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ORIGINAL RESEARCH

The inhibition of ORMDL3 prevents Alzheimer's disease through ferroptosis by PERK/ATF4/HSPA5 pathway

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[Correction added on 04 March 2023, after first online publication: In Abstract section mice was changed to patients in 3 occurrences; RMDL3 was changed as ORMDL3, and anticancer was changed as target spot; The keywords section was updated; The head levels after section 2.4 were renumbered.]

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease with high incidence and widespread attention. There is currently no clear clarification of the pathogenesis. However, ORMDL3 causes ferroptosis in AD, and the potential mechanisms remain unclear. So, this study explore the function of ORMDL3 on ferroptosis in AD and its potential regulatory mechanisms. APPswe/PS1dE9 mice and C57BL/6 mice were induced into the mice model. The murine microglial BV-2 cells also were induced into the vitro model. In serum samples of AD patients, ORMDL3 mRNA expression levels were upregulated. The serum ORMDL3 levels expression was positively related to the ADL score or MoCA score in AD patients. The serum ORMDL3 expression level was positively related to MMSE score or Hcy levels in AD patients. The mRNA expression of ORMDL3 in the hippocampal tissue of the mice model of AD was upregulated at one, four and eight months. The protein expression of ORMDL3 was upregulated in the mice model of AD. ORMDL3 promoted Alzheimer's disease, and increased oxidative response and ferroptosis in a model of AD. PERK/ATF4/HSPA5 pathway is one important signal pathway for the effects of ORMDL3 in a model of AD. Collectively, these data suggested that ORMDL3 promoted oxidative response and ferroptosis in a model of AD by the PERK/ATF4/HSPA5 pathway, which might be a novel target spot mechanism of ferroptosis in AD and may serve as a regulator of AD-induced ferroptosis.

KEYWORDS

Alzheimer's disease, ferroptosis, ORMDL3, PERK

1 | INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia, accounting for 60%–80% of all dementia patients [1]. Its prevalence increases significantly with age [2]. In more detail, the prevalence of AD among people over 65 years old is 5%–10%, while that of people over 85 years old is as high as 20%–50% [2]. Regarding the gender ratio, the prevalence of women is significantly higher than that of men. Gradually, AD has become a public health problem that cannot be ignored, which can cause a huge economic burden on families and society [3].

AD is a kind of neurodegenerative disease [4]. Most of the patients have memory impairment, vague consciousness and impaired cognitive function, and the disease often occurs in the elderly, which is also known as senile dementia. At present, there is no effective treatment to slow down, treat or reverse AD [5]. The main pathological hypotheses are: β-Amyloid protein, (Aβ) Senile plaques formed by deposition and neurofibrillary tangles formed by abnormal p-Tau protein lead to the loss and death of neurons [6]. However, a large number of clinical trials of targeted drugs based on these two assumptions around the world ended in failure, and the research focus of AD is shifting to the field of substitution or innovation [7].

As a neurodegenerative disease, the clinical manifestation of AD is progressive memory impairment [8]. With the increasing aging of the Chinese population, China has become the country with the largest number of AD patients [9]. Senile

plaque with toxic β-amyloid protein (Aβ) as the core component is a typical pathological feature of AD [10].

Ferroptosis is a regulatory cell death mode discovered in recent years [11]. It is driven by iron-dependent lipid peroxidation and is the result of the imbalance of cell metabolism and redox homeostasis [12]. It can inhibit lipid peroxidation and iron death by directly consuming iron through pharmacological or genetic means. Ferroptosis is characterised by iron imbalance, ROS accumulation, reduced glutathione level and inactivation of glutathione peroxidase 4, which are also important pathological events of AD and cognitive dysfunction; After high-throughput sequencing, it was found that the differentially expressed genes related to iron death were highly enriched in the AD-related gene concentration [13–15]. Therefore, ferroptosis may provide a new research direction for the basic research and clinical treatment of AD.

According to related studies, energy metabolism disorder can lead to an imbalance of endoplasmic reticulum homeostasis [16]. The activation of protein kinase r-like endoplasmic reticulum kinase (PERK)/eukaryotic initiation factor 2α (eIF2α) pathway can trigger endoplasmic reticulum stress (ERS) and upregulation of BACE1 protein translation, resulting in excessive accumulation of Aβ [17, 18].

In 2007, the ORMDL3 gene is defined as a susceptibility gene closely related to the occurrence of childhood asthma [19]. It has been demonstrated that ORMDL3 plays an important role in the pathogenesis of inflammatory bowel disease and type I diabetes [20]. Therefore, ORMDL3 may be involved in immune system dysregulation [21]. After the stimulation of airway epithelial cells with allergens and cytokines (IL-4 or IL-13), the expression of ORMDL3 mRNA was increases [22]. The expression product of ORMDL3 can inhibit the Ca2+ ATPase (SERCA) activity of the endoplasmic reticulum and lead to the decrease in Ca2+ concentration in the endoplasmic reticulum and the increase in unfolded protein response (UPR) [22]. So, this study explores the function of ORMDL3 on ferroptosis in AD and its potential regulatory mechanisms.

2 | MATERIALS AND METHODS

2.1 | Animals experiment

APPswe/PS1dE9 mice and C57BL/6 mice were maintained in specific pathogen-free husbandry and fed a standard rodent diet. All of the animal procedures followed the guidelines and were approved by the Animal Care and Use Committee of China–Japan Union Hospital of JiLin University.

2.2 | Vitro model

The murine microglial BV-2 cells were maintained in DMEM (Bioind, Kibbuiz, Israel) supplemented with 10% FBS and 1% penicillin/streptomycin. BV-2 cells were transfected by ORMDL3 plasmid and si-ORMDL3 mimics by using Lipofectamine 2000 (Invitrogen). After 24 h of incubation, Aβ1-42 (1 μmol/L) was induced into BV-2 cells for 24 h.

2.3 | Quantitative RT-PCR and microarray analysis

Total RNA was extracted by TRIzol reagent (Invitrogen).

RNA was reverse-transcribed into cDNA using a first strand cDNA synthesis kit (Invitrogen). Quantitative RT-PCR was performed using an ABI StepOne Plus using SYBR Green® Premix Ex Taq (Takara).

The total RNA of each sample was used using an Invitrogen SuperScript double-stranded cDNA synthesis kit. Double-stranded cDNA was executed with a NimbleGen one-colour DNA labelling kit and then executed for array hybridisation by using the NimbleGen hybridisation system and washing with the NimbleGen wash buffer kit. Axon GenePix 4000 B microarray scanner (Molecular Devices) was used for scanning.

2.4 | Patients experiment

Serum of AD patients and Healthy volunteers were obtained from our hospital. Informed consents were obtained from all the subjects. ADL score, MMSE score, or MoCA score, and Hcy levels were analyzed by case report. [Correction added on 22 February 2023, after first online publication: Section 2.4 was newly added.]

2.5 | Immunohistochemistry

Cells were fixed with 4% paraformaldehyde, permeabilised with 0.5% Triton X-100 in PBS for 15 min at room temperature and blocked with 5% BSA for 30 min at 37°C. Cells were treated with primary antibodies at 4°C overnight: ORMDL3 (ab211522, 1:200, Abcam) and p-PERK (ab192591, 1:200, Abcam). Cells were then incubated with Cy3-conjugated goat anti-rabbit (A0516, Beyotime, 1:500) or goat anti-mouse IgG DyLight 488-conjugated secondary antibodies (A0428, Beyotime, 1:500) for 2 h at 37°C. Nuclei were stained with DAPI and cells were observed under a fluorescent illumination microscope (Olympus IX71).

2.6 | Morris water maze

Morris water maze (MWM) was executed by Yang et al [23]. MWM was executed for 24 h after the FST, conducted in a pool (120 cm diameter) filled with water at 24 ± 2°C and imaginarily divided into four quadrants. The mice were placed in the contralateral quadrant, and the time spent in the target quadrant was recorded and calculated with Ugo Basile software.

2.7 | Elisa

MDA, GSH, GSH-px and SOD activity levels were measured using the ELISA kit (Beyotime) according to the manufacturer's protocol. MDA, GSH, GSH-px and SOD activity levels were detected using an ELISA reader (Infinite® M200, Tecan trading AG) at 450 nm.

2.8 | Cell viability determination and LDH activity levels

Cell viability was determined with Cell Counting Kit-8 (CCK-8). A total of 2 × 103 cells/wells were seeded in a 96-well plate after 48 h of transfection. Cell viability was detected using an ELISA reader (Infinite® M200, Tecan trading AG) at 450 nm.

Then, LDH activity levels were determined using the LDH activity kit. A total of 2×10^3 cells/wells were seeded in a 96-well plate after 48 h of transfection. LDH activity levels were detected using an ELISA reader (Infinite® M200, Tecan trading AG) at 450 nm.

