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Knockdown of ferroportin accelerates erastin-induced ferroptosis in neuroblastoma cells

N. GENG[1], B.-J. SHI[1], S.-L. LI[1], Z.-Y. ZHONG[1], Y.-C. LI[1], W.-L. XUA[1], H. ZHOU[1], J.-H. CAI[2]

[1]Department of Pediatric Surgery, Second Hospital, Hebei Medical University, Hebei, Shijiazhuang, China
[2]Hebei Medical University, Hebei, Shijiazhuang, China

Abstract. – OBJECTIVE: Ferroptosis is a new-found iron-dependent form of non-apoptotic regulated cell death (RCD), which is activated on therapy with several antitumor agents, but the potential mechanism remains unclear. Erastin, exhibiting selectivity for RAS-mutated cancer cells, induces ferroptosis by increasing iron and lipid reactive oxygen species (ROS) levels in cell. Ferroportin (Fpn), the sole iron export protein, participates in the regulation of intracellular iron concentration. In this study, we investigated the role of Fpn on ferroptosis induced by erastin in SH-SY5Y cells.

MATERIALS AND METHODS: The cell viability was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit. The activity of caspase-3 was measured by ELISA kit. qRT-PCR was performed to examine the mRNA expression of Fpn. Western blot assay was conducted to examine the expression level of marker proteins. Specific commercial kits were used to examine the levels of MDA, ROS and iron in cells, respectively.

RESULTS: Ferroptosis was evaluated by intracellular lipid ROS level and iron concentration. Hepcidin could prevent erastin-induced ferroptosis by degrading Fpn. Erastin (5 μg/mL) was observed to induce ferroptosis in neuroblastoma cells at 6 hours, which was promoted by knockdown of Fpn. The expression of Fpn gene and protein was decreased in SH-SY5Y cells treated with erastin. After treatment with erastin, Fpn siRNA transfection in SH-SY5Y cells was able to accelerate ferroptosis-associated phenotypic changes. Fpn acted as a negative regulator of ferroptosis by reducing intracellular iron concentration. Knockdown of Fpn enhanced anticancer activity of erastin.

CONCLUSIONS: These results suggested that knockdown of Fpn accelerated erastin-induced ferroptosis by increasing iron-dependent lipid ROS accumulation, highlighting Fpn as a potential therapeutic target site for neuroblastoma. Thus, Fpn inhibitors may provide new access for chemosensitization of neuroblastoma.

Key Words:
Neuroblastoma, Ferroportin, Ferroptosis, ROS, Iron, Lipid peroxidation.

Introduction

Neuroblastoma is one of the most common extracranial malignancies in central nervous system threatening children's health, for which chemotherapy drug options are of limited effects[1-3]. The poor prognosis of neuroblastoma is due to multidrug-resistant and palindromia[4]. The key of high effective chemotherapy regimen is accelerating programmed cell death (PCD) in cancer cells. However, some of the key hallmarks in the extrinsic apoptotic pathway are not expressed in neuroblastoma cells of advanced stage[5,6]. According to the inducer, intact PCD in cancer cells occurs in various ways, besides apoptosis and necrosis, ferroptosis is also noted[7-9].

As a newly discovered form of non-apoptotic PCD, ferroptosis is characterized by increase of both lipid and cytosolic ROS, and its regulator has been targeted with small molecules to induce preferential lethality in cancer cells[8,10]. Iron-mediated lipid ROS are well recognized as the major signaling pathway mediating ferroptosis and mainly based on the increase of lipid peroxidation and GSH depletion in cytoplasm but not caspases activation in apoptosis[8,10,11]. It has been proved that erastin could induce ferroptosis in some cancer cells by overexpressing oncogenic RAS, one of the possible mechanisms is the accumulation of lipid ROS derived from iron metabolic dysfunction[8,11,12]. Therefore, further investigation on the potential mechanism of erastin-induced

Corresponding Author: Jianhui Cai, MD; e-mail: gengnsjz@sina.com

ferroptosis may provide new drug targets for regulating cell death to overcome chemotherapeutics resistance in neuroblastoma cells.

Ferroportin (Fpn), the sole iron export protein, plays an indispensable role in iron transmembrane exportation and regulates iron homeostasis[13-15]. Alterations in the expression of ferroportin may result in iron deficiency or iron overload[16-18]. Endocellular iron accumulation in iron-exporting tissues involves in lack of Fpn and contributes to ferroptosis through increasing lipid ROS production by Fenton's reaction[19]. Fpn-mediated iron export is highly regulated and maintains coordination between iron acquisition, utilization and storage[20]. Fpn is an independent risk factor for breast cancer, and it is related to the reduction of distant metastasis survival in tumor patients[21]. Previous researches[22,23] have shown that the expression of Fpn was decreased in cancer cells. However, the potential role of Fpn in erastin-induced ferroptosis of neuroblastoma cells remains to be explored.

