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Nuclear receptor coactivator 4-mediated ferritinophagy drives proliferation of dental pulp stem cells in hypoxia

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ABSTRACT

Nuclear receptor coactivator 4 (NCOA4)-mediated ferritinophagy has been implicated in the ferroptosis in cancer cells and hematopoiesis in the bone marrow. However, the role of iron metabolism, especially NCOA4-mediated degradation of ferritin, has not been explored in the proliferation of mesenchymal stem cells. The present study was designed to explore the role of NCOA4-mediated ferritinophagy in hypoxia-treated dental pulp stem cells (DPSCs). Hypoxia treatment increased ROS generation, boosted cytosolic labile iron pool, increased expression of transferrin receptor 1 and NCOA4. Moreover, colocalization of LC3B with NCOA4 and ferritin was observed in hypoxia-treated DPSCs, indicating the development of ferritinophagy. Hypoxia promoted the proliferation of DPSCs, but not ferroptosis, under normal serum supplement and serum deprivation. NCOA4 knock-down reduced ferritin degradation and inhibited proliferation of DPSCs under hypoxia. Furthermore, the activation of hypoxia inducible factor 1α and p38 mitogen-activated protein kinase signaling pathway was involved in the upregulation of NCOA4 in hypoxia. Therefore, our present study suggested that NCOA4-mediated ferritinophagy promoted the level of labile iron pool, leading to enhanced iron availability and elevated cell proliferation of DPSCs. Our present study uncovered a physiological role of ferritinophagy in the proliferation and growth of mesenchymal stem cells under hypoxia.

1. Introduction

Dental pulp stem cells (DPSCs) play an essential role in the continuous replenishment of the odontoblasts throughout the lifespan [1]. DPSCs are an attractive source of mesenchymal stem cells (MSCs), showing high proliferation capability and multilineage differentiation potential [1,2]. Moreover, they may exert immunomodulatory effects by secreting cytokines [3]. However, the exact mechanisms by which MSCs, such as DPSCs and periodontal ligament stem cells (PDLSCs), regulate the intricate balance between proliferation, differentiation, and self-renewal remains remain to be fully understood.

Iron is indispensable for cell growth and differentiation, participating in the DNA synthesis and a wide variety of catabolic and anabolic processes [4]. Iron deprivation inhibits cell proliferation due to lack of ribonucleotide reductase and other enzymes [5], while iron overload triggers oxidative stress and inhibits self-renewal of stem cells [6]. Iron acts as a critical cofactor for a host of enzymes that participate in the cell proliferation and differentiation, especially in the iron-sulfur cluster assembly enzymes [7]; however, the mechanism by which stem cells orchestrates intracellular iron homeostasis in MSCs is not clear.

Intracellular iron homeostasis is regulated by ferroportin-mediated iron export and transferrin receptor (TFR) -mediated import [8]. Transferrin-bound ferric iron (Fe^{3+}) is transferred to the endosomes by TFR1, where it is reduced into ferrous iron (Fe^{2+}) for release into the cytosol. Most of the cytosolic iron is stored in the ferritin, a macromolecule with a protein shell of 24 units of heavy chain (FTH) and light chain (FTL) and an iron core of more than 4000 Fe^{3+} ions [9], while minimal part of free iron exists in the cytosol as the labile iron pool (LIP), which serves as a crossroad of cell iron metabolism [10]. Upon cold stress [11], cigarette smoke [12–14] and LPS stimulation [15,16], iron can be released from ferritin by lysosomal degradation, leading to increased Fe^{2+} in the LIP [17]. Nuclear receptor coactivator 4 (NCOA4) is the selective

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cargo receptor that can bind ferritin and deliver it to autophagosome, leading to the selective autophagy of ferritin, which is called as ferritinophagy [18].

MSCs, including bone marrow MSCs and DPSCs, showed significantly increased proliferation rate when cultured under hypoxia and supplied with plenty of supply of nutrients [19]. However, once in a serum deprivation state, MSCs may lower and switch their metabolism into a quiescent state to conserve their stemness and to survive [20]. Hypobaric hypoxia increases transferrin receptor and decreased L-ferritin levels in heart, liver, spleen, and kidney of rats [21].

