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Suppression of lncRNA LINC00472 Prevents Amyloid-β-induced Neurotoxicity in PC12 Cells via Regulating miR-141-3p/Foxo3 Axis

Min Guo
Aerospace Central Hospital: Central Hospital of China Aerospace Corporation
Jilai Li
Aerospace Central Hospital: Central Hospital of China Aerospace Corporation
Peifu Wang
Aerospace Central Hospital: Central Hospital of China Aerospace Corporation
Di Jin (Mlxu_research@163.com)
Aerospace Central Hospital: Central Hospital of China Aerospace Corporation https://orcid.org/0000-0002-9337-0603

Research Article

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Abstract

Alzheimer's Disease (AD) is characterized by the accumulations of amyloid-β (Aβ) plaques and neurofibrillary tangles in brain, dysfunctions and loss of synaptic connections, exaggerated inflammation, and eventually neuronal death. Oxidative damage may explain the key features and suggest the therapeutic potential to prevent AD progression, yet it remains largely elusive the occurrence and thus the interventions of oxidative damage. Besides, miRNA dysregulation is tightly associated with AD progression. By recruiting 30 AD patients and 30 age-matched control, we demonstrated the upregulations of LINC00472, pro-inflammatory cytokines and ROS in the serum of AD subjects. Similar results were reproduced in Aβ treated SH-SY5Y cells. In addition, through luciferase reporter and RNA pull-down assays, we revealed direct interactions between LINC00472 and miR-141-3p, and between miR-141-3p and FOXO3 and the signalling loop LINC00472/miR-141-3p/FOXO3 loop is involved in Aβ administration induced oxidative stress. Moreover, we raised evidence that knocking down LINC00472 in SH-SY5Y cells ameliorated the Aβ administration induced oxidative stress. Thus, normalization of LINC00472 may be therapeutic to retard AD progression via ameliorating oxidative stress. Future works may further strengthen the pharmacological potentials of interventions for AD progression by targeting LINC00472/ miR-141-3p/ FOXO3 loop.

Introduction

Alzheimer's Disease (AD) is one of the most prevalent age-associated dementia and has no therapy in the clinic. AD is characterized by the accumulations of amyloid-β (Aβ) plaques and neurofibrillary tangles in brain, dysfunctions and loss of synaptic connections, exaggerated inflammation, and eventually neuronal death (1). Mechanistically, oxidative damage, at least in part, contributes to the AD features such as dysfunctions and loss of synaptic connections, inflammation, and neuronal death, and suggest the therapeutic potential to prevent AD progression (2). However, it remains largely elusive the occurrence and thus the interventions of oxidative damage in AD.

Besides, miRNA dysregulation is tightly associated with AD progression (3). It was reported downregulation of miR-141-3p in AD subjects (4), as wells as upregulation of Long intergenic non-protein-coding RNA 472 (LINC00472) (5). Besides, cellular assays have shown nuclear translocation of forkhead box O3 (FOXO3), a critical factor for longevity, ageing and oxidative stress (6), may contribute to inflammation and mitophagy (7), and FOXO3 knocking down reduces mitochondrial reactive oxygen species (ROS) (7, 8). Interestingly, databases such as Starbase predicts a possible binding between LINC00472 and miR-141-3p, while miR-141-3p may target FOXO3. Both miR-141-3p and FOXO3 have been shown to induce oxidative stress (6, 9~11). Therefore, the signalling loop that involves LINC00472/ miR-141-3p/ FOXO3 may take part in oxidative damage in AD progression.

Here we compared the expression of LINC00472, miR-141-3p, FOXO3, pro-inflammatory cytokines, and ROS in AD subjects or Aβ treated cells with control and evaluated the potential of LINC00472/ miR-141-3p/ FOXO3 in retarding oxidative stress in AD progression. We demonstrated the upregulations of

LINC00472, pro-inflammatory cytokines, and Reactive oxygen species (ROS) in the blood of AD subjects. Similar results were reproduced in Aβ treated SH-SY5Y cells. In addition, through luciferase reporter and RNA pull-down assays, we revealed direct interactions between LINC00472 and miR-141-3p, and between miR-141-3p and FOXO3 and the signalling loop LINC00472/miR-141-3p/FOXO3 loop is involved in Aβ administration induced oxidative stress. Moreover, we raised evidence that knocking down LINC00472 in SH-SY5Y cells ameliorated the Aβ administration induced oxidative stress. Thus, normalization of LINC00472 may be therapeutic to retard AD progression via ameliorating oxidative stress. Future works may further strengthen the pharmacological potentials of interventions for AD progression by targeting LINC00472/ miR-141-3p/ FOXO3 loop.

