perfomed as previously described [12]

2.5. Cell viability assay

Cell viability was measured using the CellTiter 96 AQueous Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

2.6. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

LC-MS/MS analysis, database search and bioinformatics analysis was performed as described previously [12].

2.7. RNA isolation and quantitative RT-PCR

Total RNA was extracted from SH-SY5Y cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. cDNA was generated by reverse transcription using the Superscript RT kit (Invitrogen). Quantitative RT-PCR was done using SYBR select mastermix (Life Technologies) using the Applied Biosystems 7500 Real-Time PCR System. PCR primer sequences were as follows: human HO-1, 5′- C C A G G C A G A G A A T G C T G A G T T C -3′ and 5′- A A G A C T G G G C T C T C C T T G T T G C -3′; human Keap1, 5′-C A A C T T C G C T G A G C A G A T T G G C -3′ and 5′- T G A T G A G G G T C A C C A G T T G G C A -3′; human GCLM, 5′- T C T T G C C T C C T G C T G T G T G A T G-3′ and 5′- T T G G A A A C T T G C T T C A G A A A G C A G -3′; human Nrf2, 5′- C A C A T C C A G T C A G A A A C C A G T G G -3′ and 5′- G G A A T G T C T G C G C C A A A A G C T G -3′; human 18S rRNA, 5′- C G G C T A C C A C A T C C A A G G A A -3′ and 5′- G C T G G A A T T A C C G C G G C T -3′; Relative mRNA expression level was normalized to 18S rRNA and calculated by the $2^{-\Delta\Delta Ct}$ method.

2.8. Reporter gene assay

Cells were lysed with Glo Lysis Buffer (Promega) and luciferase activity was determined using the Steady-Glo Luciferase Assay System (Promega) with a Wallac 1420 Multilabel Counter (PerkinElmer). To normalize luciferase activity with transfection efficiency, β-galactosidase activity was measured using chlorophenol Red-β-D-galactopyranoside (Roche) and reaction buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgSO_4$, 10 mM KCl, 50 mM 2-mercaptoethanol, pH 7.0).

2.9. Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in PBS containing 2% SDS with protease inhibitors cocktail and phosphatase inhibitors (Roche). Cell lysates were sonicated for 20 sec, and protein concentration in the lysates was quantified using BCA Protein Assay Reagent (Thermo Scientific). Cell lysates were analyzed by Western blot analysis, using anti-Keap1 (catalog #8047, Cell Signaling), anti-HO-1 (catalog #A303–662A, Bethyl), anti-Nrf2 (catalog #EP1808Y, Abcam) or anti-β-actin (catalog #A5316, Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated anti-rabbit (catalog #HAF008, R&D Systems) or anti-mouse antibody (catalog #HAF007, R&D Systems). Band intensity was measured using ImageJ (NIH). Nuclear and cytoplasmic fractions were isolated using Nuclear Extract kit (Active motif) according to the manufacturer's instructions. To analyze the efficiency of fractionation, antibodies against alpha-tubulin (cytosol) and lamin B (nucleus) were used for Western blot analysis.

2.10. Measurement of neurite length

ReNcell VM cells transduced with lentivirial vectors as indicated were imaged using an Axiovert 2000 fluorescent

microscope (Carl Zeiss). Images were imported into Image J and converted to 8 bit grayscale. The length from the perimeter of the cell body to tip of the neurite was measured using Neuron J plugin as described previously [16]. Twenty neurons co-transduced with lentiviral vectors expressing miR (RFP reporter) and shRNA (GFP reporter) were measured for each sample.

2.11. Measurement of intracellular hydroperoxides level

Intracellular hydroperoxides levels were measured with coumarin boronic acid (CBA), a fluorescent probe as described previously [17,18]. Briefly, the cells were incubated with 100 μM of CBA for 30 min at 37 °C. Then, cells were washed with PBS. The fluorescence intensities were measured using excitation and emission filters of 355 nm and 460 nm respectively in a Wallac1420 Victor 2 microplate reader (Perkin Elmer).

2.12. Glutathione assay

Total glutathione concentration and GSH /GSSG ratio was measured using GSH/GSSG-Glo Assay kit (Promega) according to supplier's protocol. Concentration of glutathione was normalized to protein concentration.

2.13. Statistical Analysis

Data are presented as means ± S.E.M. and were analyzed by two-way ANOVA followed by Bonferroni's posthoc test unless otherwise stated. Level of significance was set at $p < 0.05$.

3. Results

3.1. MiR-7 increases HO-1 and GCLM gene expression through activating Nrf2 pathway

Previously, we showed that miR-7 protects against MPP+-induced cell death. To identify molecular target pathway through which miR-7 exerts its neuroprotective effects, we performed iTRAQ ((Isobaric tags for Relative and Absolute Quantification) based proteomics analysis to reveal differentially expressed proteins between miR-SC and miR-7-transfected cells. Our initial focus was to investigate the genes whose expression is down-regulated by the expression of miR-7 as miRs usually function by repressing gene expression [12]. However, we also observed that a number of proteins (181 proteins) were upregulated by overexpression of miR-7 (miR-7/miR-SC ratio, ≥ 1.2, $p \leq 0.05$) (Supplementary Table 1). Interestingly, heme oxygenase 1 (HO-1) and glutamate-cysteine ligase, modifier subunit (GCLM), which are transcriptional target genes of Nrf2, were significantly upregulated by the overexpression of miR-7 (Fig. 1A). We confirmed that overexpression of miR-7 led to about 2-fold increase in HO-1 expression using Western blot analysis (Fig. 1B). To examine whether the increase in protein level results from an increase in mRNA levels of the respective proteins, we performed quantitative PCR analysis to determine the levels of HO-1 and GCLM mRNAs. MiR-7 dramatically increases the mRNA levels of HO-1 and GCLM (Fig. 1C), suggesting a possible increase in the transcription of these genes. We further confirmed the transcriptional regulation of miR-7 on HO-1 gene using a luciferase reporter construct under the regulation of HO-1 promoter. Luciferase activity is significantly increased by overexpression of miR-7, demonstrating that miR-7 increases transcription of HO-1 gene (Fig. 1D).