2.9 | Western blot

Proteins were extracted from tissues or cells in RIPA assay and protein concentration was determined by a BCA protein assays kit (Thermo Scientific). Protein were separated into 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.). The PVDF membranes were incubated by ORMDL3 (ab211522, 1:1000, Abcam), p-PERK (3179, 1:1000, Cell Signaling Technology, Inc.), PERK (5683, 1:1000, Cell Signaling Technology, Inc.), p-eiF2α (ab32157, 1:1000, Abcam), eiF2α (ab169528, 1:1000, Abcam), ATF4 (ab184909, 1:1000, Abcam), HSPA5 (ab21685, 1:1000, Abcam), GPX4 (ab125066, 1:1000, Abcam) and β-actin (ab8226, 1:10000, Abcam) at 4°C overnight. PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (A0208, A0216, Beyotime, 1:5000) for 2 h. The signal was tested with the chemiluminescence system (Amersham Pharmacia).

2.10 | Statistics and data collection

Statistics were performed using GraphPad Prism 7 (Graph Pad Software Inc). Data are shown as the mean ± SD. Two-way ANOVA was used for multiple comparisons, and *t*-tests (two-tailed) were used for single comparisons. A p value of less than 0.05 was considered statistically significant.

3 | RESULTS

3.1 | ORMDL3 expression levels in a model of AD

The experiment address that the possible role of ORMDL3 in a model of AD, ORMDL3 mRNA expression, was measured using Quantitative RT-PCR. In serum samples of AD patients, ORMDL3 mRNA expression levels were upregulated (Figure 1a). The serum ORMDL3 levels expression was positively related to the ADL score or MoCA score in AD

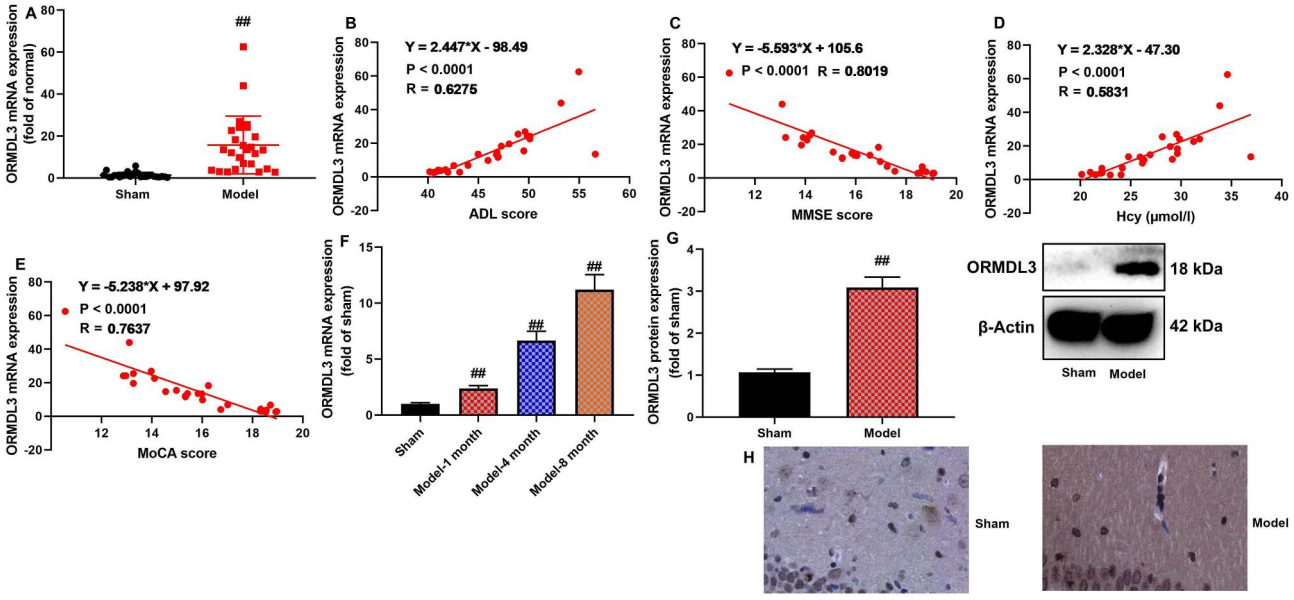

FIGURE 1 ORMDL3 expression levels in model of AD. Serum ORMDL3 mRNA expression (a), the serum ORMDL3 levels was related to ADL score (b) or MMSE score (c) or MoCA score (d) or Hcy levels (e) in patients; mRNA expression and protein expression of ORMDL3 in hippocampal tissue (f) and (g), ORMDL3 expression in hippocampal tissue (Immunohistochemistry), (h) in mice model of AD. Sham, sham control group; model, mice with AD. ##$p < 0.01$ compared with normal volunteers group or sham control group. [Correction added on 22 February 2023, after first online publication: In Figure 1 caption "ORMDL3 expression levels in mice model of AD" was changed as "ORMDL3 expression levels in model of AD." "(e), mRNA expression and protein expression of ORMDL3 in hippocampal tissue" was changed as "(e) in patients; mRNA expression and protein expression of ORMDL3 in hippocampal tissue"]

patients (Figure 1b,d). Interestingly, the serum ORMDL3 expression level was positively related to the MMSE score or Hcy levels in AD patients (Figure 1c,e). The mRNA expression of ORMDL3 in hippocampal tissue of the mice model of AD was upregulated at one, four and eight months (Figure 1f). The protein expression of ORMDL3 was upregulated in the mice model of AD (Figure 1g). Immunohistochemistry showed ORMDL3 expression in the hippocampal tissue of the AD mice model (Figure 1h). Collectively, these findings suggested that ORMDL3 promoted the cognitive decline of AD, and it may participate in the disease progression of AD. [Correction added on 22 February 2023, after first online publication: In the following sentence in section 5.1 "AD mice" was changed to "AD patients" in 3 instances.]

3.2 | ORMDL3 promoted Alzheimer's disease and increased oxidative response in a model of AD

To explore the function of ORMDL3 on the cognitive decline of AD, its possible mechanisms were analysed. The ORMDL3 gene increased the number of entering the dark compartment, heightened the time of escape latency and path length, reduced timelatency and the frequency of passing through the goal, heightened the number of entering the dark compartment and inhibited time spent in the target quadrant in AM mice of AD (Figure 2a–g). The ORMDL3 gene increased the MDA activity levels and reduced GSH, GSH-px and SOD activity levels in the hippocampal tissue of the AD mice model (Figure 2h–k).

Next, ORMDL3 plasmid increased ORMDL3 mRNA expression, and sh-ORMDL3 reduced the expression of ORMDL3 mRNA in vitro model of AD (Figure 3a–d). Overexpression of ORMDL3 increased MDA levels and reduced SOD, GSH and GSH-px levels in vitro model (Figure 3c–f). Downregulation of ORMDL3 reduced MDA levels and increased SOD, GSH and GSH-px levels in vitro model (Figure 3g–j).

Overexpression of ORMDL3 lessened cell growth, and promoted lipid ROS levels and ROS production levels in vitro model of AD (Figure 4a–c). Downregulation of ORMDL3 promoted cell growth and reduced lipid ROS levels and ROS production levels in vitro model of AD (Figure 4d–f). Overexpression of ORMDL3 suppressed the GPX4 protein expression and downregulation of ORMDL3 induced GPX4 protein expression in vitro model (Figure 4g–h). Therefore, the data proved that ORMDL3 promoted oxidative response and ferroptosis to accelerate the cognitive decline of AD, which regulated oxidative response in mice with AD.

3.3 | PERK/ATF4/HSPA5 pathway is one important signal pathway for the effects of ORMDL3 in a model of AD

The study extended the study of the precise mechanism of ORMDL3 on neurological damage in a model of AD.