Methods

Participates

Approval for the assays involving 30 AD patients (determined by the DSM-IV-TR criteria) and 30 age-matched healthy controls was obtained from the institutional review board of Aerospace Center Hospital (Approval Number: [KY-E-2019-12-17]). In our research, the patients with AD did not received any chemotherapy or radiotherapy. According to the Declaration of Helsinki, the informed consent was signed by all participate patients.

Blood samples

Blood samples were taken from all subjects and serum was obtained by centrifuge and stored at −80℃ prior to the analysis. Concentrations of TNF-α, IL-8, and IL-1β were checked by ELISA kits (Catalog # BMS223HS, # KHC0081, and FK-F10189).

Luciferase assay

Luciferase assays were taken out under the guidance lines of commercial Luciferase assay kit with HEK293 cell lines (CAS No. 9014-00-0).

ROS examination

Reactive oxygen species (ROS; including SOD, CAT, MDA and total ROS) were measured spectrophotometrically using commercially available assay kits in line with the manufacturer’s protocol (Jiancheng, China) (9).

Aβ treatment

As reported (15) with minor modification, SH-SY5Y cells were treated with 20 μM Aβ (1-42) for 24 hours before further assays.

Transfection and overexpression

Experiments with sh-LINC00472 were taken out in the stable SH-SY5Y cell lines with sh-LINC00472 transfected with Lentivirus. Other plasmids were transfected via Lipofectamine™ 2000 Transfection Reagent (#11668019).

RT-PCR

RT-PCR was taken out following the commercial kit (Biolabs, #E5315S)

RNA pull-down

RNA pull-down was taken out under the guidance of commercial pull-down kits from Guangzhou CHINA.

Western blotting

Briefly, the cells of interest were scrapped down from the dish and treated in the lysate buffer (Beyotime, Shanghai, China) for 30 minutes and homogenized for 20 minutes before centrifuged at 800g for 30 minutes at 4°C. Protein extracts were dissolved in sample buffer and equal amounts of protein (30 mg) were resolved on a 10% Bis-Tris gel before transferred onto a polyvinylidene difluoride membrane. After blocking for 60 minutes at room temperature in 5% skim milk solution, membranes were incubated at 4°C with primary antibodies (FOXO3: Millipore #07-702, 1:1000 dilution; GAPDH: Proteintech #60004-1-Ig, 1:2000 dilution) overnight and secondary antibody at room temperature for 1 hour. Membranes were exposed by the Bio-Rad imaging system and analyzed by ImageJ software.

Statistical analysis

All statistics were analyzed through the Student T-test, One or Two-way ANOVA. Graphs were made using GraphPad Prism 6 (GraphPad, La Jolla, CA, USA). Error bars mean ± standard error mean (SEM). P-value < 0.05 was considered to be statistically significant. In all figures, */# P<0.05, **/##P<0.01, ***/###P<0.001.

Results

Upregulations of LINC00472 and proinflammatory cytokines in AD subjects.

RT-PCR was utilized to analyze the blood expression of LINC00472 in AD subjects and age-matched healthy individuals. As reported (5), LINC00472 was upregulated in AD subjects (Fig. 1A). Compared to healthy individuals, LINC00472 expression is 1.5-2 times higher in AD subjects (Fig. 1A). In addition, ELISA assays revealed significant upregulations of proinflammatory cytokines in serum such as TNF-α, IL-8, and IL-1β (12) (Fig. 1B; AD vs control, TNF-α is 150 vs 64 pg/ml, IL-8 is 120 vs 62 pg/ml, and IL-1β is 12 vs 2 pg/ml). Moreover, AD subjects have significantly reduced serum expression of superoxide dismutase (SOD) and catalase (CAT) and increase of malondialdehyde (MDA) (9) (Fig. 1C; AD vs control, SOD is 0.17 vs 0.22U/mol, MDA is 18 vs 9 nmol/L, and CAT is 1.64 vs 2.52 U/ml).

LINC00472 downregulation ameliorates Aβ induced oxidative stress.