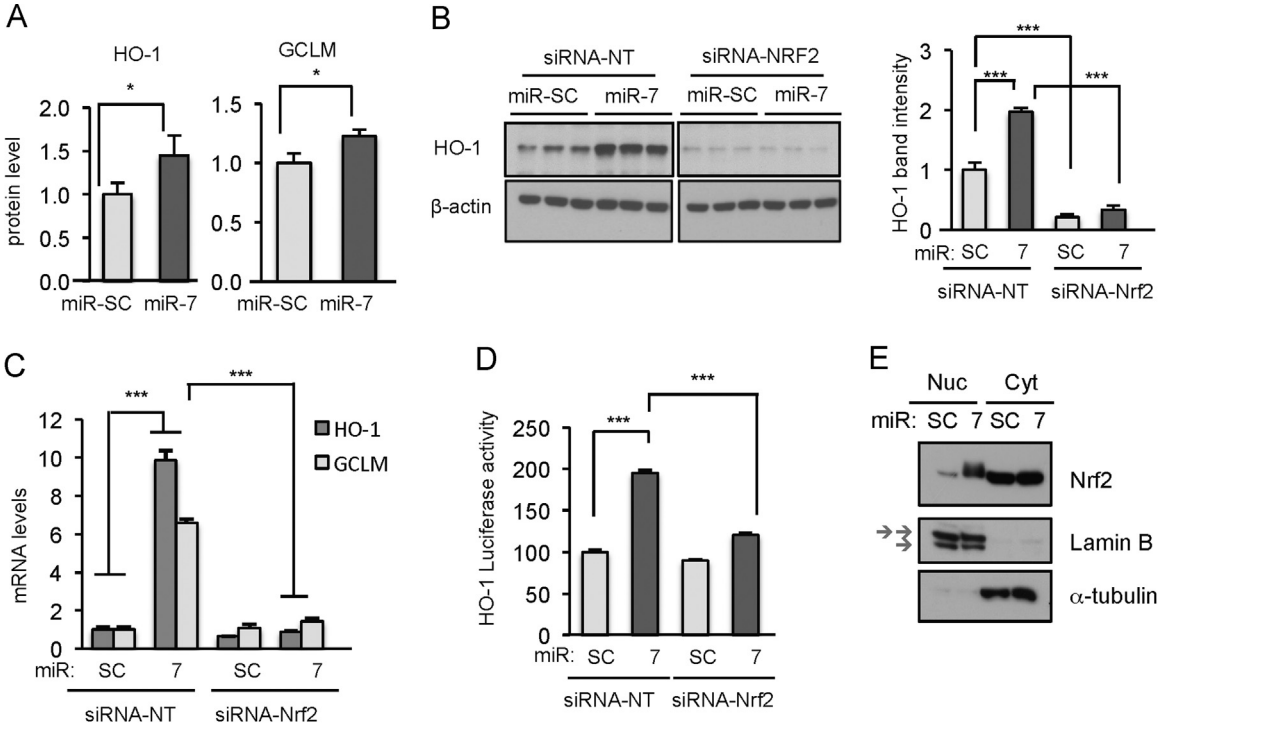

Fig. 1. MiR-7 promotes Nrf2 pathway. SH-SY5Y cells were transfected as indicated. Pre-miRs and siRNAs were transfected at a concentration of 50 nM for 48 h. (A) Quantification of HO-1 and GCLM protein levels from proteomic study. Data were analyzed using Student's t-test. (B) A representative Western blot showing the expression levels of HO-1 and β-actin. Band intensities were calculated from three samples by normalizing to β-actin using NIH Image J Software. (C) Quantitative RT-PCR analysis showing the mRNA levels of HO-1 and GCLM. mRNA levels were normalized to 18S rRNA and are shown as a ratio compared with the cells transfected with miR-SC and siRNA-NT. (D) Relative HO-1 promoter luciferase activities are shown as a ratio compared with the cells transfected with miR-SC and siRNA-NT. A luciferase reporter construct under the regulation of HO-1 promoter was co-transfected with pre-miRs and siRNAs as indicated, along with pSV- β-galactosidase plasmid. Luciferase activity was normalized against β-galactosidase activity. (E) Overexpression of miR-7 enhances nuclear translocation of Nrf2. SH-SY5Y cell lysates were fractionated to nuclear and cytoplasmic fractions. Western blotting was performed with the following antibodies: anti-Nrf2, anti-Lamin B1 for nuclear marker, anti-alpha-tubulin for cytoplasmic marker. Two arrows (→→) denote full-length Lamin B1, and one arrow (→) a cleaved form of Lamin B1. Experiments were done in triplicates. Data are shown as means ± SEM. *$p < 0.05$, ***$p < 0.001$.