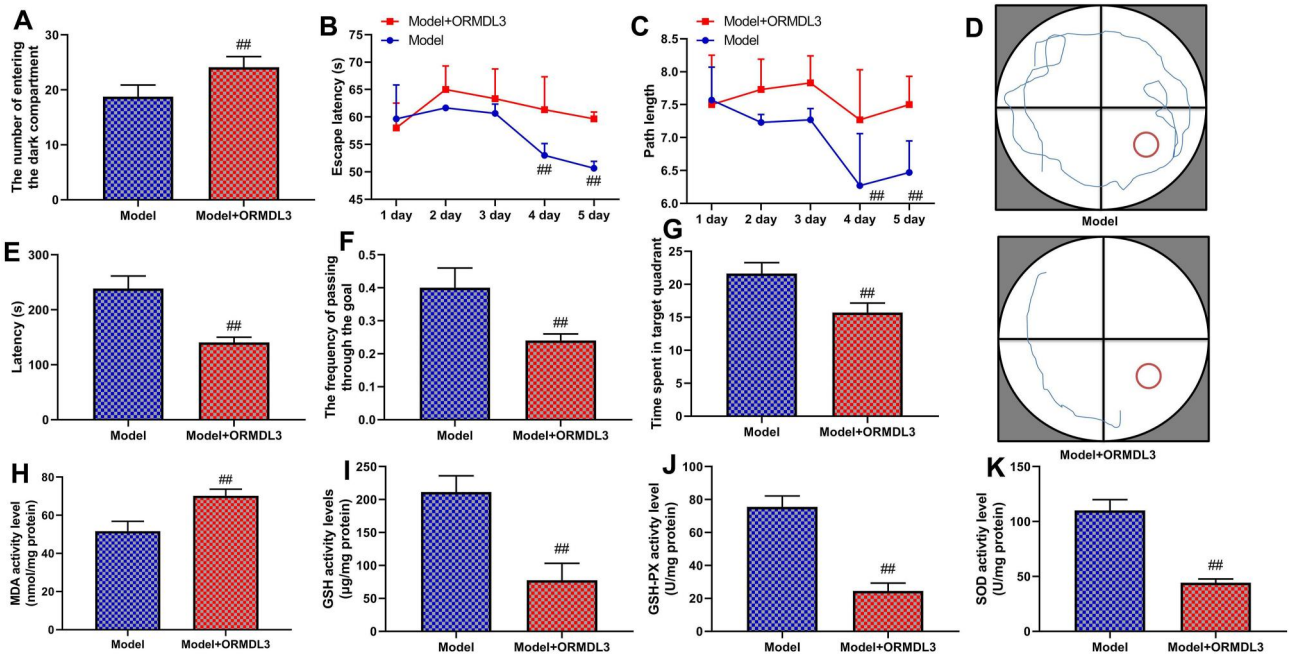

FIGURE 2 ORMDL3 promoted Alzheimer's disease and increased oxidative response in mice model of AD. The number of entering the dark compartment (a), the time of escape latency (b) and path length (c), Morris water maze (MWM) (d), the time of latency (e), the frequency of passing through the goal (f), the number of entering the dark compartment (g), MDA activity level (h), GSH, GSH-px and SOD activity levels (i, j, and k) in hippocampal tissue of AD mice. Model, mice with AD; Model+ORMDL3, mice with AD by ORMDL3 virus. ##$p < 0.01$ compared with Model, mice with AD.

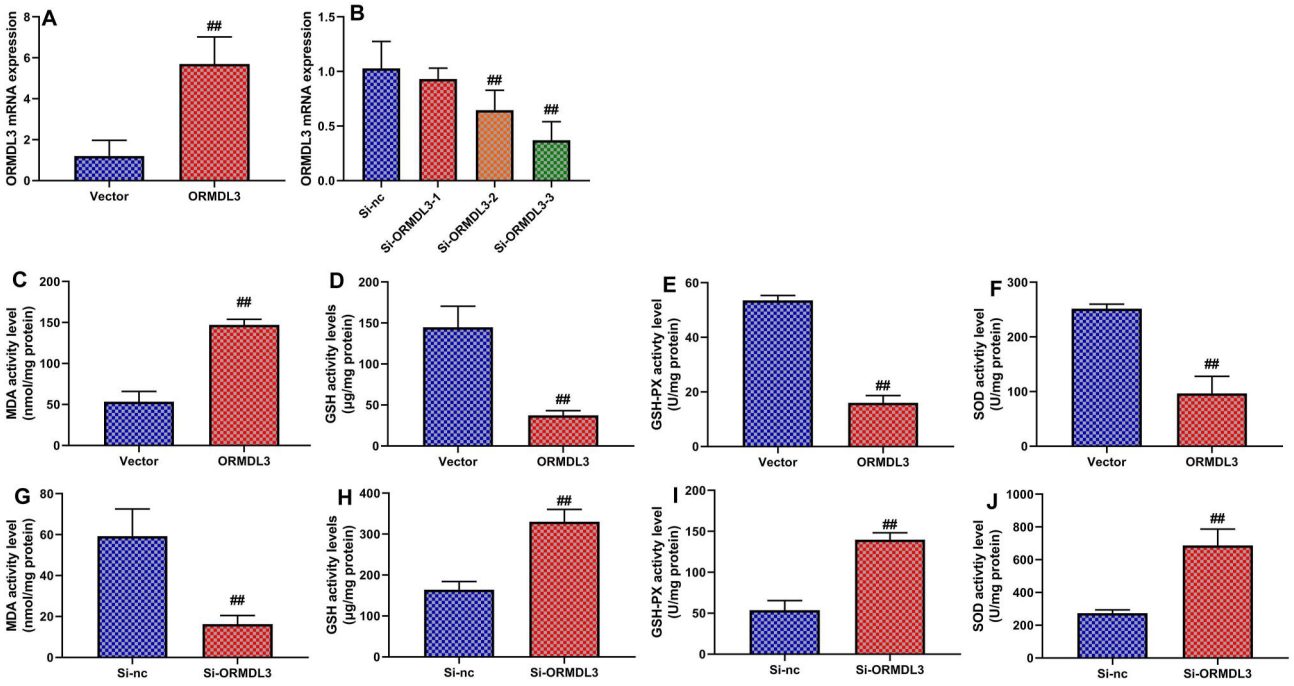

FIGURE 3 ORMDL3 increased oxidative response in vitro model of AD. ORMDL3 mRNA expression (a) and (b), MDA, GSH, GSH-px and SOD activity levels (c, d, e, and f) in vitro model by ORMDL3 over-expression; MDA, GSH, GSH-px and SOD activity levels (g, h, i, and j) in vitro model by ORMDL3 down-regulation. Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 over-expression group; Si-ORMDL3, ORMDL3 down-regulation group. ##$p < 0.01$ compared with normal volunteers group or sham control group.

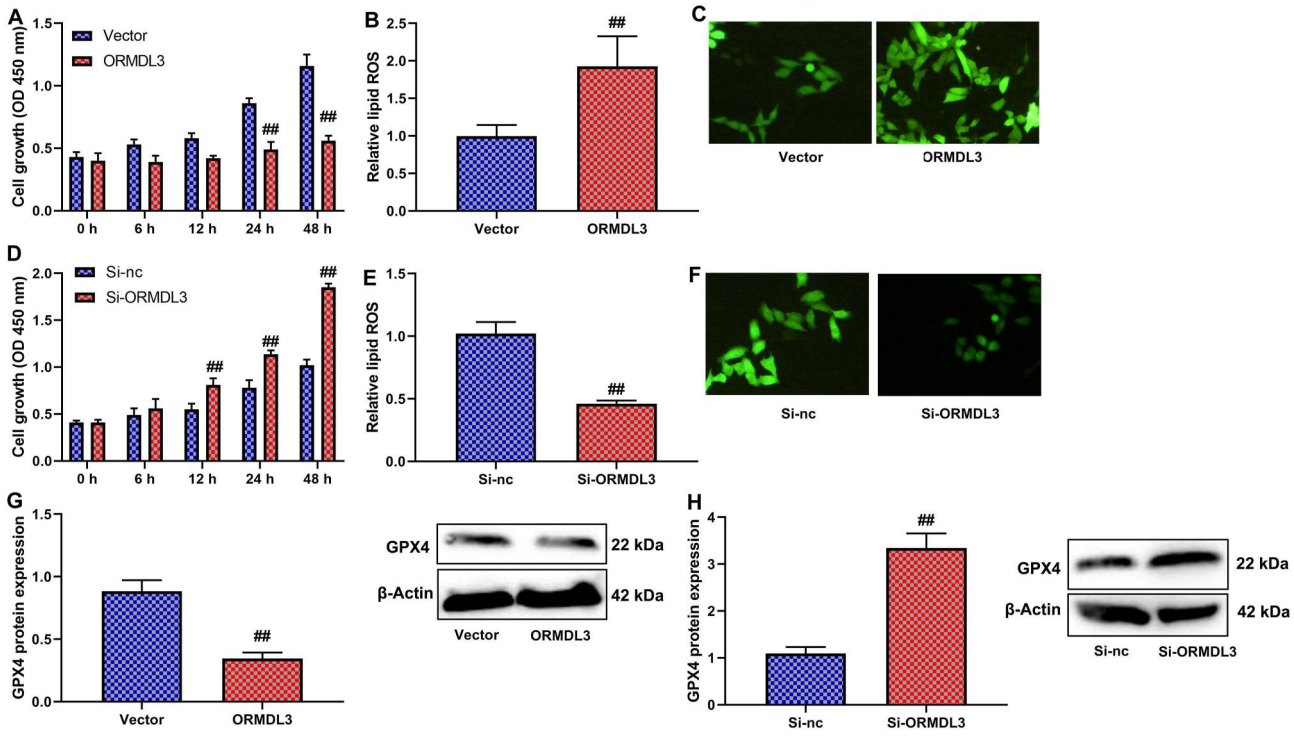

FIGURE 4 ORMDL3 promoted ferroptosis in vitro model of AD. Cell growth (a), lipid ROS level (b, and c) in vitro model by ORMDL3 over-expression; Cell growth (d), lipid ROS level (e, and f) in vitro model by ORMDL3 down-regulation; GPX4 protein expression in vitro model by ORMDL3 over-expression (g); GPX4 protein expression in vitro model by ORMDL3 down-regulation (h). Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 over-expression group; Si-ORMDL3, ORMDL3 down-regulation group; ##$p < 0.01$ compared with vector control or si-negative group.