In the present study, we investigated whether erastin induced neuroblastoma cell ferroptosis, and examined the role of Fpn in regulating ferroptosis induced by erastin in neuroblastoma cells.

Materials and Methods

Reagents

Erastin, ferrostatin-1, deferoxamine (DFO), N-acetylcysteine (NAC), hepcidin, Z-VAD-FMK, Necrostatin-1, 3-Methyladenine (3-MA), and ponasterone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ferroportin-1 antibody, ferroportin-1 siRNA (h) (sc-60633), control siRNA (sc-37007) and siRNA Reagent System (sc-45064) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 and TRIzol reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The reverse transcription system and One Step SYBR PrimeScript™ RT-PCR Kit were obtained from TaKaRa (Otsu, Shiga, Japan). Caspase-3 activity assay kit was obtained from Abcam (Cambridge, MA, USA).

Cell Culture and Transfection

SH-SY5Y cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) medium supplemented with 10% fetal bovine serum (FBS), 100 μg /ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. Transient transfections of SH-SY5Y cells with ferroportin-1 siRNA (h) (sc-60633) and control siRNA (sc-37007) were performed with siRNA reagent system following the manufacturer's protocol.

Cell Viability Assay and Caspase-3 Activity Assay

Cell growth inhibition was evaluated with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, Wi, USA) according to the manual. SH-SY5Y cells were seeded in 96-well plates and treated with erastin for an indicated time. Then, 20 μl of the combined MTS/PMS solution were pipetted into each well. After incubating for 2 hours, the absorbance of plate was measured at 490 nm using ELISA plate reader to calculate percentages of survival cells. Caspase-3 activity was detected using Caspase-3 Activity Assay Kit.

Quantitative RT-PCR Analysis

The total RNA was prepared from SH-SY5Y cells using TRIzol reagent. Reverse transcription was carried out using the primers sequences as follows: Fpn, 5'-AGGCAAGATGGCACTAAGCAC-3' (sense) and 5'-TCTATGTTATGCAACAGACAT-3' (antisense), GAPDH, 5'-CAAGGTCATCCATCCATGACAACTTTG-3' (sense) and 5'-GTCCACCACCCTGTTGCTGTAG-3' (antisense). Real-time PCR was performed in a 96-well optical reaction plate using qPCR Kit on ABI Prism 7500.

Western Blot Analysis

Membrane proteins and whole-cell proteins of SH-SY5H cell were extracted respectively using CelLytic™ MEM Protein Extraction Kit and ProteoPrep® Universal Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). Proteins extracted from cell were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4°C with anti- ferroportin-1 and β-actin antibodies, and subsequently incubated with goat anti-rabbit or mouse IgG horseradish peroxidase (HRP)-conjugate. The membranes were scanned by using the Odyssey Fc System. The relative intensity of bands was measured using Quantity One software.

Lipid Peroxidation and Cytosolic ROS Assay

After incubated with different agents for 12 hours, SH-SY5H cells were treated for detecting. The malondialdehyde (MDA) amount in the supernatants lysed from SH-SY5H cells was assessed using lipid peroxidation (MDA) assay kit (MAK085, Sigma-Aldrich, St. Louis, MO, USA) according to the technical instruction. Fluorescence intensity was measured at 532 nm using FLx800 fluorescence reader (Bio Tek, Winooski, VT, USA). The cytosolic ROS was examined by using Fluorometric Intracellular ROS Kit (MAK145, Sigma-Aldrich, St. Louis, MO, USA). The measurement of intracellular ROS was performed using fluorescence reader.

Iron Assay

The intracellular iron concentration was measured using Iron Assay Kit (MAK 025, Sigma) according to the instruction. To measure total iron, samples were added with 5 μL of iron reducer to reduce Fe^{3+} to Fe^{2+} and incubated for 30 min at room temperature in darkness. The reaction was then terminated by adding 100 μL iron probe; thus, absorbance at 593 nm was measured.

Statistical Analysis

Data were expressed as mean ± SD. Statistical analysis was evaluated by ANOVA and SNK-q test. Statistical significance was accepted at $p < 0.05$.

Results

Erastin Induces Ferroptosis in SH-SY5Y Cells

Since erastin induces iron-dependent ferroptosis in different cancer cells carrying oncogenic RAS, we firstly detected whether erastin induced ferroptosis in SH-SY5Y cells. Cell viability was assessed to evaluate erastin-induced cell death in the presence or absence of ferrostatin-1 (ferroptosis inhibitor), DFO (iron chelator), NAC (antioxidant), Z-VAD-FMK (apoptosis inhibitor), Necrostatin-1 (necrosis inhibitor), or 3-methyladenine (autophagy inhibitor). The cell viability was reduced in erastin, and could be prevented by ferrostatin-1, DFO, and NAC (Figure 1A). It was found that ferrostatin-1, DFO, and NAC blocked erastin-induced ferroptotic cell death whereas necrostain-1 and 3-methyladenine failed to protect against erastin-induced cell death. Previous study found that accumulation of iron and lipid ROS are keys of cytotoxicity in ferroptosis. To explore the cell death manner, we further measured the level of intracellular lipid peroxidation and the concentration of iron. Both ferrostatin-1 and DFO significantly inhibit erastin-induced lipid ROS generation and iron accumulation (Figure 1B and C). The treatment with NAC also effectively decreased lipid ROS level but not iron concentration (Figure 1B and C). Furthermore, erastin did not markedly improve the caspase 3 activity, an apoptotic marker (Figure 1D). These results suggested that erastin did induce iron-dependent ferroptosis but not other forms of cell death, such as apoptosis, necrosis and necroptosis in neuroblastoma cells.