Since expressions of both HO-1 and GCLM gene are both positively regulated by transcription factor, Nrf2, we next examined whether miR-7-induced increases in these gene expression result from the activation of Nrf2 pathway. Indeed, miR-7-induced increase in the level of HO-1 protein was significantly compromised in Nrf2-knock down cells (Fig. 1B). In addition, miR-7-induced increase in the level of HO-1 and GCLM mRNAs was significantly diminished after silencing Nrf2 expression (Fig. 1C). Consistently, miR-7-induced increase in the HO-1 promoter luciferase activity was also attenuated in cells with silenced Nrf2 expression (Fig. 1D). The knockdown of Nrf2 mediated by siRNA-Nrf2 was confirmed by quantitative RT-PCR analysis (Supplementary Fig 1A). Next, we examined whether miR-7 increases the nuclear localization of Nrf2. SH-SY5Y cells transfected with either pre-miR-SC or pre-miR-7 were fractionated into cytosolic and nuclear fractions, and subjected to western blotting for Nrf2. In the presence of miR-7 overexpression, Nrf2 levels were significantly increased in the nucleus (Fig. 1E), indicating that miR-7 expression promotes the translocalization of Nrf2 into the nucleus. These results suggest that miR-7 activates Nrf2 and subsequently increases HO-1 and GCLM gene expression.

3.2. MiR-7 targets the 3'-UTR of Keap1 mRNA

Next, we examined the mechanism by which miR-7 activates Nrf2 pathway. Interestingly, miR-7 expression increases the level of Nrf2 protein by about 30% (Fig. 2A), whereas miR-7 did not augment the level of Nrf2 mRNA (Supplementary Fig 1B), suggesting that miR-7 increases the level of Nrf2 protein post-transcriptionally. The primary point of regulation in the Nrf2 pathway is Keap1, an inhibitory protein that binds to Nrf2 and promotes its degradation by the ubiquitin proteasome pathway [5]. Therefore, we hypothesized that miR-7 targets Keap1 mRNA, and consequently prevents the degradation of the Nrf2 protein and increases the steady-state levels. To prove our hypothesis, SH-SY5Y cells were transiently transfected with pre-miR-7 or pre-miR-SC, and Keap1 protein and mRNA levels were examined by Western blot and qRT-PCR analysis, respectively. As shown in Figs. 2A and 2B, the expression levels of Keap1 protein were significantly decreased, but Keap1 mRNA levels were not reduced by transfection of pre-miR-7, indicating that miR-7 might inhibit the translation of Keap1 mRNA. To investigate whether miR-7 targets the 3′-UTR of Keap1 mRNA, a luciferase reporter construct in which Keap1 3'-UTR sequence is placed downstream of the luciferase gene, was