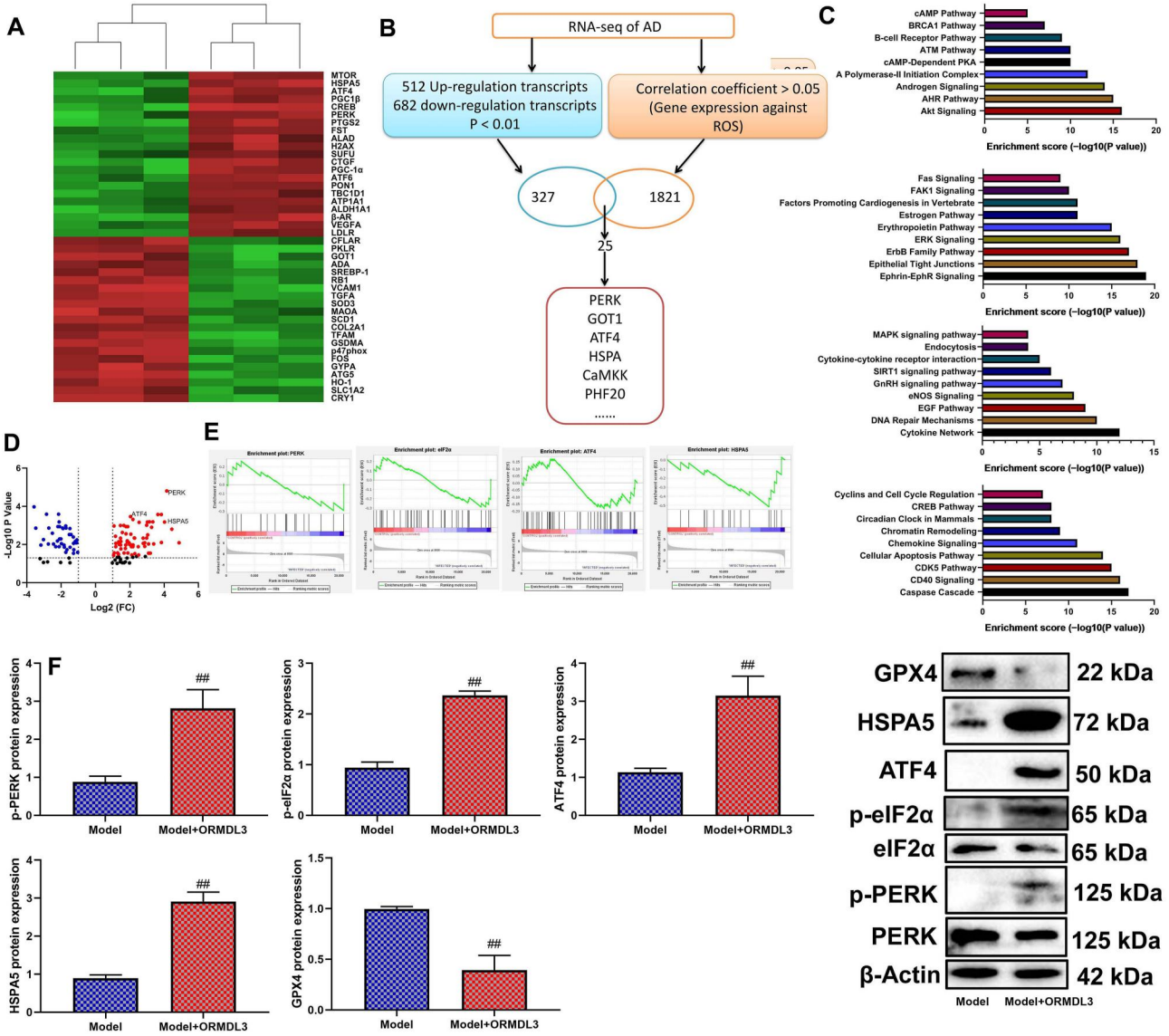

FIGURE 5 PERK/ATF4/HSPA5 pathway is one important signal pathways for the effects of ORMDL3 in mice model of AD. Heat map (a), technological process (b), signaling pathway (c), Volcanic map (d), KEGG terms (e), p-PERK/p-eiF2α/ATF4/HSPA5/GPX4 protein expression levels (f). Model, mice with AD; Model+ORMDL3, mice with AD by ORMDL3 virus. ##$p < 0.01$ compared with Model, mice with AD.

Microarray analysis was used to analyze the regulation of possible genes by ORMDL3 in a model of AD (Figure 5a). PERK/ATF4/HSPA5 pathway might be one important signal pathway for the effects of ORMDL3 on neurological damage in a model of AD (Figure 5b–e). The ORMDL3 gene induced p-PERK, p-eiF2α, ATF4 and HSPA5 protein expression levels and suppressed the GPX4 protein expression level in the hippocampal tissue of the mice model of AD (Figure 5f).

ORMDL3 induced p-PERK, p-eiF2α, ATF4 and HSPA5 protein expression levels in vitro model of AD (Figure 6a,c). Downregulation of ORMDL3 suppressed p-PERK, p-eiF2α, ATF4 and HSPA5 protein expression levels in vitro model of AD (Figure 6b,d). Meanwhile, we found that ORMDL3 reduced PERK protein Ubiquitination in vitro model of AD, and downregulation of ORMDL3 promoted PERK protein Ubiquitination in vitro model of AD (Figure 6E). Immunofluorescence showed that overexpression of ORMDL3 promoted p-PERK expression in vitro model (Figure 6f). Consequently, the PERK/ATF4/HSPA5 pathway is one important signal pathway for the effects of ORMDL3 in model of AD.

3.4 | PERK participated in the regulation of ORMDL3 in a model of AD

The experiment determined the role of PERK in the regulation of ORMDL3 in a model of AD. PERK Agonist (CCT020312, 5 μM) induced p-PERK, p-eiF2α, ATF4 and

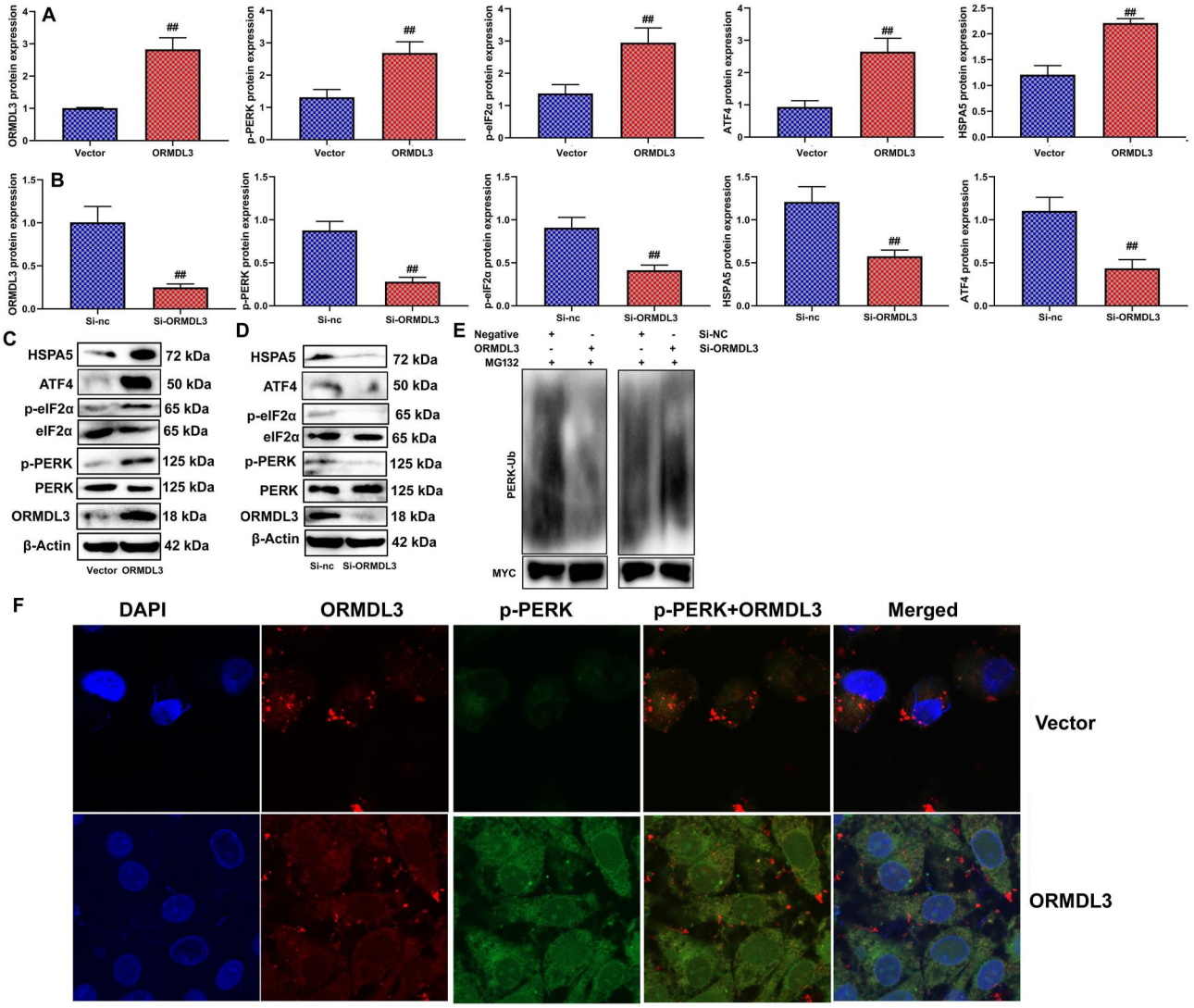

FIGURE 6 ORMDL3 regulated PERK/ATF4/HSPA5 pathway in vitro model of AD. p-PERK/p-eiF2α/ATF4/HSPA5 protein expression levels in vitro model by ORMDL3 over-expression (a, and c); p-PERK/p-eiF2α/ATF4/HSPA5 protein expression levels in vitro model by ORMDL3 downregulation (b, and d); PERK Ubiquitination (e); PERK and ORMDL3 expression (Confocal, f). Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 over-expression group; Si-ORMDL3, ORMDL3 down-regulation group. ##$p < 0.01$ compared with vector control or si-negative group.