Erastin Regulates Fpn Expression in SH-SY5Y Cells

To determine the role of erastin in Fpn expression *in vitro*, SH-SY5Y cells were treated with erastin (20 μM) for 6, 12 and 24 hours. The total protein and messenger RNA levels of Fpn were detected. The level of Fpn mRNA began to reduce at 6 hours and a significant reduction was observed after 12 hours of erastin treatment (Figure 2A); meanwhile, Fpn protein began to decrease at 12 hours and a striking decrease was evident after 24 hours of erastin incubation (Figure 2B). Furthermore, erastin decreased mRNA and protein levels of Fpn in a time-dependent manner. The expression of Fpn was up-regulated by ferrostatin-1, DFO and NAC in the presence of erastin (Figure 2C). However, the level of Fpn was not regulated by Z-VAD-FMK, Necrostatin-1, and 3-Methyladenine in the presence of erastin. These findings suggested that erastin reduced the expression of Fpn in SH-SY5Y cells.

Knockdown of Fpn Promotes Erastin-Induced Ferroptosis

Because decreased cytosolic iron concentration resulted from Fpn-dependent iron export rather than prevention of iron uptake[24], we ascertained the role of Fpn in erastin-induced ferroptosis. SH-SY5Y cells were transfected with ferroportin 1 siRNA or control siRNA and incubated with erastin for 12 hours; cell viability, lipid ROS level, and iron concentration were assessed. The level of Fpn mRNA was efficiently reduced by transfection of ferroportin 1 siRNA in the presence or absence of