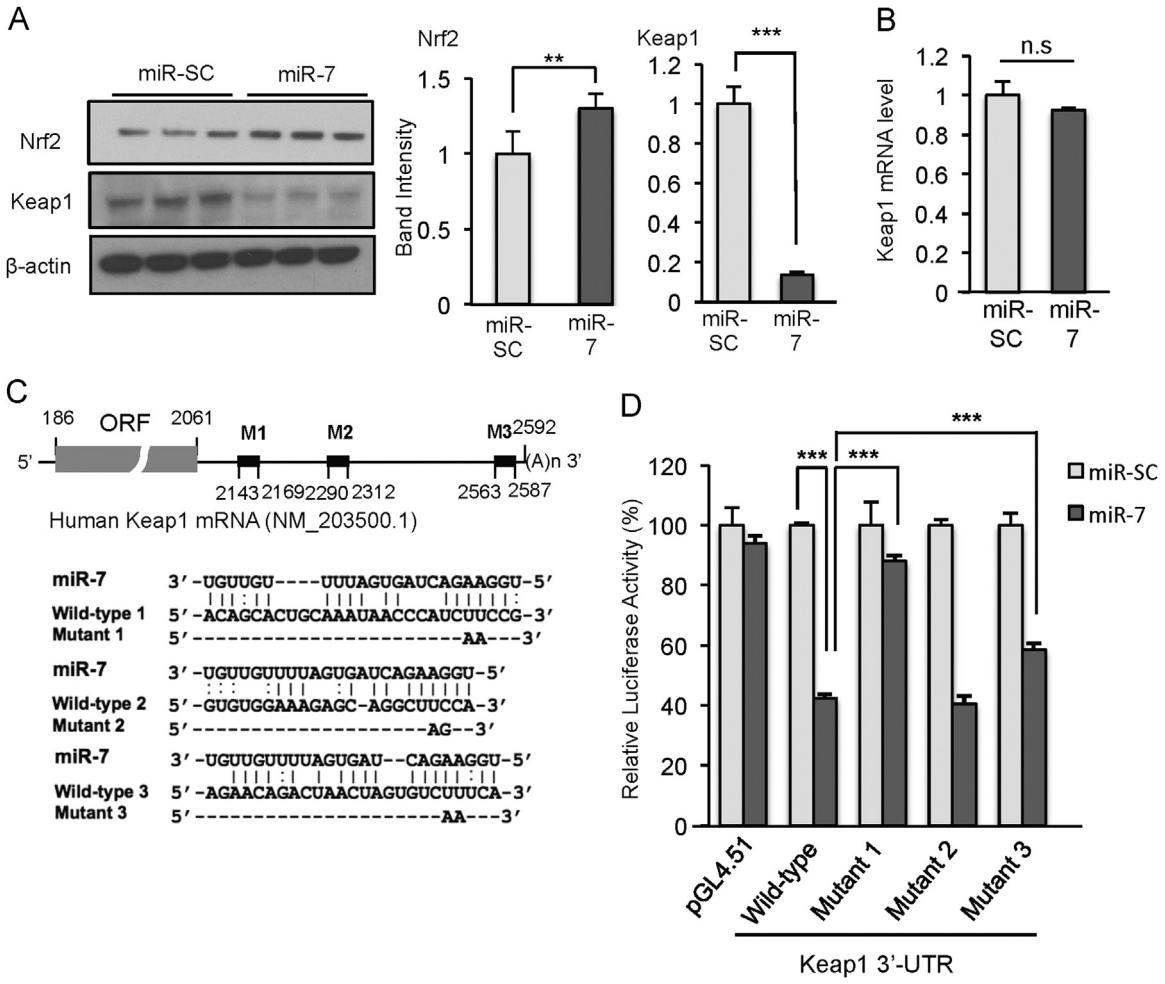

Fig. 2. MiR-7 targets the 3′-UTR of Keap1 mRNA. SH-SY5Y cells were transfected as indicated. Pre-miRs were transfected at a concentration of 50 nM for 48 h. (A) A representative Western blot showing Nrf2 and Keap1 expression. Band intensities were calculated from three samples by normalizing to β-actin using NIH Image J Software. Data were analyzed using Student's t-test. (B) Quantitative RT-PCR analysis of Keap1 mRNA after overexpression of miR-7. mRNA levels were normalized to 18S rRNA and are shown as a ratio compared with the cells transfected with miR-SC. (C) Schematic drawing of Keap1 mRNA containing three potential miR-7 binding sites in the 3'-UTR. All three targeting sequences in Keap1 mRNA were shown along with respective mutants. (D) Reporter gene assay using Keap1 3′-UTR and its potential miR-7 binding site mutants. SH-SY5Y cells were co-transfected with miR-SC or miR-7 along with the luciferase construct harboring the Keap1 3'-UTR or the mutants and pSV-β-galactosidase. Cells were harvested after 48 h. Luciferase activity was normalized against β-galactosidase activity. Experiments were done in triplicates. Data are shown as means ± SEM. $^{**}p < 0.01$ $^{***}p < 0.001$. n.s. denotes non-significant.

co-transfected with pre-miR-7 into SH-SY5Y cells. MiR-7 significantly decreased the luciferase activity from Keap1 3'-UTR construct, but had no effect on pGL4.51 construct lacking Keap1 3′-UTR (Fig. 2D). In the Keap1 3'-UTR sequence, we identified three potential miR-7 target sites (Fig. 2C). Computational calculation of miR-7 binding to potential target sites using RNAhybrid algorithm [19] showed extensive base pairing of 19 (target #1), 17 (target #2), 19 (target #3) out of 23 nucleotides. The minimal free energy required for each site is -16.0 kcal/mol (#1), -15.8 kcal/mol (#2) and -19.1 kcal/mol (#3), suggesting a favorable interaction. However, all these three sites do not contain perfect matched sequence against the seed region of miR-7. To identify which of the predicted target sequences are genuine binding site(s) for miR-7, the sites were mutated as shown in Fig. 2C. Compared with 60% repression of Keap1 3′-UTR luciferase activity, #1 mutated Keap1-3′-UTR construct could be repressed by only about 10% when pre-miR-7 was co-transfected. In addition, #3 mutated one was repressed by about 40%, while #2 mutated one showed the similar repression as wild-type. These results indicate that miR-7 binds strongly to #1 site, and weakly to #3 site, which contributes to Keap1 repression in response to miR-7 expression.

3.3. Endogenous miR-7 regulates Nrf2 pathway

To examine the role of endogenously expressed miR-7 in activating the Nrf2 pathway, we utilized anti-miR-7, a single-stranded oligonucleotides that specifically bind and inhibit endogenous miR-7. Western blot analysis revealed that transfection of anti-miR-7 resulted in a dramatically reduced HO-1 expression, while an increase in Keap1 expression (Fig. 3A). As expected, inhibition of endogenous miR-7 also led to a significant decrease in HO-1 mRNA levels (Fig. 3B) as well as HO-1 promoter activity (Fig. 3C). These results suggest that endogenous miR-7 targets Keap1 mRNA and consequently upregulates the Nrf2 pathway leading to an increase in basal HO-1 expression under physiological condition.

3.4. MiR-7 protects cells against MPP+-induced death by activating Nrf2 pathway

Previously, we showed that overexpression of miR-7 leads to cell protection against MPP+ [12,13]. To study whether miR-7-mediated reduction of Keap1 level underlies the cytoprotective effect of miR-7 against MPP+, SH-SY5Y cells were transfected with plasmid containing Keap1 cDNA without its 3′-UTR, along with pre-miR-7. This approach restores Keap1 levels despite downregulation of endogenous Keap1 by miR-7. Overexpression of Keap1 partially mitigated the protective effect of miR-7 against MPP+ in SH-SY5Y cells (Fig. 4A). This result demonstrates that the cytoprotective effect of miR-7 against MPP+, in part, requires the down-regulation of Keap1 level. Functional integrity of Flag-Keap1 plasmid was validated by detecting the reduced activity of luciferase from HO-1 promoter luciferase construct (Supplementary Fig 1C).

As miR-7 activates Nrf2 pathway by targeting the 3′-UTR of Keap1 mRNA, we also examined whether miR-7-induced cell protection requires the involvement of Nrf2 pathway. Indeed, knocking down the expression of Nrf2, in part, abolished the cytoprotective effect of miR-7 against MPP+ in SH-SY5Y cells (Fig. 4B). Also, miR-7 protected SH-SY5Y cells against hydrogen peroxide, and this protection was significantly impaired when Nrf2 expression was silenced (Supplementary Fig 1D), suggesting that miR-7-induced increase in Nrf2 pathway contributes to its cytoprotective effect against oxidative stressors. To further confirm the effect of miR-7 on Nrf2 pathway in post-mitotic neuron-like