Several key issues remain in the iron metabolism of MSCs. For example, the level of labile iron pool and expression of iron metabolism-related genes has never been reported in proliferative MSCs. Moreover, until now, the degradation of ferritin in the lysosome, a process called ferritinophagy, is mainly implicated in tumor cells [22] and pathological states including cigarette-induced chronic obstructive pulmonary diseases [12,13], cold stress [11] and sepsis [15]. Ferritinophagy induces excessive generation of iron-derived reactive oxygen species (ROS), depletion of cytosolic glutathione and onset of lipid peroxidation, leading to ferroptosis [8]. However, whether NCOA4-mediated ferritinophagy plays a physiological role in the proliferation of stem cells has not been reported.

Autophagy can be triggered in various stressful conditions, such as hypoxia and nutrition deprivation [23,24]. Hypoxia has been observed to promote the proliferation of various MSCs, including DPSCs [25,26].Therefore, we inferred that DPSCs may initiate ferritinophagy to dynamically orchestrate the availability of labile iron pool during hypoxia. And we found that NCOA4-mediated ferritinophagy enhanced cellular free iron levels and promoted proliferation of DPSCs in hypoxia.

2. Materials and methods

2.1. Isolation of DPSCs

Human DPSCs were isolated from the third molars of participants at the age of 18–20 years, and written informed consent was obtained from the patients. Cells were seeded in a T-25 tissue culture flask with 10% fetal bovine serum (FBS), and incubated in 5% CO_2 95% air at 37 °C. Fresh medium was changed every 3 days until confluence [25].

2.2. Cell culture and treatments

DPSCs of passage 3–5 was cultured in DMEM supplemented with 10% FBS. A hypoxic chamber was applied to achieve hypoxia (1% O_2), while DMEM without FBS was used to mimic the condition of serum starvation. Cells were also visualized and photographed with inverted phase contrast microscope (Nikon, Japan).

2.3. Cell viability assay

Cell counting kit (CCK-8, Biosharp, China) was used to evaluate the proliferation ability of DPSCs. OD values at 450 nm were measured by a SpectraMax M3 (Sunnyvale, CA, USA).

2.4. Carboxyfluorescein succinimidyl ester (CFSE) assay

2×10^5 cells were cultured in each well of 6-well plates. Before treatment, cells were incubated with 2 mL CFSE solution. And later the staining was quenched by addition of equal volumes of media contained 1% FBS. After the cells were treated, cells were trypsinized and washed twice by resuspending in the fresh DMEM. Cells were analyzed by FACSCalibur™ Flow Cytometer (BD Biosciences, USA) with 488 nm excitation.

2.5. EDU proliferation assay

Proliferation of DPSCs was assessed by incorporation of 5′-ethynyl-2′-deoxyuridine (BeyoClick™ EdU-647, Beyotime, China) following the protocol of the manufacturer. After CSFE and DAPI staining, cells were observed with a confocal microscopy (Nikon, Japan).

2.6. Cytotoxicity assay

The LDH assay was performed according to the manufacturer's instructions (Promega, USA). The optical density was read using a SpectraMax M3 (Molecular Devices, Sunnyvale, CA, USA).

2.7. ROS detection

The ROS level was detected by a reactive oxygen species assay kit (Beyotime, China). All procedures completely complied with the manufacturer's instructions. The mean fluorescence intensity (MFI) was detected with a BD FACSCalibur™ flow cytometer.

2.8. Cellular labile iron pool quantification

The intracellular LIP was visualized using a Calcein-AM assay [27]. After sample treatment, cells were collected and resuspended in 500 nM Calcein-AM, and incubated for 15 min at 37 °C. Subsequently, cells were washed twice and resuspended in 1 mL PBS before dividing each sample into two flow cytometry tubes, to which 100 μM 2′,2′-bipyridyl (BIP) was added. BIP tubes were incubated for 15 min. Finally, samples were analyzed on a flow cytometer. The LIP = MFI(BIP) − MFI(NoBIP) was normalized against the control samples to calculate the relative labile iron pool.