In SH-SY5Y cells, we aimed to evaluate the therapeutic potential of LINC00472 downregulation in treating AD. The widely used Aβ was added into culture medium and the expression of LINC00472, proinflammatory cytokines, and ROS were compared with or without LINC00472 downregulation. Consistent with AD subjects, Aβ administration elevates LINC00472 in SH-SY5Y cells (Fig. 2A). Moreover, the Aβ treated cells revealed upregulations of TNF-α, IL-8, and IL-1β (Fig. 2B), reduced SOD and CAT (Fig. 2C), and increased MDA (Fig. 2C) and ROS (Fig. 2D). sh-LINC00472 transfection dramatically downregulated cellular LINC00472 (Fig. 2A), and accordingly, reversed the anomalies of TNF-α, IL-8, IL-1β (Fig. 2B), SOD, CAT, MDA (Fig. 2C), and ROS (Fig. 2D).

miR-141-3p is the target gene of LINC00472 and targets the FOXO3 gene.

Searching databases such as Starbase, we noticed the predicted bindings between LINC00472 and miR-141-3p (Fig. 3A), and miR-141-3p and FOXO3 (Fig. 4A). Luciferase assay revealed suppression of enzymatic activity by miR-141-3p overexpression, which was released by an intact but not mutated form of LINC00472 (Fig. 3B). Importantly, the miR-141-3p significantly pull down LINC00472 in RNA-pull down assay, and vice versa (Fig. 3C). In Aβ treated cells, miR-141-3p expression was significantly reduced, and could be attenuated by co-transfect sh-LINC00472 (Fig. 3D). Similarly, blood expression of miR-141-3p is reduced in AD subjects (Fig. 3E) and negatively correlated with LINC00472 expression (Fig. 3F). In addition, the suppression of enzymatic activity by miR-141-3p overexpression in luciferase assay was also released by an intact but not mutated form of FOXO3 (Fig. 4A, B). Cellular expression of FOXO3 was elevated after Aβ administration and was reversed by co-transfection with miR-141-3p mimic (Fig. 4C). Moreover, AD subjects have higher blood expression of FOXO3 by RT-PCR (Fig. 4D). Interestingly, blood expression of FOXO3 has a negative correlation with miR-141-3p (Fig. 4E) and, at the same time, a positive correlation with LINC00472 (Fig. 4F).

LINC00472 regulates oxidative stress through miR-141-3p/FOXO3

So far, we've shown increased oxidative stress in AD subjects and Aβ treated cells, as well as accumulated LINC00472. Then we wondered if specifically manipulating LINC00472/miR-141-3p/FOXO3 benefit controlling oxidative stress in AD. Indeed, in the Aβ treated cells, FOXO3 is accumulated (Fig. 5A). Consistent with the correlation assays, knocking down LINC00472 normalized the accumulation of FOXO3, and the normalization was attenuated by FOXO3 overexpression or miR-141-3p inhibitor (Fig. 5A). Similarly, Aβ administration induced upregulations of TNF-α, IL-8, and IL-1β were reversed by knocking down LINC00472 (Fig. 5B). Again, the knocking down effects were dismissed by FOXO3 overexpression or miR-141-3p inhibitor (Fig. 5B). Moreover, Aβ administration induced similar reductions of SOD and CAT and increase of malondialdehyde MDA as AD subjects (13), which have been partially rescued by knocking down LINC00472 (Fig. 5C). FOXO3 overexpression or miR-141-3p inhibitor treatment dismissed the rescue effects (Fig. 5C). Furthermore, the knocking down LINC00472 induced normalization of ROS was blocked by FOXO3 overexpression or miR-141-3p inhibitor treatment (Fig. 5C).

Discussion And Conclusion

Collectively, we reported that targeting LINC00472/miR-141-3p/FOXO3 may help prevent AD progression by minimizing oxidative damage. We demonstrated that the increased LINC00472 (5) and FOXO3 and the reduction of miR-141-3p (4) may contribute to the oxidative stress in AD subjects and Aβ treated cells. Importantly, LINC00472/miR-141-3p/FOXO3 may serve as a therapeutic target for AD progression. However, there are several limitations. Obviously, Aβ treated cells cannot be equalled to AD conditions in vivo and another major AD feature-neurofibrillary tangles-has not been evaluated in this study. Future investigations with models of Tau pathology will definitely strengthen our conclusion. Besides, although we dismissed the molecular defects in cultured cells, we lack behavioural assays to further evaluate the therapeutic potentials. Systemically behavioural studies are worthwhile in the future. Nevertheless, human induced pluripotent stem cell-derived neuronal cells (iPSC) from sporadic AD patients who have no Aβ and tau pathology also revealed the oxidative stress and altered mitochondrial protein expression (14). Therefore, our finding that LINC00472/miR-141-3p/FOXO3 regulates oxidative stress in AD progression might be a therapeutic target for general AD populations.