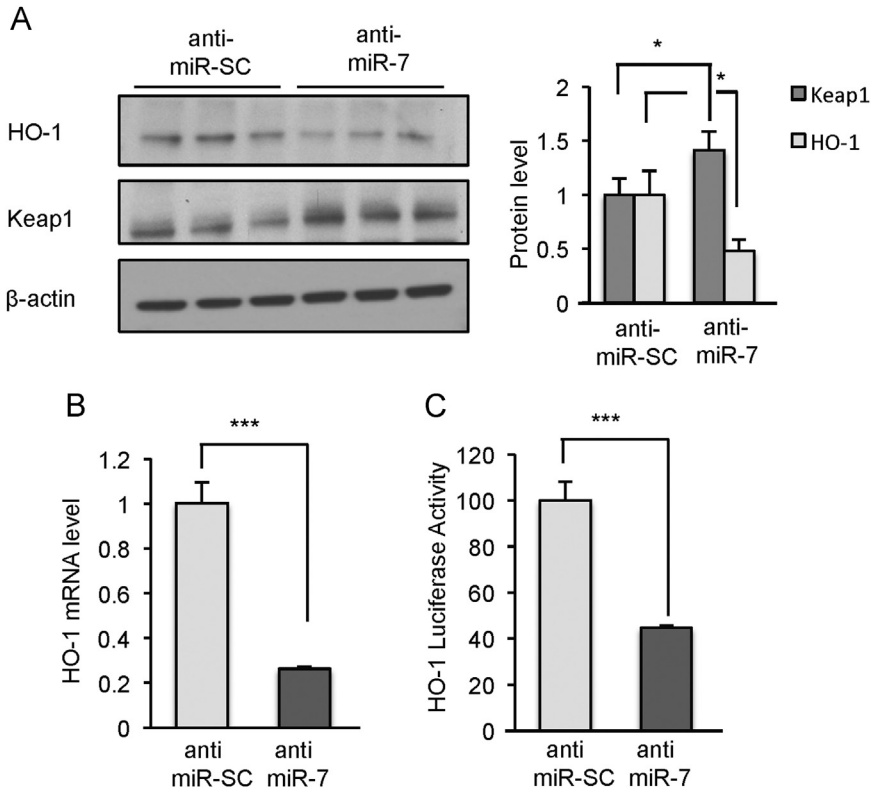

Fig. 3. Endogenous miR-7 represses Keap1 expression and upregulates HO-1 expression. (A) SH-SY5Y cells were transfected with 50 nM anti-miR-7 for 48 h, and followed by Western blot analysis showing HO-1, Keap1 and β-actin. Band intensities were normalized to β-actin using NIH Image J software. (B) Quantitative RT-PCR analysis of HO-1 mRNA in SH-SY5Y cells after transfection of anti-miR-7. Relative mRNA levels were normalized to 18S rRNA and are shown as a ratio compared to the cells transfected with anti-miR-SC. Data were analyzed using Student's t-test. (C) Relative HO-1 promoter luciferase activity in SH-SY5Y cells upon transfection with anti-miR-SC or anti-miR-7. Experiments were done in triplicates. Data were analyzed using Student's t-test. Data are shown as means ± SEM. *$p < 0.05$, ***$p < 0.001$.