2.9. Quantitative real-time PCR

Total RNA for real-time PCR was extracted from DPSCs and reverse transcribed into cDNA. The actin gene was used as an internal control. The PCR primer sequences were attached to Supplementary Material.

2.10. Immunofluorescence and confocal tmicroscopy

After treatment, DPSCs were washed and fixed with 4% paraformaldehyde. Afterwards, cells were incubated with primary antibodies against LC3B (1:400; CST, USA), NCOA4 (1:400; Abnova, USA) or Ferritin (1:400; Affinity, USA)) overnight at 4 °C. Then cells were incubated with secondary antibodies, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse and Alexa Fluor 647 goat anti-mouse (1:2000; Invitrogen, USA) for 1.5 h in the dark. DAPI (New Cell & Molecular Biotech, China) was added for 10 min, and dishes were then observed with a confocal microscopy.

2.11. Western blotting

DPSCs lysate was collected and centrifuged to remove cellular debris. Protein concentrations were determined using a Nanodrop (Termo Fisher, USA). Protein was electrophoresed on a 4–12% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% BSA and incubated with anti-NCOA4 & anti-LC3B (ABclonal, China), anti-Ferritin & anti-JNK (Affinity, USA), anti-TFR1& anti-SLC40A1 (Abcam, USA), anti-p62& anti-ACSL4 (Proteintech, China), anti-GPX4 (Bioss, China), anti-LPCAT3 (Cloud-