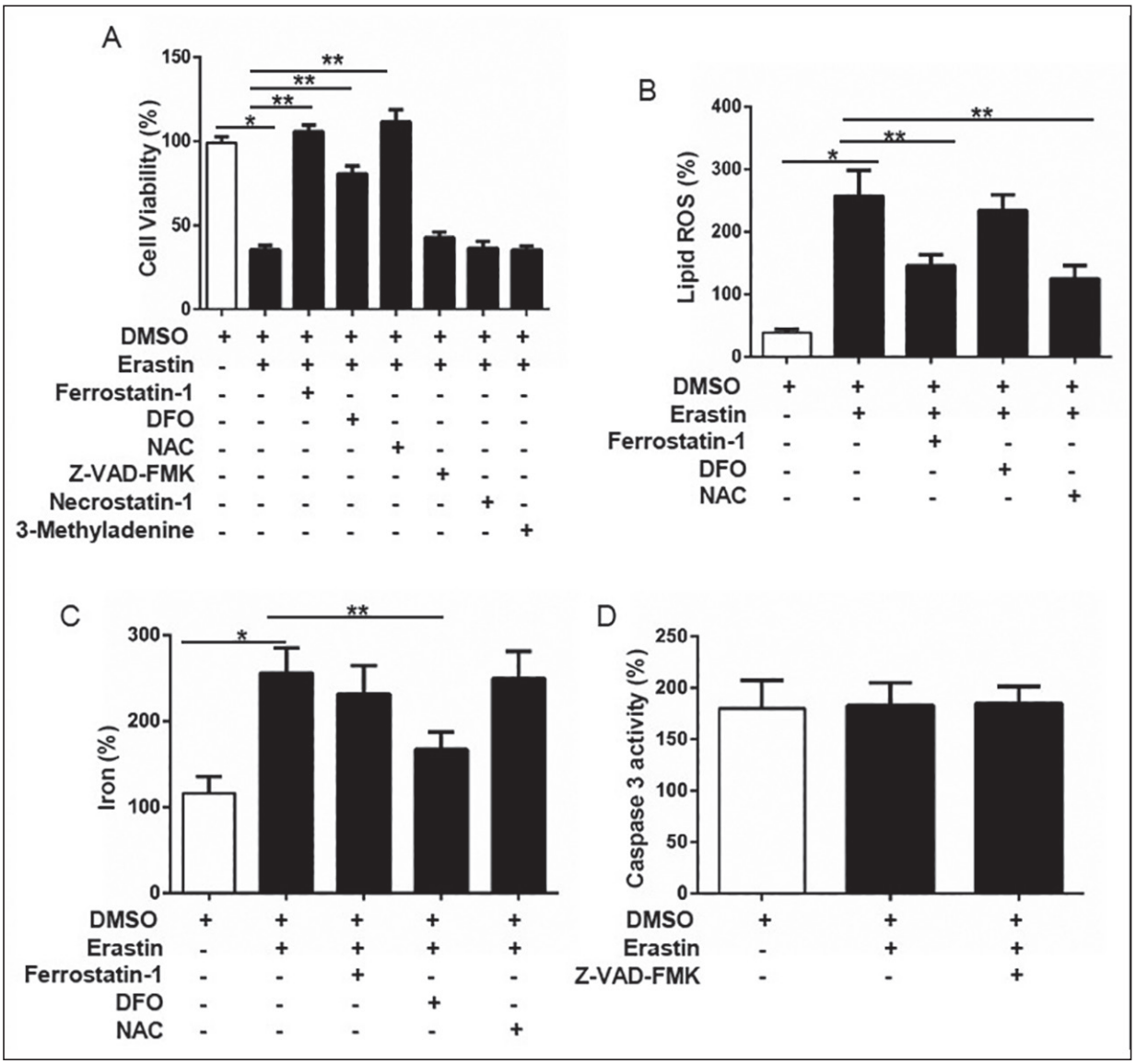

Figure 1. Erastin induces ferroptosis in SH-SY5Y cells. The SH-SY5Y cells were treated with erastin (20 μM) in the presence or absence of ferrostatin-1 (2 μM), DFO (100 μM), NAC (20 mM), Z-VAD-FMK (10 μM), Necrostatin-1 (10 μM) and 3-Methyladenine (0.5 nM) for 24 hours. ***A,*** Cell viability was measured using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (n = 6). ***B,*** Lipid peroxidation was analyzed by flow cytometry using lipid peroxidation (MDA) assay kit (n = 6). ***C,*** Intracellular iron was assayed using iron assay kit (n = 6). ***D,*** Caspase 3 activity was detected using Caspase-3 Activity Assay Kit (n = 6). Values are mean ± SD. *$p < 0.05$ *vs.* DMSO, **$p < 0.01$ *vs.* erastin.

erastin (Figure 3A). The downregulation of Fpn increased erastin-induced growth inhibition in SH-SY5Y cells, suggesting that the Fpn might be a negative regulator of erastin-induced ferroptosis (Figure 3B). Knockdown of Fpn equally increased erastin-induced intracellular concentration of iron (Figure 3C) and lipid ROS (Figure 3D). Moreover, treatment with ferrostatin-1, DFO and NAC significantly increased the cell viability of cells compared with erastin-treated only, and no similar effect was found in cells treated with Z-VAD-FMK, Necrostatin-1, and 3-Methyladenine (Figure 3E). The result showed that inhibitor of ferroptosis and antioxidant prevented erastin-induced growth inhibition in SH-SY5Y cells with Fpn knockdown. Thus, these findings indicate that the inhibition of Fpn expression specifically prompted erastin-induced ferroptosis but not other types of cell death in SH-SY5Y cells.