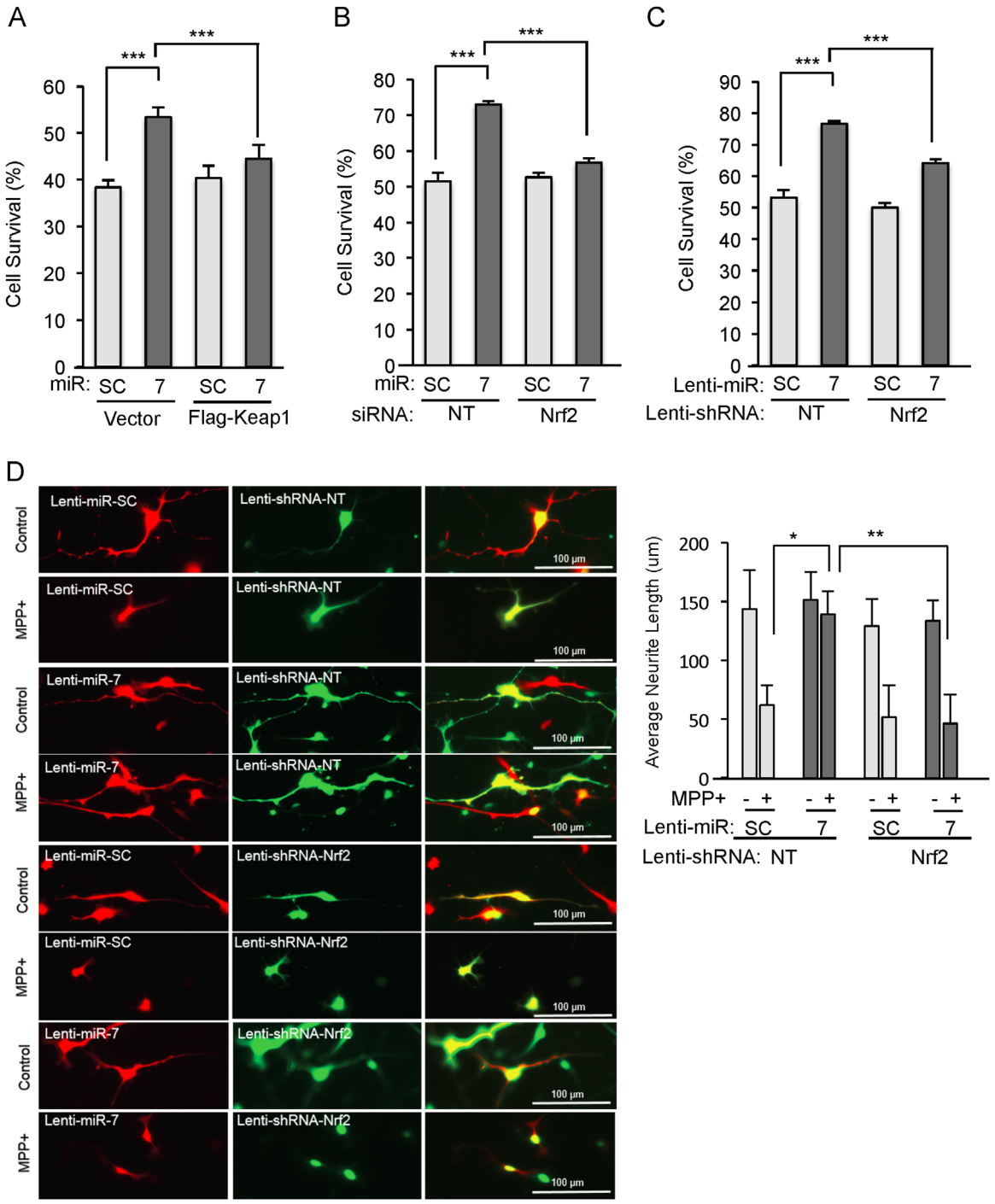

Fig. 4. MiR-7-induced protective effect against MPP+ requires the targeting of Keap1. (A) Overexpression of Keap1 cDNA without its 3'-UTR partly abrogates miR-7-mediated cytoprotection. SH-SY5Y cells were co-transfected with Flag-Keap1 in the presence of miR-7 or miR-SC. After 48 h, cells were challenged with 2 mM MPP+ for 24 h, and cell survival was assessed. (B) Silencing the Nrf2 expression inhibited the protective effect of miR-7 against MPP+. SH-SY5Y cells were co-transfected with either miR-SC or miR-7 along with siRNA-NT or siRNA-Nrf2. After 48 h, cells were challenged with 2 mM MPP+ for 24 h, and cell survival was assessed. (C) Differentiated ReNcell VM cells were transduced with lentiviral vectors as indicated. After 72 h, cells were challenged with 1 mM MPP+ for 24 h, and cell survival was assessed. Cell survival was calculated relative to each group of transfected cells without MPP+ treatment. Experiments were done in triplicates. (D) Differentiated ReNcell VM cells were cotransduced with either lenti-miR-SC or lenti-miR-7 along with lenti-shRNA-NT or lenti-shRNA-Nrf2. After 72 h, cells were challenged with 1 mM MPP+ for 24 h, and neurite length was measured. Differentiated ReNcell VM cells infected with lenti-miR-SC or lenti-miR-7 are shown as tRFP-positive (red), and cells infected with lenti-shRNA-NT or lenti-shRNA-Nrf2 are shown as eGFP-positive (green). Neurite length was only measured in 20 double-positive (yellow) cells. Data are shown as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cells, we employed ReNcell VM cells which were differentiated into tyrosine hydroxylase (TH)-positive neuronal cells. Differentiated ReNcell VM cells were transduced with lentivirus expressing miR-7 (lenti-miR-7) or control (lenti-miR-SC) along with either shRNA targeting Nrf2 (lenti-shRNA-Nrf2) or non-targeting shRNA (lenti-shRNA-NT). Functional integrity of lenti-shRNA-Nrf2 was confirmed by detecting the dramatic reduction in Nrf2 mRNA level after infecting SH-SY5Y cells (Supplementary Fig 1E). As expected, differentiated ReNcell VM cells infected with lenti-miR-7 survived significantly better in response to MPP+ treatment than

the cells infected with lenti-miR-SC. However, silencing the Nrf2 expression also partly mitigated the protective effect of miR-7 against MPP+ (Fig. 4C). Neurotoxicity was also examined by measuring the neurite length. Treatment with MPP+ led to a significant decrease in neurite length in differentiated ReNcell VM cells (Fig. 4D) that were transduced with control lenti-miR-SC and lenti-siRNA-NT (1st row vs 2nd row). Certainly, miR-7-expressing differentiated ReNcell VM cells were protected from neurotoxic effect of MPP+ as evidenced by significantly longer neurites compared to miR-SC-expressing cells (4th row vs 2nd row), which is consistent with our previous report [12]. However, when expression of Nrf2 was silenced by lenti-shRNA-Nrf2, a significant decrease in neurite length was observed in the miR-7-expressing cells (4th row vs 8th row) treated with MPP+, indicating that Nrf2 pathway is required for miR-7-induced neuroprotection. Based on these results, we conclude that the protective effect of miR-7 requires the downregulation of Keap1 and the subsequent increase in the Nrf2 activity.

3.5. MiR-7 relieves oxidative stress through targeting Keap1

Next, we measured the intracellular hydroperoxides level using CBA, a fluorescent probe. Expectedly, MPP+ treatment led to an increase in fluorescence signal in SH-SY5Y cells (Supplementary Fig 2A and Fig. 5A). As CBA probe is known to interact with peroxynitrite as well as hydrogen peroxide to generate fluorescence [18], we wanted to ascertain the nature of oxidant that we measure in this experimental condition. MPP+-induced increase in fluorescence signal was completely abrogated by the inclusion of N-acetyl cysteine (NAC), a well-known antioxidant and glutathione precursor, while cotreatment of S-methyl-L-thiocitrulline (MTC), a competitive inhibitor of nitric oxide synthase, did not change the oxidation of CBA (Supplementary Fig 2A). This result suggests that MPP+ treatment leads to the production of hydroperoxides, and CBA successfully detects the level of hydroperoxides in SH-SY5Y cells. Remarkably, overexpression of miR-7 dramatically decreased the hydroperoxides level by nearly 50%, and this degree of reduction in hydroperoxides level was maintained even after MPP+ treatments (Fig. 5A). To determine whether miR-7-induced drop in hydroperoxides level requires Keap1 targeting, we overexpressed Flag-Keap1 plasmid along with miR-7. MiR-7-induced decline in hydroperoxides level was almost mitigates by overexpressing Keap1 (Fig. 5A), suggesting miR-7 decreases hydroperoxides level by targeting Keap1 expression.