HSPA5 protein expression levels in vitro model of AD by si-ORMDL3 (Figure 7a–f). The PERK inhibitor (PERK-IN-4-d3, 0.1 nM) suppressed p-PERK, p-eiF2α, ATF4 and HSPA5 protein expression levels in vitro model of AD by ORMDL3 (Figure 7–g–l).

The PERK inhibitor increased cell growth, inhibited the lipid ROS level and MDA activity level, promoted SOD, GSH and GSH-px levels in vitro model of AD by ORMDL3 (Figure 8a–g). PERK Agonist reduced cell growth, increased the lipid ROS level and MDA activity level and reduced SOD, GSH and GSH-px levels in vitro model of AD by si-ORMDL3 (Figure 8h–n).

The PERK inhibitor (AMG PERK 44, 100 mg/kg po) decreased the number of entering the dark compartment, inhibited the time of escape latency and path length, increased time latency and the frequency of passing through the goal, heightened the number of entering the dark compartment and promoted the time spent in the target quadrant in AM mice of AD by the ORMDL3 gene (Figures S1a–g). The PERK inhibitor decreased the MDA activity levels and increased GSH, GSH-px and SOD activity levels in the hippocampal tissue of the AD mice model by the ORMDL3 gene (Figures S1h–k). These findings indicate that ORMDL3-regulated PERK expression may facilitate ferroptosis resistance in a model of AD.

3.5 | ATF4/HSPA5 participated in the regulation of ORMDL3 in model of AD

We next addressed the role of ATF4 in the regulation of ORMDL3/PERK in model of AD. ATF4 plasmid increased

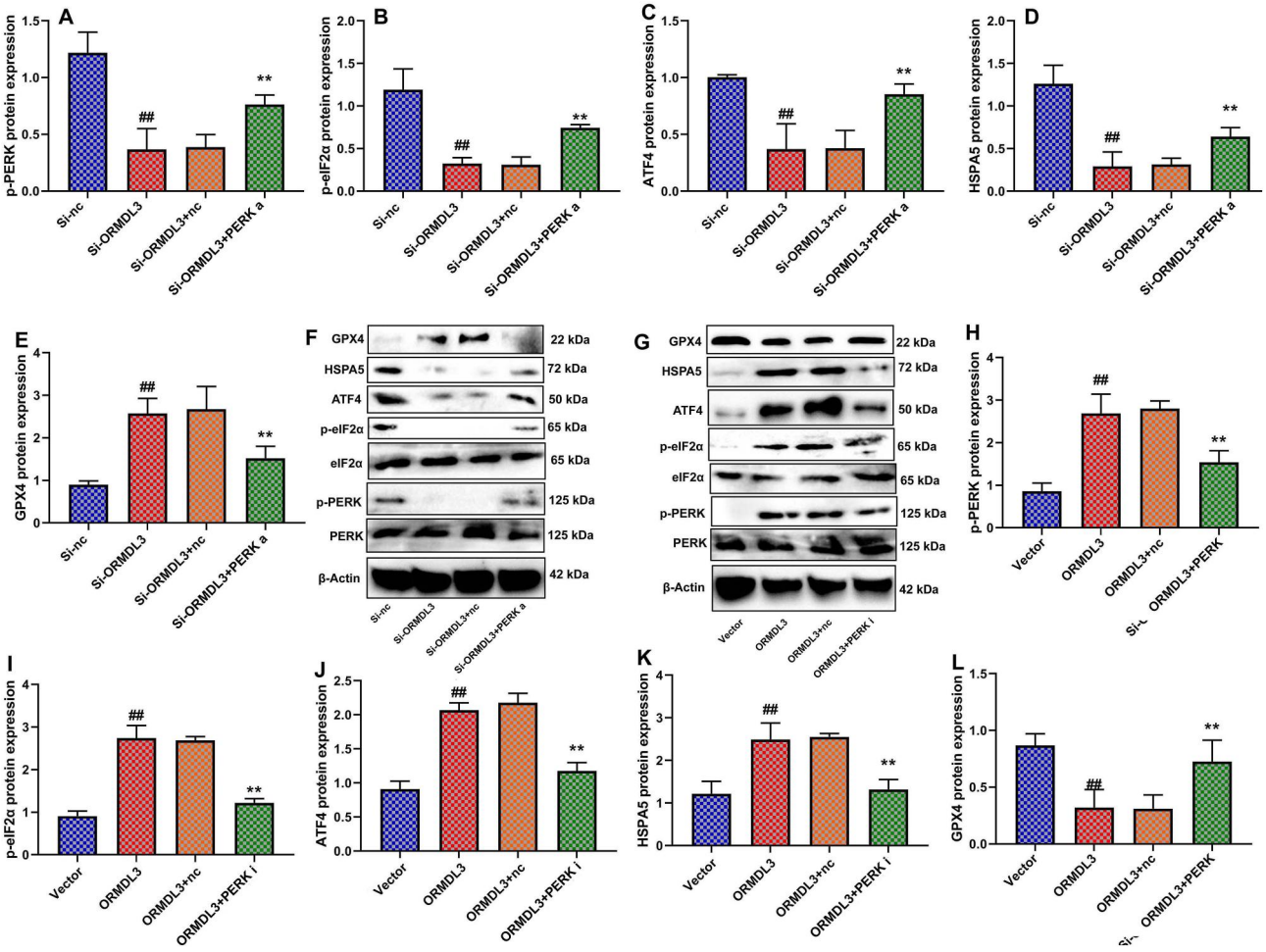

FIGURE 7 PERK participated in the regulation of ORMDL3 in model of AD. p-PERK/p-eiF2α/ATF4/HSPA5/GPX4 protein expression levels in vitro model by ORMDL3 down-regulation + PERK (a, b, c, d, e, and f); p-PERK/p-eiF2α/ATF4/HSPA5/GPX4 protein expression levels in vitro model by ORMDL3 over-expression + PERK i(g, h, i, j, k, and l). Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 overexpression group; Si-ORMDL3, ORMDL3 downregulation group; PERK a, PERK Agonist; PERK i, PERK inhibitor. ##$p < 0.01$ compared with vector control or si-negative group; ##$p < 0.01$ compared with ORMDL3 overexpression group or ORMDL3 downregulation group.

ATF4 and HSPA5 protein expression levels and reduced GPX4 protein expression level in vitro model of AD by si-ORMDL3 (Figure 9a–c). Si-ATF4 suppressed ATF4 and HSPA5 protein expression levels and induced GPX4 protein expression level in vitro model of AD by ORMDL3 (Figure 9f–f). Then, HSPA5 plasmid increased HSPA5 protein expression levels and reduced GPX4 protein expression level in vitro model of AD by si-ORMDL3 (Figure 9g–h). Si-HSPA5 suppressed HSPA5 protein expression levels and induced GPX4 protein expression level in vitro model of AD by ORMDL3 (Figure 9i–j).

Si-ATF4 increased cell growth, inhibited lipid ROS level and MDA activity level and promoted SOD, GSH and GSH-px levels in vitro model of AD by ORMDL3 (Figure 10a–g). ATF4 plasmid also reduced cell growth, increased lipid ROS level and MDA activity level and reduced SOD, GSH and GSH-px levels in vitro model of AD by si-ORMDL3 (Figure 10h–n).

Meanwhile, si-HSPA5 increased cell growth, inhibited lipid ROS level and MDA activity level, promoted SOD, GSH and GSH-px levels in vitro model of AD by ORMDL3 (Figure 11a–g). HSPA5 plasmid also reduced cell growth, increased lipid ROS level and MDA activity level and reduced SOD, GSH and GSH-px levels in vitro model of AD by si-ORMDL3 (Figure 11h–n).