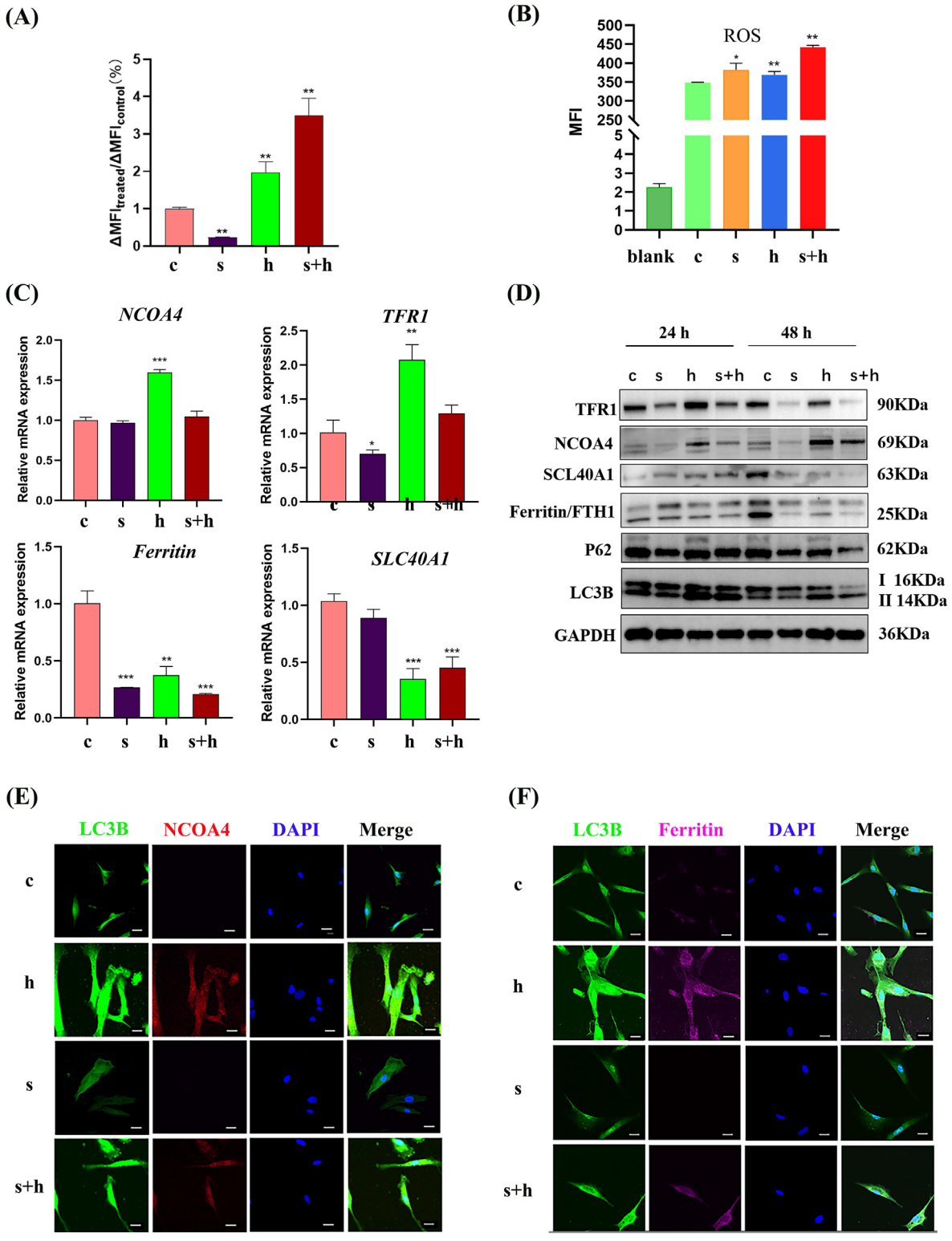

Fig. 1. Hypoxia affected iron metabolism and ferritinophagy. (A) The intracellular labile iron pool (LIP) was detected using a Calcein-AM assay and determined by flow cytometry at 4 h (n = 4). (B) ROS level in DPSCs was determined by flow cytometry at 4 h (n = 4). (C&D) mRNA transcription at 2 h and protein expression at 24 h were determined by quantitative real-time PCR and Western blot (n = 3). (E&F) Representative images of colocalization of NCOA4 (red) with LC3B (green), and ferritin (red) with LC3B (green) in DPSCs. DPSCs were treated with various condition for 24 h *$p < 0.05$, **$p < 0.001$ *vs*. the control. * abbreviations were as followed: c (control), s (starvation), h (hypoxia) and s + h (starvation + hypoxia). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