As Nrf2 positively regulates the expression of catalytic and the regulatory subunits of glutamate cysteine ligase, an enzyme required in the rate-limiting step in the biosynthesis of glutathione [3], we analyzed the total level of glutathione (GSH plus GSSG) after miR-7 overexpression in SH-SY5Y cells. Overexpression of miR-7 led to a 2.8-fold increase in total glutathione level (Fig. 5B). However, overexpression of Keap1 decreased the basal level of total glutathione by 65% (1st bar vs 3 rd bar). Interestingly, overexpression of Keap1 also significantly diminished the miR-7-induced increase in total glutathione level (1.3-fold increase in Flag-Keap1-overexpressing cells vs 2.8-fold increase in Flag vector-overexpressing cells). Also, miR-7-induced increase in total glutathione level was similarly attenuated when Nrf2 expression was silenced (1.5-fold increase in siRNA-Nrf2-transfected cells vs 2.7-fold increase in siRNA-NT-transfected cells) (Fig. 5B). This result suggests that miR-7 increases the total level of glutathione by activating Nrf2 pathway. It is noteworthy that the increase of total glutathione level in response to miR-7 overexpression results from the increase in reduced form of glutathione (GSH), and the level of oxidized glutathione (GSSG) is kept low throughout all experimental conditions (Fig. 5B). As GSH /GSSG ratio has been commonly used to denote cellular redox status [20], data in Fig. 5B was also calculated to show GSH/GSSG ratio (Supplementary Fig 2B). Overexpression of miR-7 again led to a significant increase in GSH/GSSG ratio, and this increase was dramatically abrogated by either overexpression of Keap1 or knockdown of Nrf2 expression. Collectively, miR-7 reduces oxidative stress through activating Nrf2 pathway by targeting Keap1 expression.

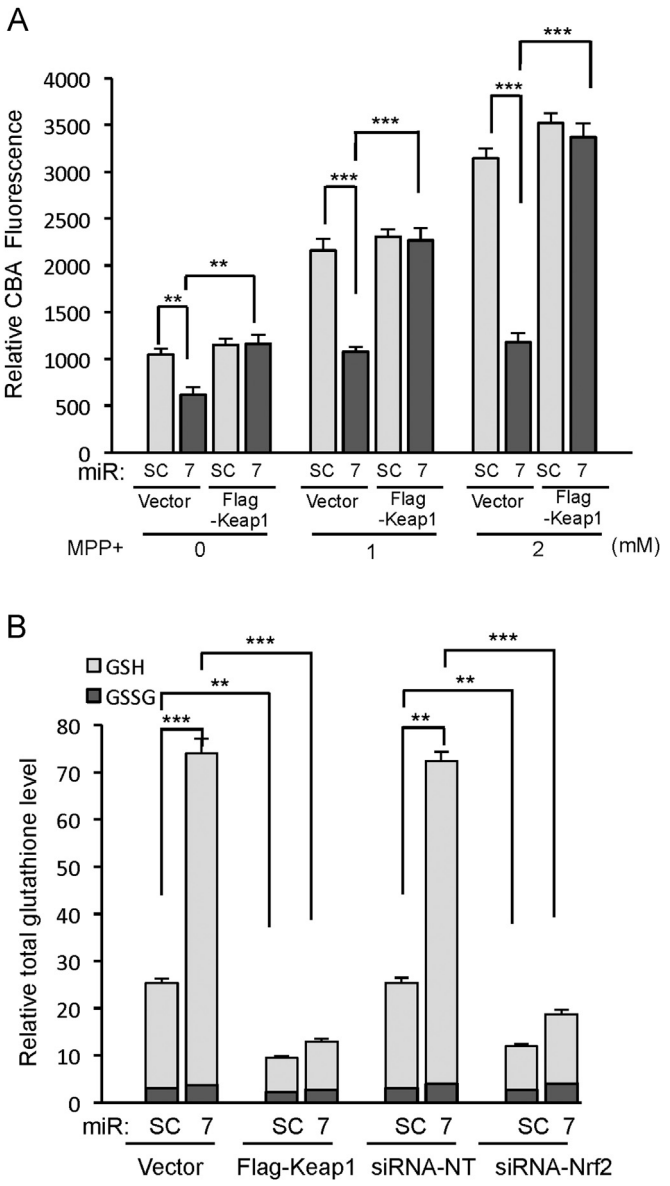

Fig. 5. MiR-7 relieves oxidative stress through activating Nrf2 pathway. (A) Overexpression of Keap1 abolishes miR-7-induced decreases in intracellular hydroperoxides levels. Intracellular hydroperoxides levels were measured with CBA in SH-SY5Y cells transfected as indicated. Cells were treated with MPP+ for 12 h. (B) Intracellular total glutathione levels (GSH plus GSSG) were measured in SH-SY5Y cells transfected as indicated. Experiments were done in triplicates. Data are shown as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