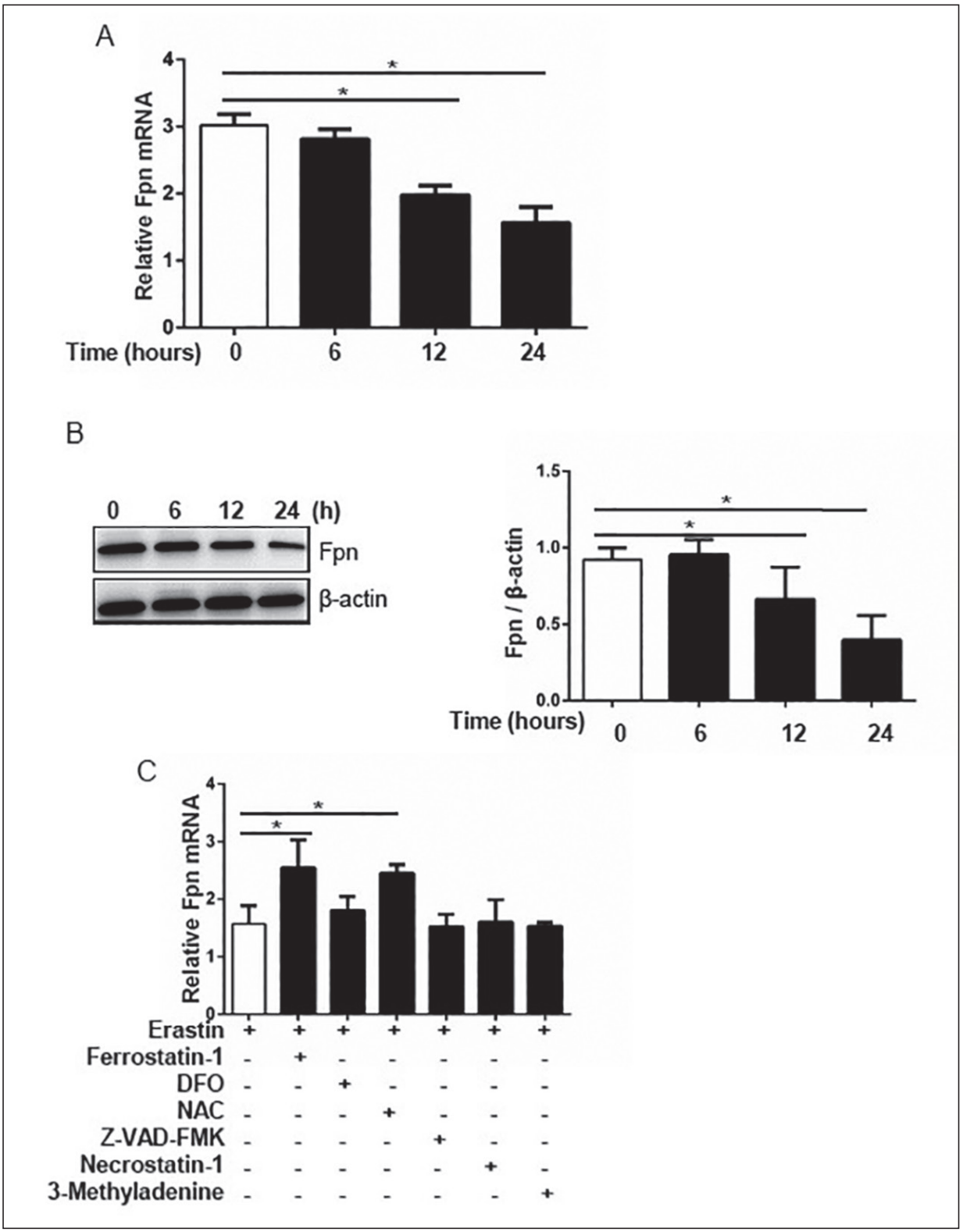

Figure 2. Erastin regulates Fpn expression in SH-SY5Y cells. The SH-SY5Y cells were treated with erastin (20 μM) for 6, 12 and 24 hours. ***A,*** The expression level of Fpn mRNA was analyzed by RT-qPCR (n=6). ***B,*** The expression levels of Fpn protein were analyzed by Western blot (n = 6). ***C,*** Effects of ferrostatin-1 (2 μM), DFO (100 μM), NAC (20 mM), Z-VAD-FMK (10 μM), Necrostatin-1 (10 μM) or 3-Methyladenine (0.5 nM) in the presence of erastin on the expression of Fpn mRNA were detected by RT-qPCR (n = 6). Values are expressed as mean ± SD. *$p < 0.05$ *vs.* 0 hour group.