Declarations

ETHICAL STATEMENT:

Ethics approval and consent to participate

Approval for the assays involving 30 AD patients (determined by the DSM-IV-TR criteria) and 30 age-matched healthy controls was obtained from the institutional review board of Aerospace Center Hospital (Approval Number: [KY-E-2019-12-17]). In our research, the patients with AD did not received any chemotherapy or radiotherapy. All the clinical procedures were in accordance with the declaration of Helsinki.

Consent to participate

The written INFORMED CONSENTS were collected from all the participants of the study. All the protocols were reviewed and approved by the committee for human experimentations, Aerospace Center Hospital, China.

Consent for publication

All authors consent to publication.

Availability of data and materials

All supporting data of this work, which are not available in public because of the ethical restrictions are available from the corresponding author upon request.

Competing interests

The authors report no conflicts of interest in this work.

Funding

There is no funding source in this work.

Authors' contributions

Min Guo designed the project and collected data. Jilai Li analyzed the data and drafted the manuscript. Peifu Wang did almost all the experiments and were involved in data collection and analysis. Di Jin conducted methodology and validation and was responsible for investigation and data curation. All the authors revised and corrected the manuscript.

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Figures

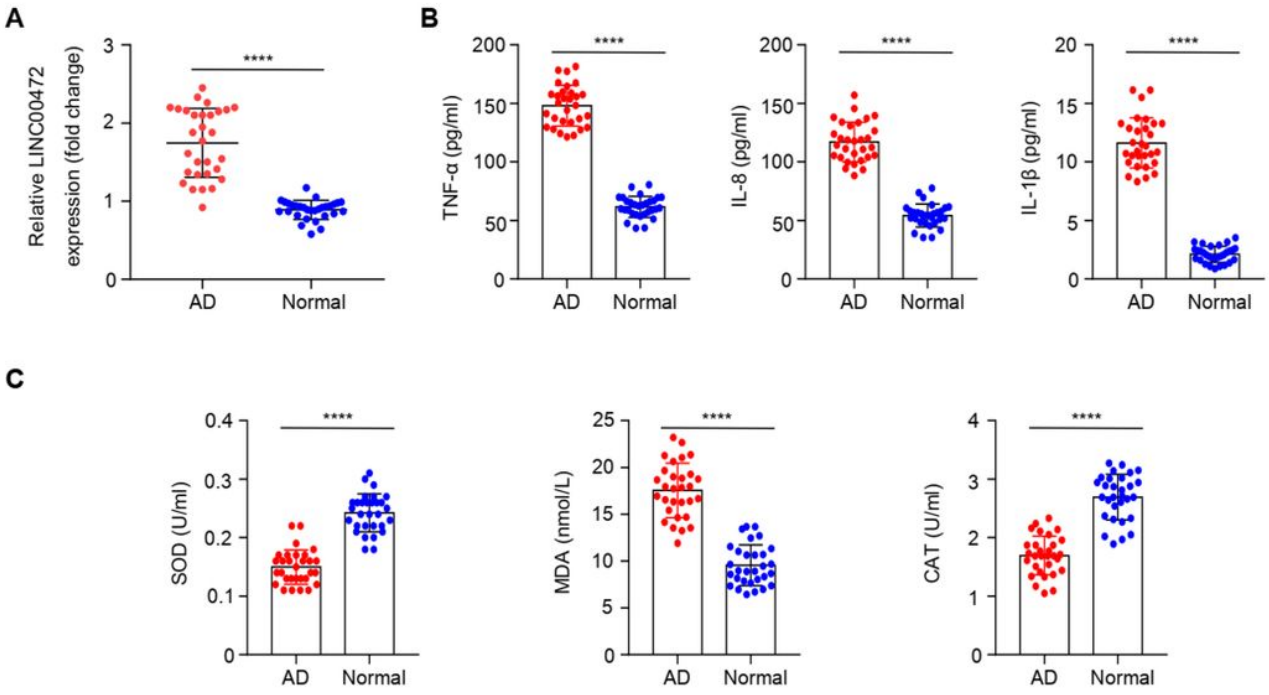

Figure 1

Expression of LINC00472 and inflammatory factors in the blood of Alzheimer's patients. A. Serum expression of LINC00472 by RT-PCR (n=30 individuals). B. Serum expression of TNF-α, IL-8, and IL-1β in by ELISA. (AD vs control, TNF-α: 150 vs 64 pg/ml, IL-8: 120 vs 62 pg/ml, IL-1β: 12 vs 2 pg/ml). C. Serum expression of SOD, MDA, and CAT (AD vs control, SOD: 0.17 vs 0.22U/mol, MDA: 18 vs 9 nmol/L, and CAT: 1.64 vs 2.52 U/ml).