In the present study, we have elucidated the mechanism by which miR-7 regulates the anti-oxidant Nrf2 pathway. Accumulating evidence suggest the detrimental role of oxidative stress in various disease conditions. In PD, dopaminergic neurons are highly sensitive to oxidative stress as dopamine can undergo oxidation that generates ROS and electrophilic quinone molecules, which

could lead to the demise of dopaminergic neurons [21]. Nrf2 is a transcription factor involved in oxidative and xenobiotic stress responses, and provides the cellular protection against stressors. In the absence of stressor, Keap1 binds to Nrf2 and promotes its degradation by the ubiquitin proteasome pathway [4]. Exposure to oxidative stress causes the dissociation of Keap1-Nrf2 complex, resulting in the translocation of Nrf2 protein to the nucleus and subsequent activation of Nrf2-regulated anti-oxidant gene transcription. Further, it was reported that Nrf2 activity is increased through interaction of Keap1 and p62 [22]. Excess p62 binds Keap1, and prevents it from inhibiting Nrf2 activity. As such, Keap1 works as a central regulatory player in Nrf2 pathway. Consequently, elucidating the mechanisms to regulate Keap1 expression could provide crucial therapeutic opportunities to modulate the activity of the Nrf2 pathway.

We have previously reported the protective role of miR-7 in the cellular models of PD [11–13]. As miRs function by targeting a multitude of mRNAs, we performed a proteomics study in order to identify the downstream targets of miR-7. Among the proteins that were found to be significantly upregulated were HO-1 and GCLM, two prominent transcriptional targets of Nrf2, suggesting that miR-7 activates the Nrf2 pathway. In this study, we report the novel function of miR-7 regulating the activation of Nrf2 by targeting the Keap1 mRNA.

Nrf2 pathway can be regulated through several mechanisms in addition to Keap1. Expression of Nrf2 mRNA can be regulated by transcription factor such as ATF4 [23] and c-Jun [24]. Additionally, Nrf2 activity could also be affected through interaction with other proteins inside the nucleus such as Maf [25] and BRCA1 [26]. MiRs have reportedly provided another layer to the regulatory mechanism of Nrf2 pathway. It was reported that several miRs such as miR-153, miR-27a, miR-142-5p and miR-144 regulate Nrf2 pathway by directly downregulating the Nrf2 level through its 3'-UTR of mRNA, which renders cells to be sensitive to oxidative-stress induced cell death [27]. In addition, miR-200a has been shown to target Keap1 mRNA in the human breast cancer cell lines, leading to increased Nrf2 activation [28]. However, it is unlikely that miR-200a is involved in Nrf2 activation in human brain, as expression level of miR-200a is negligibly low [29]. In contrast, miR-7 is highly expressed in the human brain [29] including the dopaminergic neurons of substantia nigra [11, 30]. In addition, miR-7 expression was shown to be downregulated in the midbrain of MPTP-intoxicated mice [11], suggesting that reduced expression of miR-7 could contribute to the degeneration of dopaminergic neurons in this model. Therefore, it is likely that miR-7 exerts its neuroprotective functions through activating Nrf2 pathway in the brain.

Occasionally, miRs exert their functional role via targeting multiple mRNAs which are in the same cellular pathway [31]. We previously reported that miR-7 targets the 3'-UTR of RelA mRNA, and protects cells against MPP+-induced cell death by promoting glycolysis [12,13]. Interestingly, RelA reportedly inhibits Nrf2 pathway either by selectively depriving CREB binding protein (CBP) from Nrf2, which results in inactivation of Nrf2, or directly interacting with Keap1, which leads to nuclear localization of Keap1 and subsequent inhibition of Nrf2 activity [32,33]. Therefore, in addition to directly targeting and downregulating Keap1, we could postulate that miR-7 activates Nrf2 pathway by targeting the expression of RelA as well.

Several lines of evidence suggest that dysfunctional Nrf2 pathway contributes to the pathogenesis of neurodegenerative diseases including PD. Ramsey et al. showed that there was increased nuclear localization of Nrf2 in the dopaminergic neurons of substantia nigra of PD patients [34]. This observation has several implications. First, nuclear localization of Nrf2 is probably an expected outcome of sustained oxidative stress. Second, neurons that we observe in post-mortem tissue are the ones that are still surviving at the time of autopsy. These neurons were able to prolong their survival in the face of sustained oxidative stress probably due to the activity of active (nuclear) Nrf2, while neurons having insufficient Nrf2 activity were already lost. Third, Nrf2 pathway can be finely regulated by miR-7 while both are co-expressed in neurons. In addition, significant decrease in the level of glutathione, a vital Nrf2-regulated antioxidant, was observed in PD patients, further indicating the significant lack of Nrf2 activation [35]. Involvement of Nrf2 pathway in the pathogenesis of neurodegenerative disorders was also corroborated in the studies utilizing cellular and animal models [36–43]. Nrf2 knockout mice reportedly displayed increased vulnerability of dopaminergic neurons in the substantia nigra to 6-OHDA [39] and MPTP [44], which was rescued by expression of Nrf2. Additionally, cerebellar granule neurons derived from transgenic mice overexpressing HO-1 are more resistant to glutamate- and H_2O_2-mediated oxidative damage in vitro [45]. HO-1 also confers the neuroprotection in animal models following traumatic [46,47] and excitotoxic brain damage [48,49] and spinal cord injury [50]. Consequently, in various pathological conditions in which Nrf2 activity is insufficient or diminished, increasing miR-7 expression could provide significant therapeutic benefit by increasing Nrf2 activation.

In conclusion, our current study demonstrates a novel mechanism by which miR-7 exerts its protective effect against MPP+-induced toxicity through targeting Keap1 mRNA and activating Nrf2 pathway.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.09.010.

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