Next, sh-ATF4 virus decreased the number of entering the dark compartment, inhibited the time of escape latency and path length, increased time latency and the frequency of passing through the goal, heightened the number of entering the dark compartment and promoted the time spent in the target quadrant in AM mice of AD by the ORMDL3 gene (Figures S2a–g). The sh-ATF4 virus decreased the MDA activity levels and increased GSH, GSH-px and SOD activity levels in the hippocampal tissue of the AD mice model by ORMDL3 gene (Figures S2h–k).

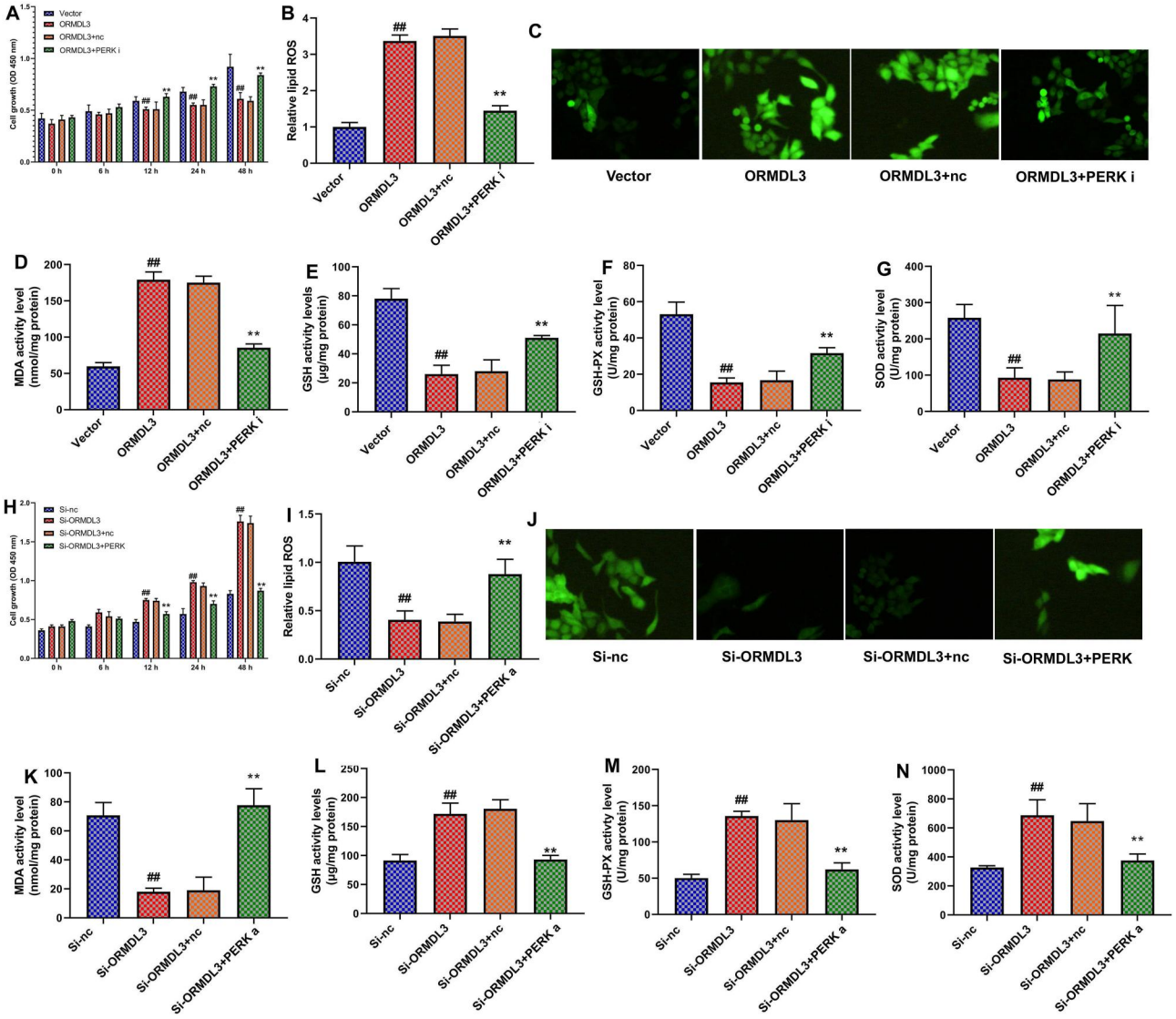

FIGURE 8 PERK participated in the regulation of ORMDL3 on oxidative response and ferroptosis in model of AD. Cell growth (a); lipid ROS level (b, and c); MDA, GSH, GSH-px and SOD activity levels (d, e, f, and g); Cell growth (h); lipid ROS level (i, and j); MDA, GSH, GSH-px and SOD activity levels (k, l, m, and n). Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 overexpressio n group; Si-ORMDL3, ORMDL3 downregulation group; PERK a, PERK Agonist; PERK i, PERK inhibitor. ##$p < 0.01$ compared with vector control or si-negative group; **$p < 0.01$ compared with ORMDL3 overexpression group or ORMDL3 downregulation group.

Lastly, the sh-HSPA5 virus decreased the number of entering the dark compartment, inhibited the time of escape latency and path length, increased time latency and the frequency of passing through the goal, heightened the number of entering the dark compartment and promoted the time spent in the target quadrant in AM mice of AD by the ORMDL3 gene (Figure S3a–g).The sh- HSPA5 virus decreased the MDA activity levels and increased GSH, GSH-px and SOD activity levels in the hippocampal tissue of the AD mice model by the ORMDL3 gene (Figures S3h–k). Collectively, these findings suggest that ORMDL3 promoted ferroptosis through protecting against AD through the regulation of the PERK/ATF4/HSPA5 pathway (Figure 12).

4 | DISCUSSION

AD is a major refractory disease with a high mortality and disability rate, which ranks sixth among the main killers of human health [24]. With the increasing population aging, the number of AD patients in China has increased dramatically to nearly 9 million every year [25]. China has become the country with the largest number of AD patients in the world [26]. With responsible etiology, the course of AD involves multiple pathologies. There is currently no effective way to cure AD [26]. In the present study, the protein and mRNA expression of ORMDL3 was upregulated in the mice model of AD. Clarke et al. indicated that ORMDL3 ensured proper myelination and neurologic function [27]. These observations