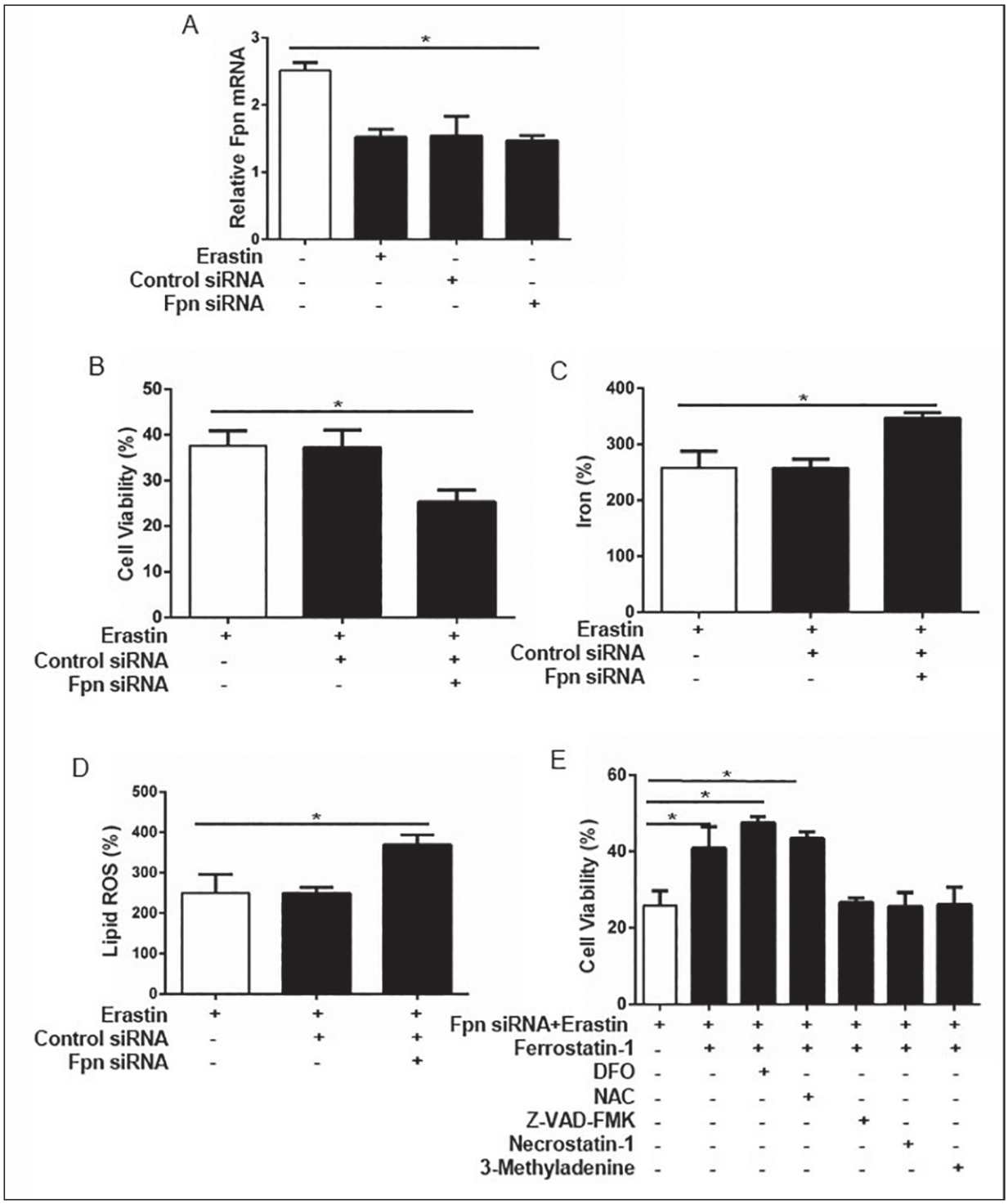

Figure 3. Knockdown of Fpn promotes erastin-induced ferroptosis. The SH-SY5Y cells were transfected with ferroportin 1 siRNA or control siRNA in the presence or absence of erastin for 12 hours. ***A,*** The level of Fpn mRNA was analyzed by RT-qPCR (n = 6). **p* < 0.05 *vs.* control. ***B,*** Effect of knockdown of Fpn on erastin-induced cell growth inhibition at 12 hours in indicated SH-SY5Y cells (n=6). ***C,*** Effect of knockdown of Fpn on erastin-induced intracellular iron was assayed using Iron Assay Kit (n=6). ***D,*** Effect of knockdown of Fpn on erastin-induced lipid ROS was analyzed by flow cytometry using lipid peroxidation (MDA) Assay Kit (n = 6). ***E,*** Effects of ferrostatin-1 (2 μM), DFO (100 μM), NAC (20 mM), Z-VAD-FMK (10 μM), Necrostatin-1 (10 μM) or 3-Methyladenine (0.5 nM) on erastin-induced growth inhibition in SH-SY5Y cells with Fpn knockdown were measured using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (n = 6). Values are expressed as mean± SD. **p* < 0.05 *vs.* erastin.

Upregulation of Fpn Inhibits Erastin Induced Ferroptosis

To further explore the function of Fpn in erastin induced ferroptosis, we investigated whether ponasterone induced Fpn overexpression would inhibit erastin induced ferroptotic cell death. After 24 hours of ponasterone (10 μM) incubation, the protein level of Fpn was significantly increased (Figure 4A). Ponasterone, a Fpn inducer, decreased erastin induced cell growth inhibition and intracellular iron concentration (Figure 4B and C). On the contrary, downregulation of Fpn by Fpn siRNA reversed the effect of ponasterone (Figure 4B and C). Meanwhile, up-regulation of Fpn by transfection of Fpn complementary DNA (cDNA) also remarkably reduced erastin induced