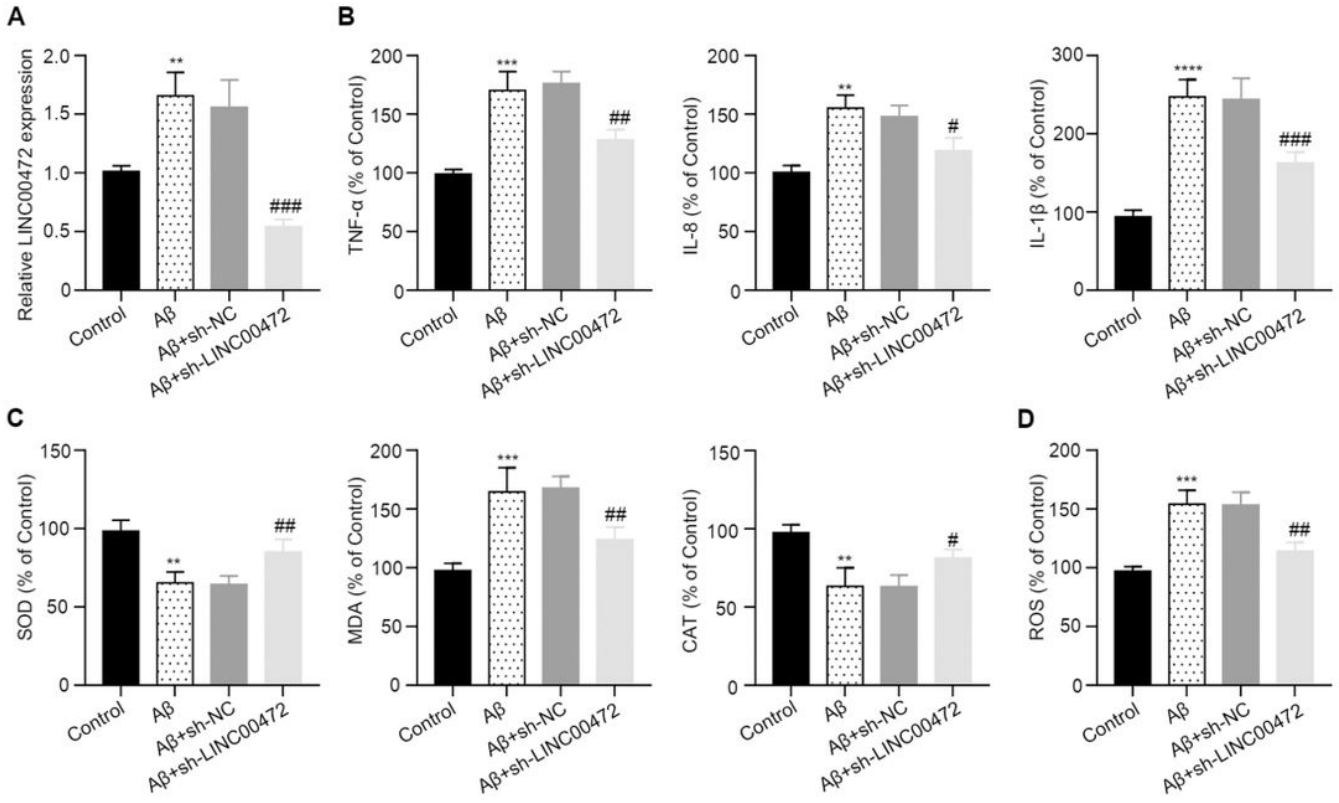

Figure 2

LINC00472 downregulation ameliorates Aβ induced oxidative stress in SH-SY5Y cells. A. Cellular expression of LINC00472 by RT-PCR. B. Expression of TNF-α, IL-8, and IL-1β in culture medium by ELISA. The upregulations of TNF-α, IL-8, and IL-1β by Aβ administration were normalized by Sh-LINC00472. C. The alternations of SOD, MDA, and CAT by Aβ administration were normalized by Sh-LINC00472. D. Aβ administration induced elevation of ROS in SH-SY5Y cells was reversed by Sh-LINC00472 treatment.