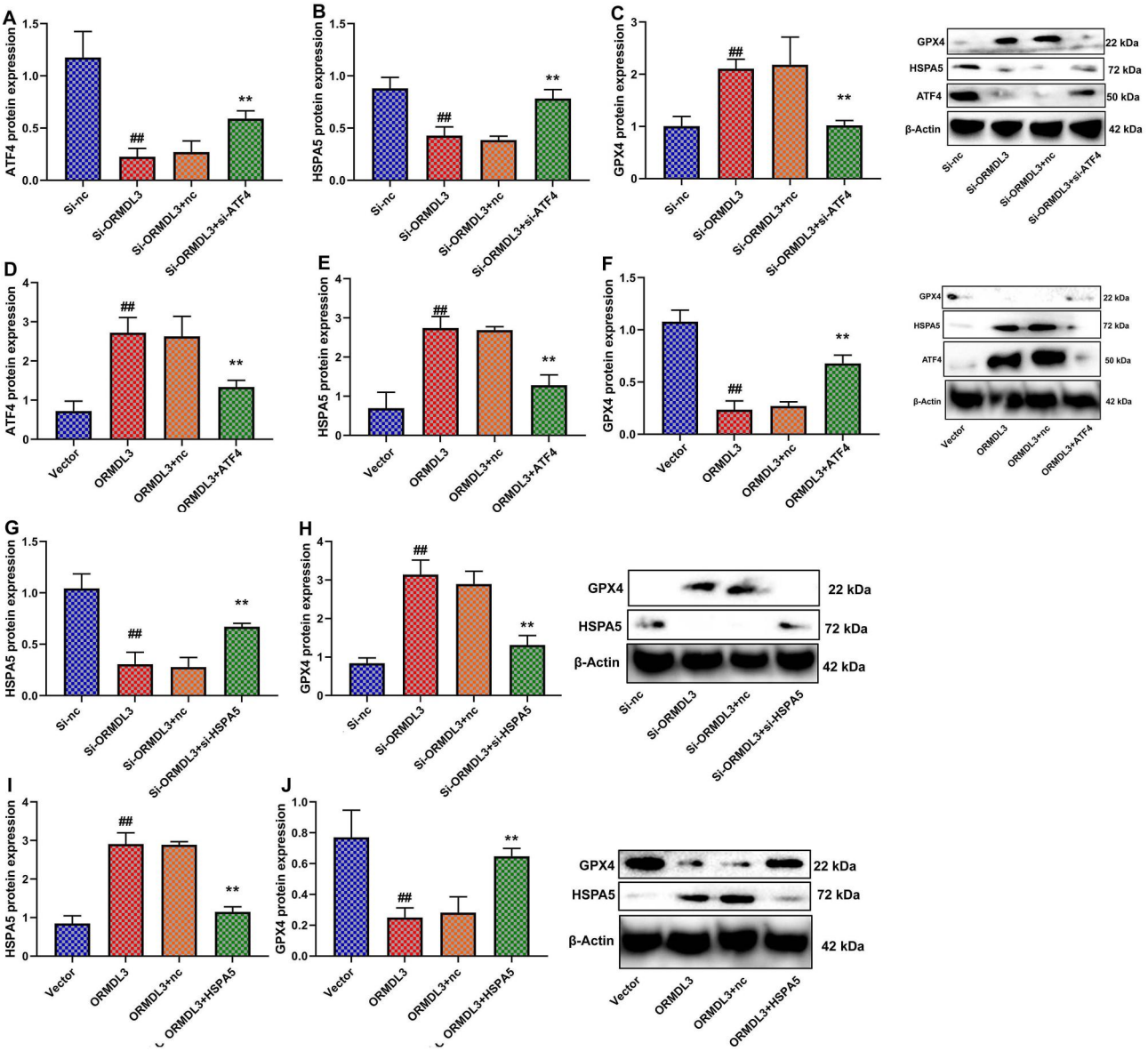

FIGURE 9 ATF4/HSPA5 participated in the regulation of ORMDL3 in model of AD. ATF4/HSPA5/GPX4 protein expression levels in vitro model by ORMDL3 downregulation + ATF4 (a, b, and c); ATF4/HSPA5/GPX4 protein expression levels in vitro model by ORMDL3 overexpression + si-ATF4 (d, e, and f); HSPA5/GPX4 protein expression levels in vitro model by ORMDL3 downregulation + HSPA5 (g, and h); HSPA5/GPX4 protein expression levels in vitro model by ORMDL3 overexpression+si- ATF4 (I and J). Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 overexpression group; Si-ORMDL3, ORMDL3 downregulation group; ATF4, overexpression of ATF4; si-ATF4, down-regulation of ATF4; HSPA5, over-expression of HSPA5; si- HSPA5, down-regulation of HSPA5. $\#\#p < 0.01$ compared with vector control or si-negative group; $**p < 0.01$ compared with the ORMDL3 overexpression group or ORMDL3 downregulation group.

indicate that ORMDL3 depends on the disease process of AD.

ER stress is a protective mechanism for cells against external damage [28]. In the course of AD, excessive accumulation of Aβ and NFTs activates ERS and triggers UPR, which in turn increases autophagy and eliminates misfolded proteins [29, 30]. This study indicated that ORMDL3 promoted Alzheimer's disease and increased oxidative response in the model of AD. Chen et al. reported that silencing ORMDL3 reduced cigarette smoke-induced ER stress [31]. Collectively, these findings indicate that ORMDL3 mediates oxidative response in a model of AD.

Ferroptosis is a new regulated method of cell death discovered in 2012. According to recent studies, ferroptosis is closely related to ERS [32]. Ferroptosis inducers can simultaneously activate the ERS response. The activation of ERS pathway inhibits ferroptosis [33]. In some pathological conditions, the activation of the ERS pathway can exacerbate the occurrence of ferroptosis [34]. The close connection between ferroptosis and ERS has become an important scientific

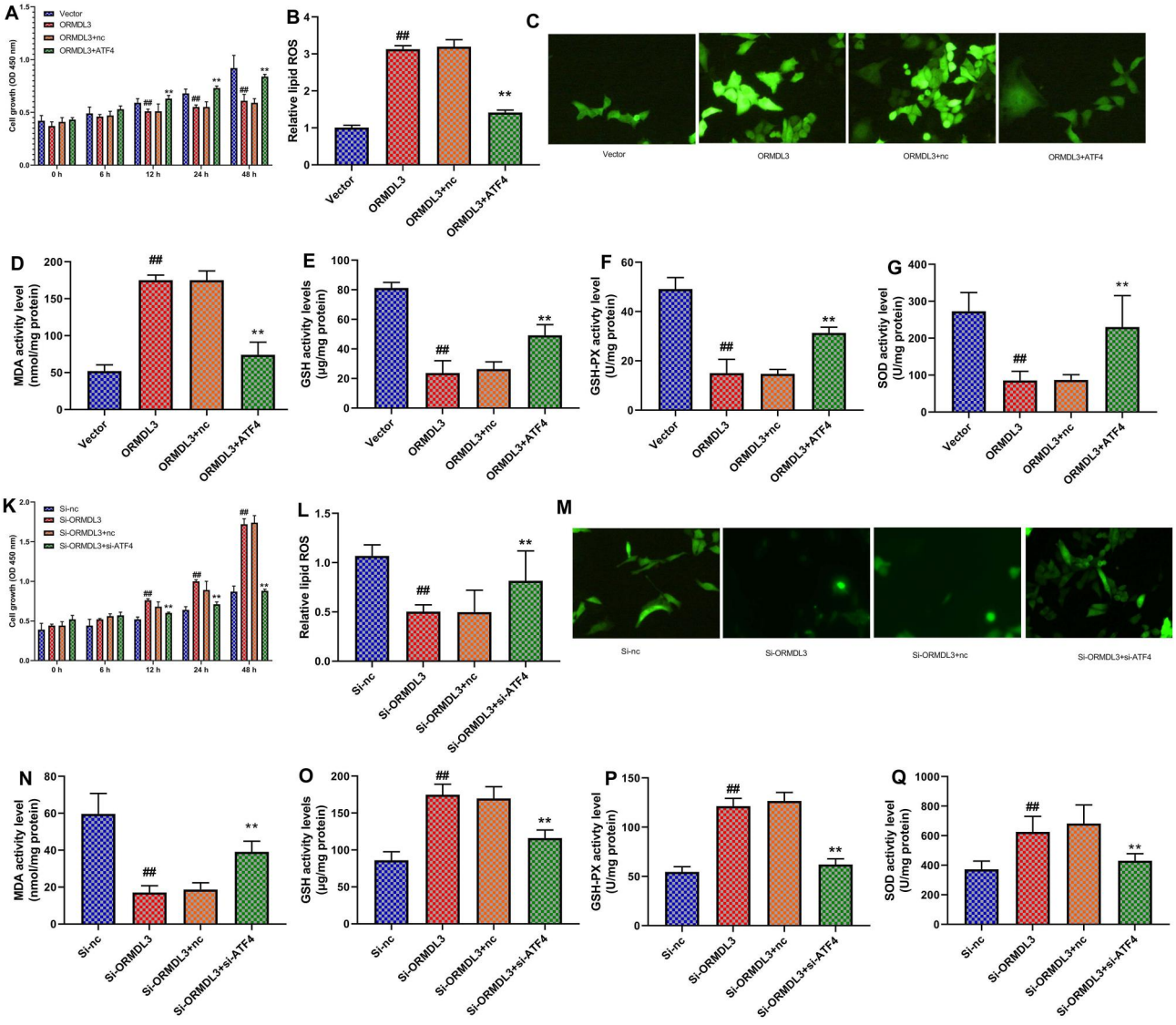

FIGURE 10 ATF4 participated in the regulation of ORMDL3 on oxidative response and ferroptosis in model of AD. Cell growth (a); lipid ROS level (b, and c); MDA, GSH, GSH-px and SOD activity levels (d, e, f, and g); Cell growth (h); lipid ROS level (i, and j); MDA, GSH, GSH-px and SOD activity levels (k, l, m, and n). Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 over-expression group; Si-ORMDL3, ORMDL3 down-regulation group; ATF4, over-expression of ATF4; si-ATF4, down-regulation of ATF4. ##$p < 0.01$ compared with vector control or si-negative group; **$p < 0.01$ compared with ORMDL3 over-expression group or ORMDL3 down-regulation group.

issue in the current research on cell death [35]. The results of this study showed that ORMDL3 promoted oxidative response and ferroptosis to accelerate the cognitive decline of AD. Ma et al. suggest that ORMDL3 promoted ox-LDL-induced cell death [36]. These findings suggest that ORMDL3 promoted oxidative response during the induction of ferroptosis in a model of AD. How ORMDL3 regulation is helpful to treat AD through ferroptosis is still debatable. We will further explore the mechanism of ORMDL3 regulation ferroptosis in a model of AD.

Energy metabolism disorder is an important pathological event in the early stage of AD [37]. Energy metabolism can cause environmental disturbances in the endoplasmic reticulum and the activation of ERS [38]. As the PERK/eIF2α pathway is activated, downstream reactions reduce the overall level of intracellular protein synthesis and endoplasmic reticulum overload stress [38]. In the early stage of AD, energy metabolism disorders can induce ERS, activate the PERK/eIF2α pathway and promote BACE1 translation. Excessive accumulation of Aβ will cause a series of toxic damages [39]. Our study found that the PERK/ATF4/HSPA5 pathway is one important signal pathway for the effects of ORMDL3 in a model of AD. Ogi et al. suggest that ORMDL3 plays an important role in regulating the proinflammatory cytokine expression via PERK phosphorylation in RBL-2H3 cells [40]. These findings indicate that the ORMDL3-regulated PERK signaling pathway may facilitate ferroptosis resistance in a model of AD.