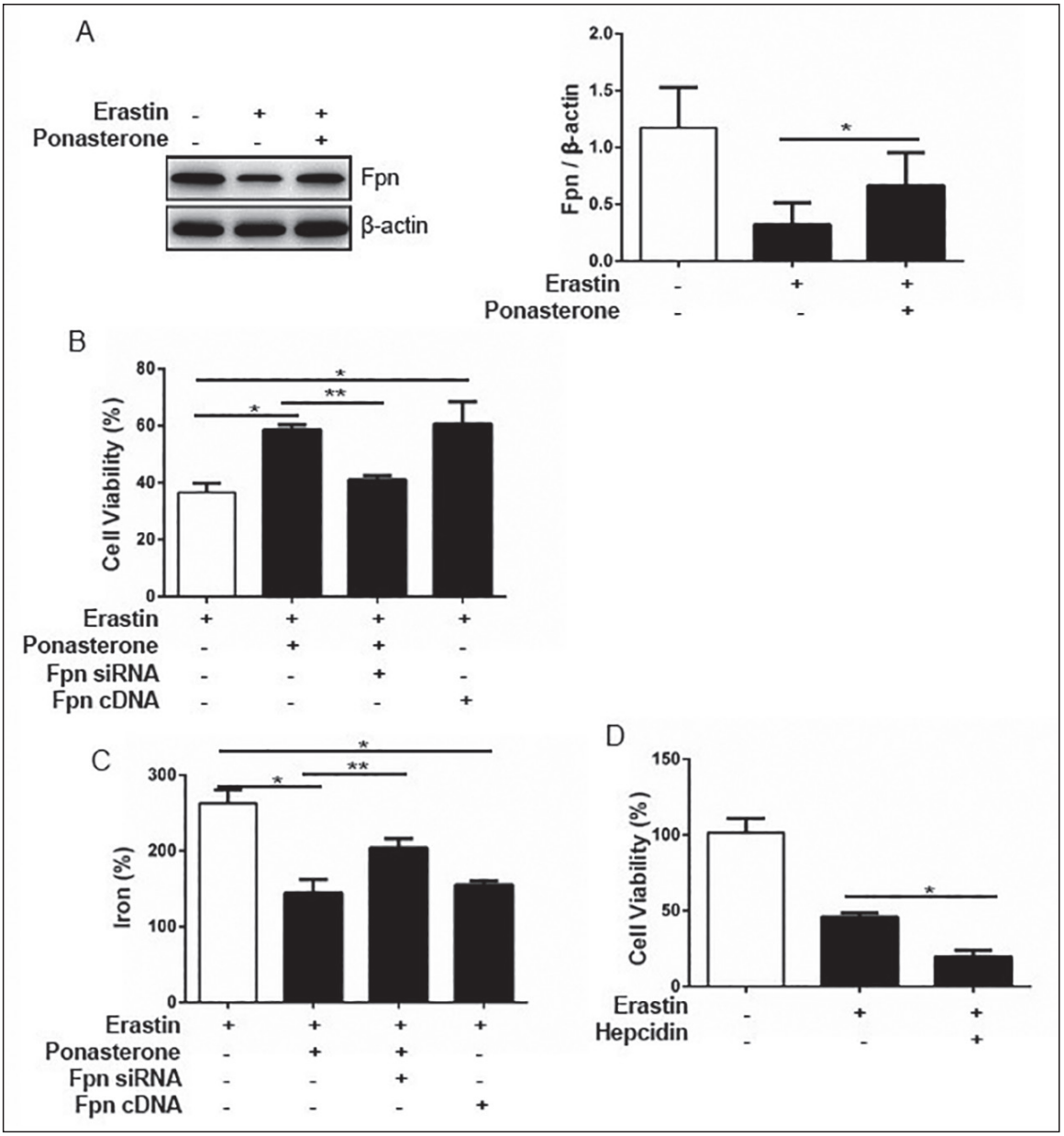

Figure 4. Upregulation of Fpn inhibits erastin-induced ferroptosis. The SH-SY5Y cells were treated with ponasterone (10 μM) for 24 hours. ***A,*** The expression levels of Fpn protein were analyzed by Western blot (n=6). ***B,*** Effect of knockdown of Fpn or Fpn overexpression by gene transfection on erastin-induced cell viability was measured using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (n = 6). ***C,*** Effect of knockdown of Fpn or Fpn overexpression by gene transfection on erastin-induced intracellular iron was assayed using Iron Assay Kit (n = 6). ***D,*** Effect of Hepcidin (0.5 μM) on erastin-induced growth inhibition was measured using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (n = 6). Values are expressed as mean± SD. *$p < 0.05$ *vs.* erastin, **$p < 0.01$ *vs.* ponasterone.

cell growth inhibition and iron concentration (Figure 4B and C). Furthermore, we incubated cells of Fpn overexpression with erastin in the presence or absence of Hepcidin, a unique ligand of Fpn as an inhibitor[25]. The cell growth inhibition was detected by cell viability assay kit, while the levels of lipid peroxidation and intracellular iron were assessed simultaneously. Hepcidin promoted erastin induced ferroptosis (Figure 4D). Taken together, these findings confirmed that Fpn is a negative regulator of erastin-induced ferroptotic cell death in SH-SY5Y cells.

Discussion

Current chemotherapy for neuroblastoma in children includes several chemotherapeutic agents, which cause myelosuppression or subsequent infection[26,27]. Targeted therapy using small molecular compound has target specificity and lower toxicity, which can improve the sensitivity of tumor cells and the treatment efficacy in drug-resistant neuroblastoma[28]. Researches[29] have focused on apoptosis. Anti-apoptotic ability of neuroblastoma cell usually lead to drug resistant to chemotherapeutic agents, exploring new types of cell death, such as iron-dependent ferroptosis, which may creates a new access for therapeutic intervention against neuroblastoma. In addition, erastin, the inducer of ferroptosis, enhances antitumor ability of chemotherapy agents[30]. In this study, we verified that erastin may lead to ferroptosis, which inhibits the proliferation of SH-SY5Y cells.