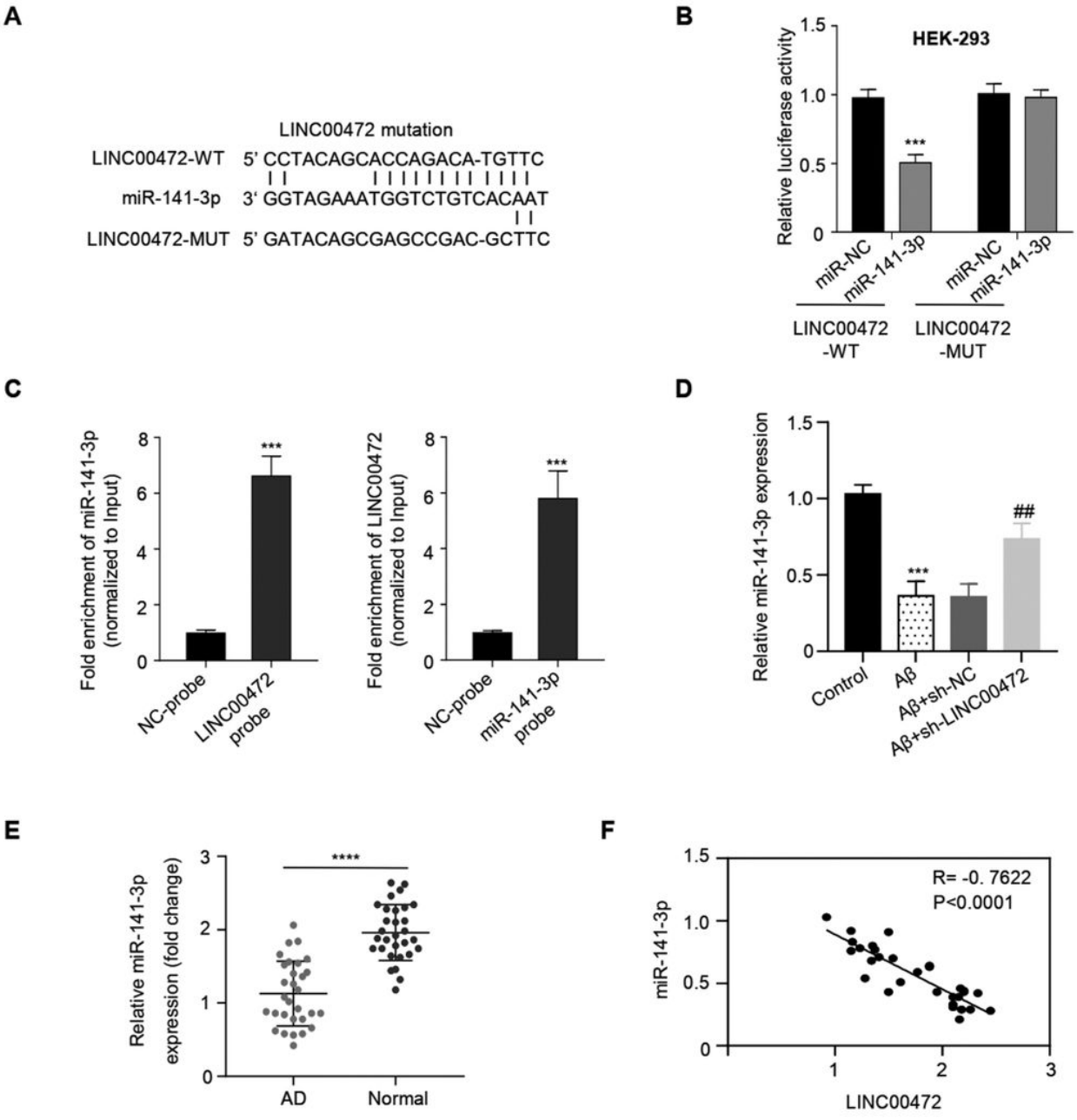

Figure 3

miR-141-3p is the target gene of LINC00472. A. The predicted binding sites of LINC00472 and miR-141-3p. B. Compared to control microRNA (miR-NC), the Luciferase activity in HEK-293T cells was inhibited by miR-141-3p overexpression, which was ameliorated by the mutations of binding sites for LINC00472 and miR-141-3p. C. The direct binding of LINC00472 and miR-141-3p in RNA-pull down assay. D. Aβ administration reduced miR-141-3p expression and Sh-LINC00472 treatment reversed it in RT-PCR. D. Blood expression of miR-141-3p in AD subjects is reduced. E. A negative correlation of blood expression of LINC00472 and miR-141-3p.