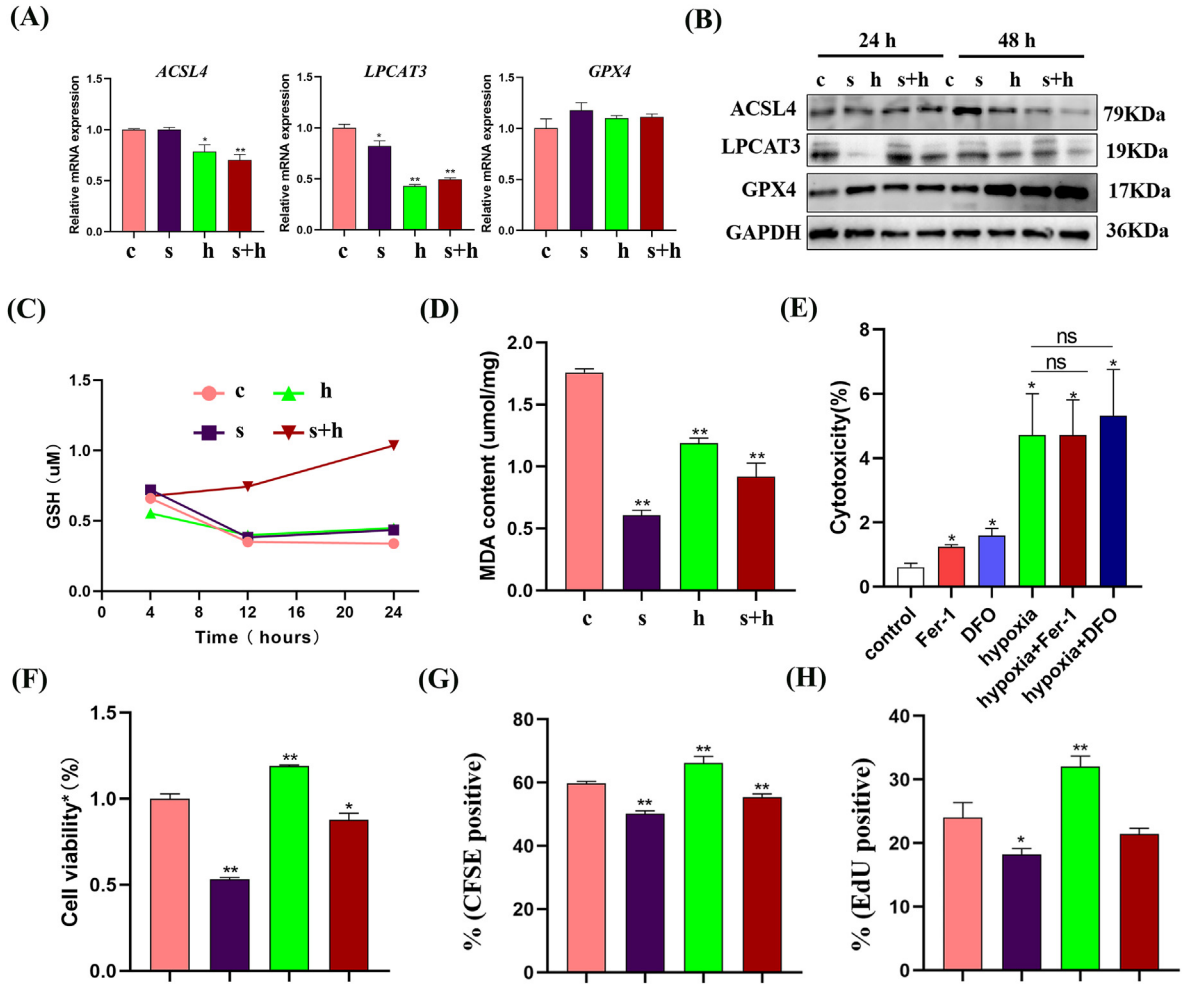

Fig. 2. Hypoxia boosted the proliferation of DPSCs with no ferroptosis. DPSCs was treated with normal condition, serum starvation, hypoxia, and hypoxia with serum deprivation. (A&B) mRNA transcription and protein expression were determined by quantitative real-time PCR at 2 h and Western blot at 24 and 48 h respectively (n = 3). (C) Changes of the glutathione (GSH) redox status after 4 h, 12 h and 24 h. (D) Analysis of malondialdehyde (MDA) generation to determine lipid peroxidation at 24 h. (E) Analysis of LDH generation after hypoxia treatment in DPSCs at 24 h after pretreatment with lipid peroxide scavenger (ferrostatin-1, 10 μM) or iron chelator (DFO, 50 μM); (F) CCK-8 assays were performed to assess viable DPSCs at 24 h; (G) DPSCs was stained with CSFE, and then detected by flow cytometry at 24 h; (H) 5′-ethynyl-2′-deoxyuridine (EDU) was utilized to stain DPSCs, and then positive cells were determined by the confocal imaging. *$p < 0.05$, **$p < 0.001$ *vs.* the control.

Clone, USA), Tubulin (Bioworld, China), anti-phospho-JNK, anti-phospho-p38, anti-p38 (CST, Germany), anti−HIF−1α (Abcam, USA), β-actin (Proteintech, China), or GAPDH (Servicebio, China), followed by secondary antibodies. The signals were detected using Tanon-5200.

2.12. Small interferencing RNA

DPSCs were transfected with a specific NCOA4 siRNA (RiboBio, China). The cells were seeded in 6-well plates. Cells were transfected with a scramble siRNA (50 nM) or NCOA4 siRNA (50 nM) for 6 h using Lipofectamine 3000 (Thermo Fisher, USA). Efficiency of NCOA4 siRNA silencing was detected by qRT-PCR and Western blot 72 h after transfection.

2.13. Lipid peroxidation and GSH assays

The generation of malondialdehyde (MDA) was used to measure lipid peroxidation(Beyotime, China). The GSH and GSSG were determined using commercially available kits (Beyotime, China). All procedures completely complied with the manufacturer's instructions.