In 2012, ferroptosis was recognized as a regulated necrosis, which is genetically, morphologically, and biochemically distinct from other forms of necrosis, apoptosis and autophagy[30-39]. It is characterized by the accumulation of lipid ROS derived from iron overload and can be prevented by iron chelators and lipid peroxidation inhibitors[30]. Ferroptotic cell death induced by erastin is mediated by iron-dependent lipid peroxidation and inhibited by antioxidants or iron chelators[20,40]. Some high-confidence genes include proteins, which involved in the regulation of intracellular iron or lipid ROS metabolism, were required for erastin-induced ferroptosis[30,41]. Iron catalyzes fenton reactions can increase lipid ROS generation and result in ferroptotic cell death[42]. In this study, we found erastin, which has been shown to be efficient in inhibiting cell growth in kidney and leukemia cancer cells, inducing neuroblastoma cell ferroptotic death. Furthermore, we found that accompany with iron overload, erastin could induce lower expression of Fpn. Thus, we investigated the effect of Fpn interference on erastin-induced ferroptosis in SH-SY5Y cells *in vitro*, providing evidence that knockdown of Fpn improved erastin-induced iron accumulation, whereas upregulation of Fpn reduced intracellular iron concentration in neuroblastoma cells. Ferroptosis is inhibited by antioxidant and iron chelator but by none of proverbial small molecule inhibitors of apoptosis, autophagy or necrosis. Those results were in line with previous study for prevention of ferroptosis. These findings suggest that iron regulation induced by erastin is changed with Fpn in neuroblastoma cells and could be a potential target site for neuroblastoma therapy.

Iron is an essential component of several proteins such as hemoglobin and myoglobin, its redox reactivity, which generates ROS and improves oxidative stress, would directly contribute to toxic side effects for cell death[43]. Investigations suggest that reduction of intracellular iron storage and increase of iron intake may cause iron overload in the process of ferroptosis; also, eliminating iron overload can prevent erastin-induced ferroptosis[11,26]. Thus, the proteins involved in iron intake and export, including transferrin and Fpn, are essential for the induction of ferroptotic cell death. Ferroportin is the main iron efflux transporter locating on the cell membrane and releases Fe2+ from cells[44]. Knockdown of Fpn leads to a serious iron deficiency by reducing absorption of ectogenic iron and iron in cycling in red cell hemoglobin[45]. Fpn downregulation helps to increase lipid peroxides and ferroptosis with either normal or low transferrin level[46]. Downregulated Fpn was found to be associated with worse prognosis in breast cancer patients and promotes cancer cells growth[18,21,47]. Previous researches also showed that Fpn expression was significantly decreased in hepatocarcinoma tissue compared with normal hepatic tissue[21,47]. Preclinical studies demonstrated that imbalance of iron metabolism promoted breast cancer growth and that misregulated Fpn had an oncogenic effect on breast cancer cells[21]. In the present work, we found that lower expression of Fpn increased intracellular iron concentration and accelerated SH-SY5Y cells ferroptosis but not apoptosis *in vitro*. In addition, iron overloaded in SH-SY5Y cells had been indicated to provide strong anti-

cancer activity. This study represents a promising starting point to the possibility of restraining breast cancer through targeting Fpn or its upstream regulatory factors. These results also confirm the key role of Fpn in deciding the fate of neuroblastoma cells through balancing iron level intracellularly.

Although the specific influence of iron in ferroptotic cell death is not clear, earlier studies[31] have demonstrated that iron-dependent lipid ROS plays an essential role in ferroptosis induced by erastin. Thus, we further elucidated the probable relationship between the Fpn and lipid ROS in neuroblastoma. We found that the expression of Fpn was decreased after treatment with erastin, knockdown of Fpn accelerated erastin-induced lipid ROS and ferroptosis. Overexpression of Fpn results in decrease of lipid ROS, leading to ferroptotic cell death. The discrepancy between our result and previous research may reflect difference in the use of cells. Our data provided evidence that overexpression of Fpn decreased the antitumor activity of erastin and avoided the increase of lipid ROS, whereas antioxidant effectively decreased the anticancer ability of erastin by reducing lipid ROS level and had no effect on iron concentration. Meanwhile, we found that iron chelator also decreased the antitumor activity of erastin regardless whether the Fpn was regulated or not. Furthermore, we found hepcidin, a small serum peptide secreted by hepatocyte that stimulates Fpn degradation and internalization to cause intracellular accumulation of iron, which increased antitumor activity of erastin *in vitro*. Inhibition of lipid ROS generation by antioxidant was able to prevent ferroptosis in neuroblastoma cells but was not responsible for ROS generated following erastin treatment. Overall, those results indicate that knockdown of Fpn promotes erastin-induced ferroptosis through iron outflow disruption, leading to the increase of lipid ROS.

Conclusions

We demonstrated that knockdown of Fpn promotes erastin-induced ferroptosis by excessive iron accumulation and increasing lipid ROS production. We suggest that Fpn could be a potential therapeutic target site for neuroblastoma and offer a new therapeutic direction to be explored to overcome apoptosis resistance in neuroblastoma.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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