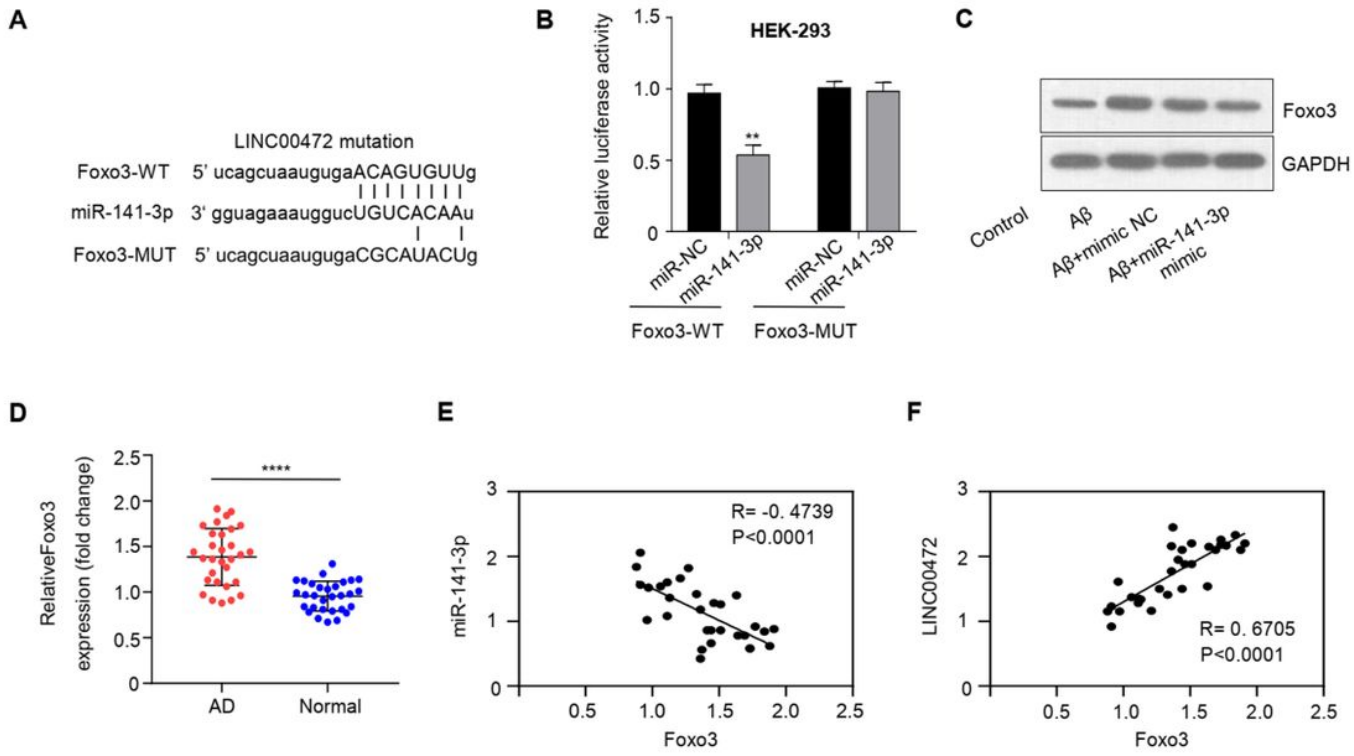

Figure 4

FOXO3 is the target gene of miR-141-3p. A. The predicted binding sites of FOXO3 and miR-141-3p. B. Compared to control microRNA (miR-NC), the Luciferase activity in HEK-293T cells was inhibited by miR-141-3p overexpression, which was ameliorated by the mutations of binding sites for FOXO3 and miR-141-3p. C. The upregulation of FOXO3 by Aβ administration was attenuated by miR-141-3p mimic treatment in western blotting. D. Blood expression of FOXO3 is increased by RT-PCR. E. A negative correlation of blood expression of FOXO3 and miR-141-3p. F. A positive correlation of blood expression of LINC00472 and FOXO3.