However, activated eIF-2α induces ATF4 mRNA translation, thereby activating the expression of the downstream

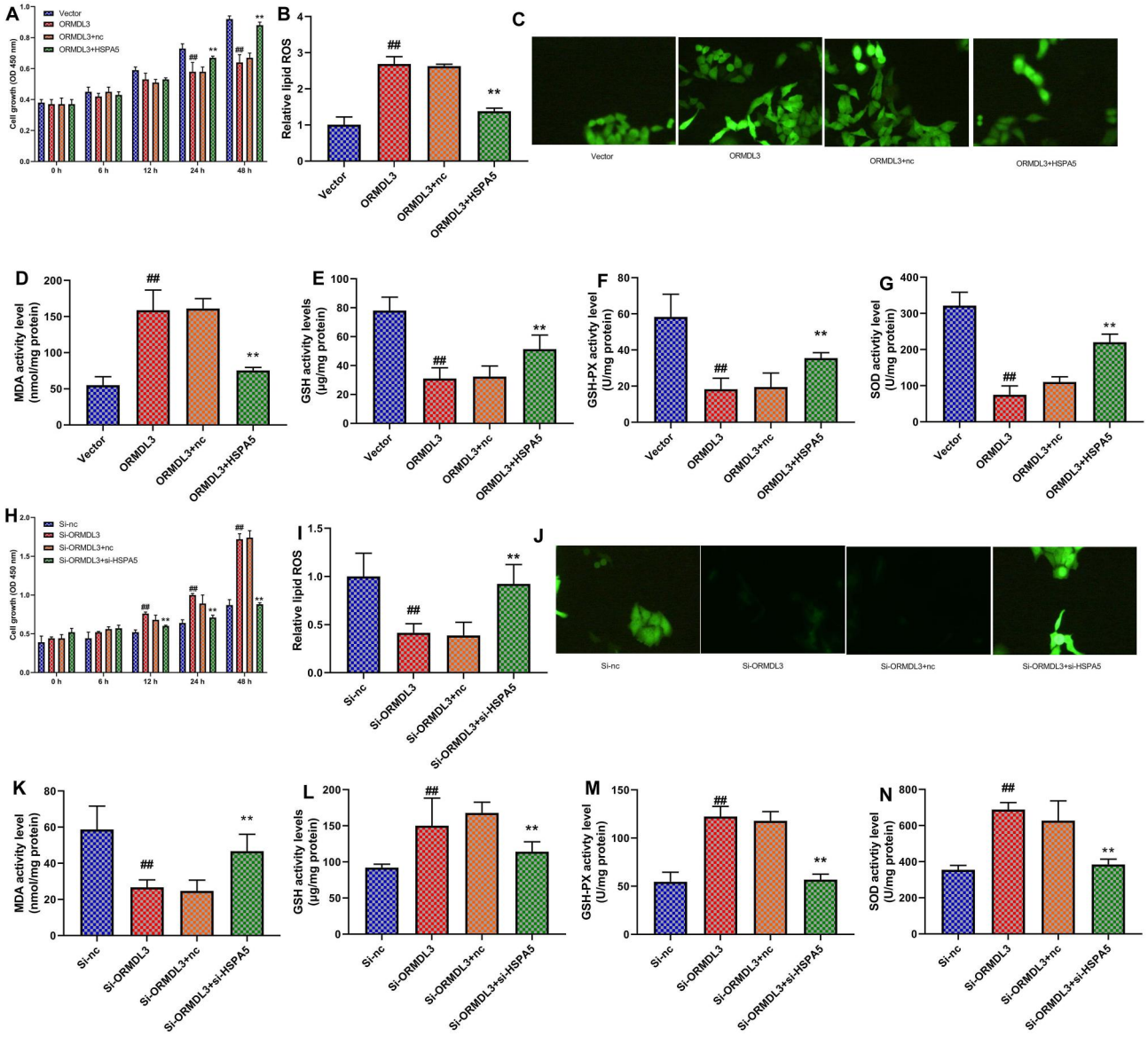

FIGURE 11 HSPA5 participated in the regulation of ORMDL3 on oxidative response and ferroptosis in model of AD. Cell growth (a); lipid ROS level (b, and c); MDA, GSH, GSH-px and SOD activity levels (d, e, f, and g); Cell growth (h); lipid ROS level (i, and j); MDA, GSH, GSH-px and SOD activity levels (k, l, m, and n). Vector, vector control group; Si-nc, si-negative group; ORMDL3, ORMDL3 overexpression group; Si-ORMDL3, ORMDL3 downregulation group; si-ATF4, downregulation of ATF4; HSPA5, overexpression of HSPA5; si- HSPA5, down-regulation of HSPA5. ##$p < 0.01$ compared with vector control or si-negative group; **$p < 0.01$ compared with ORMDL3 overexpression group or ORMDL3 downregulation group.

molecule HSPA5 [41]. In mammalian cells, HSPA5 is ubiquitously expressed, which is the main signal molecule that ER stress mediates apoptosis [42, 43]. This study found that ATF4 participated in the regulation of ORMDL3 on oxidative response and ferroptosis in a model of AD.

Persistent stimulation such as pathogens, inflammatory factors, ischemia and hypoxia can induce ERS [44]. Unfolded or misfolded proteins accumulated in the endoplasmic reticulum will compete to bind to the endoplasmic reticulum chaperone HSPA5 [45]. With the dissociation of HSPA5 from IRE1, PERK and ATF6, downstream signal transduction is activated, triggering a series of reactions to process misfolded/unfolded proteins and restore endoplasmic reticulum homeostasis [46, 47]. These reactions of this study showed that HSPA5 participated in the regulation of ORMDL3 on oxidative response and ferroptosis in a model of AD. Liu et al. showed that THP-1 monocytes overexpressing ORMDL3 expression to increase HSPA5 [48]. These findings suggest that ORMDL3-dependent PERK/ATF4/HSPA5 accelerated ferroptosis in a model of AD.

In the present study, ORMDL3 promoted oxidative response and ferroptosis in a model of AD by PERK/ATF4/HSPA5 pathway. We provided the evidence of ORMDL3 as a novel mechanism for ferroptosis in a model of AD. The

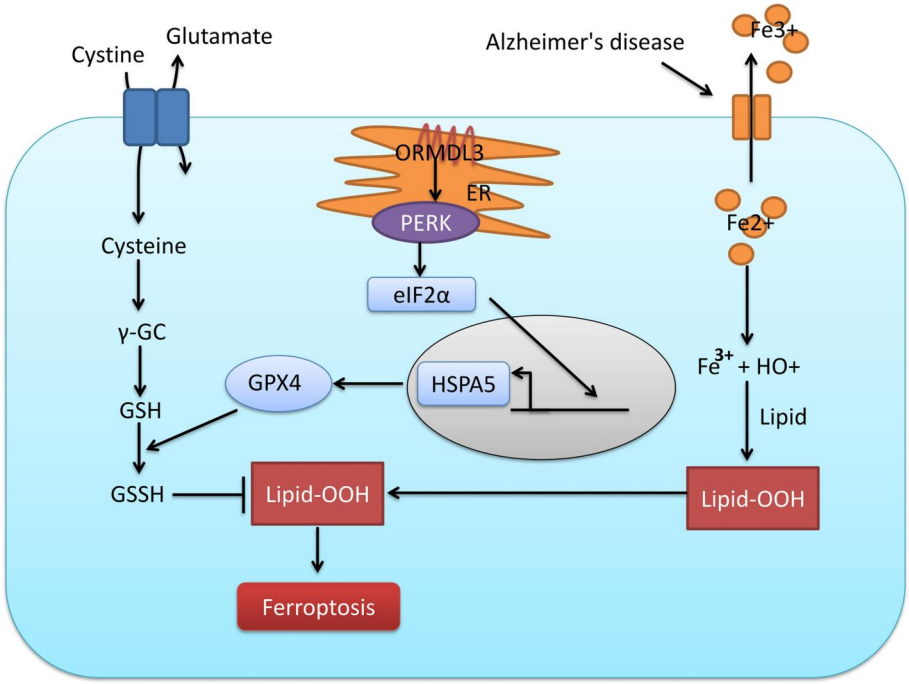

FIGURE 12 The inhibition of ORMDL3 prevents Alzheimer's disease through ferroptosis by PERK/ATF4/HSPA5 pathway.

inhibition of the ORMDL3 gene was conducted as a treatment strategy for AD. Finally, ORMDL3 can be used as a diagnosis and prognosis evaluation of AD.

AUTHOR CONTRIBUTIONS

Yankun Shao designed the experiments. Yilin Xu, Xinxiu Shi and Yingying Wang performed the experiments. Hongyu Liu and Lina Song collected and analyzed the data. Yankun Shao, Hongyu Liu and Lina Song drafted manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The current study was approved by the Animal Ethics Committee and was conducted in accordance to the relevant agreements with the China-Japan Union Hospital of JiLin University. All procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

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