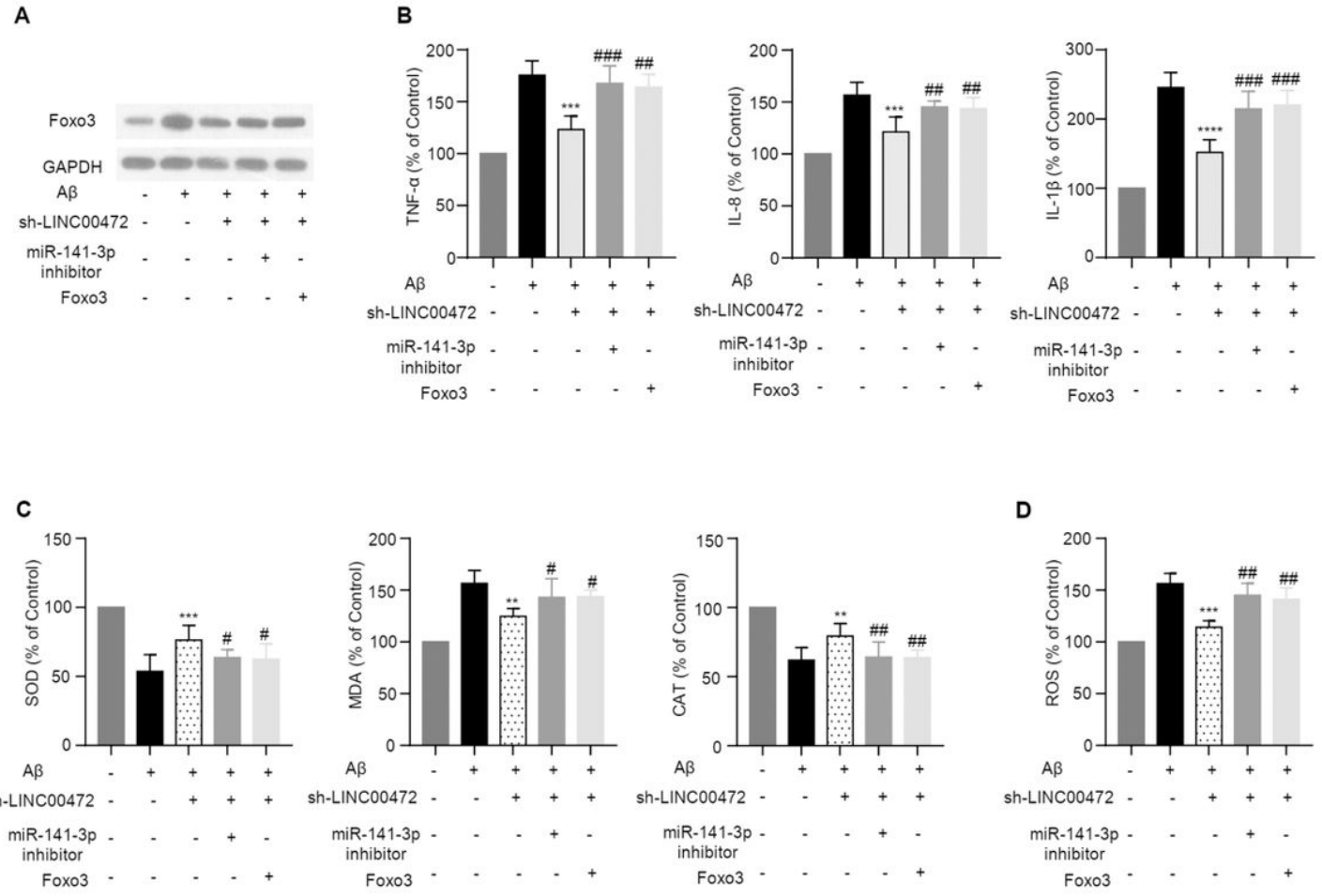

Figure 5

LINC00472 regulates oxidative stress through miR-141-3p/FOXO3. A. The downregulation of Foxo3 by sh-LINC00472 in Aβ treated cells was blocked by miR-141-3p inhibitor co-transfection or FOXO3 overexpression. B. The reductions of TNF-α, IL-8, and IL-1β by sh-LINC00472 in Aβ treated cells were attenuated by miR-141-3p inhibitor co-transfection or FOXO3 overexpression in ELISA assay. C. The alterations of SOD, MDA, and CAT by sh-LINC00472 in Aβ treated cells were reversed by miR-141-3p inhibitor co-transfection or FOXO3 overexpression. D. The reduction of ROS by sh-LINC00472 in Aβ treated cells was dismissed by miR-141-3p inhibitor co-transfection or FOXO3 overexpression.