2.14. Quantification and statistical analysis

The analysis was performed using Graph Pad Prism 8.0 software. All results are presented as the mean ± standard deviation (SD). The Student's t-test was used to analyze difference between two groups. And values of $p < 0.05$ were considered statistically significant. All in vitro experiments were independently performed as indicated.

3. Results

3.1. Enhanced labile iron pool and ferritinophagy under hypoxia in DPSCs

We first characterized changes of LIP in hypoxia-treated DPSCs. Intracellular free iron was increased in DPSCs that were treated by hypoxia in both normal and serum starvation condition (Fig. 1A). In consistence with increased LIP, ROS level was also markedly increased in DPSCs under hypoxia (Fig. 1B). TFR1 was upregulated at both mRNA and protein level after hypoxia treatment, whereas ferroportin (SLC40A1) was downregulated after hypoxia incubation (Fig. 1C and D). NCOA4 was significantly upregulated, while ferritin

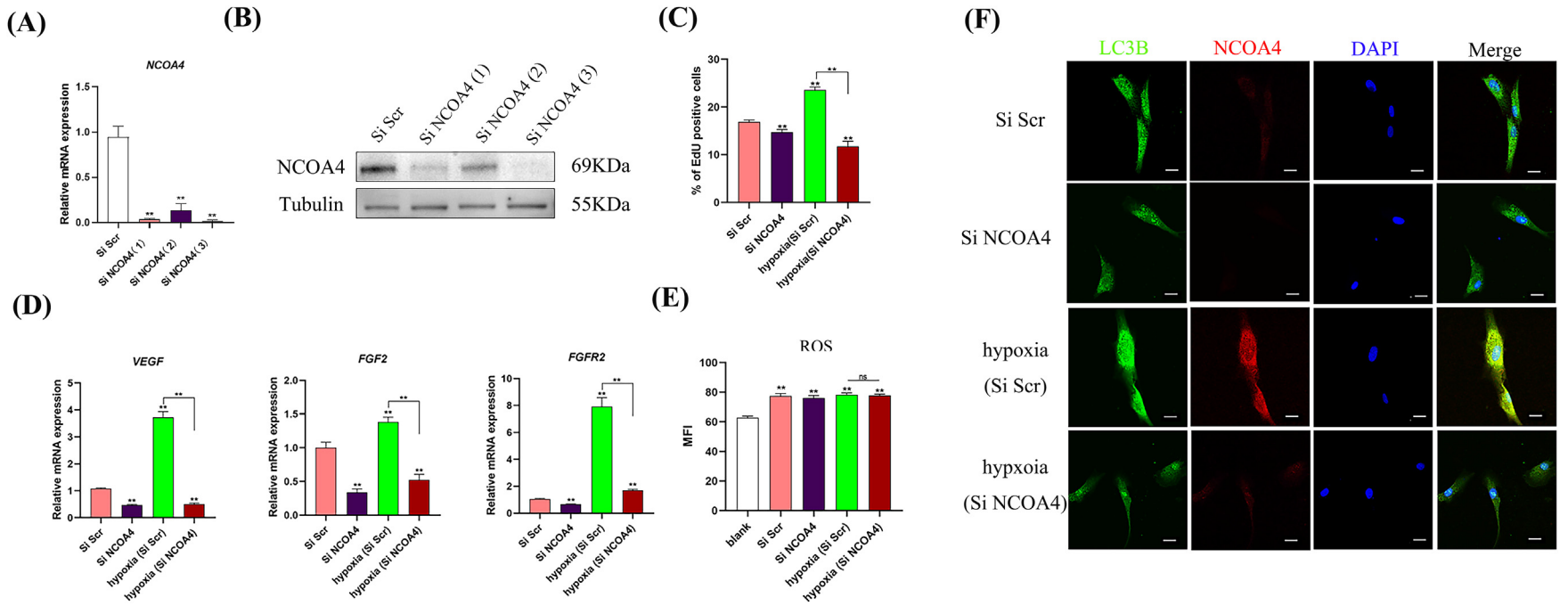

Fig. 3. NCOA4-knock down inhibited the proliferation of hypoxia-treated DPSCs. (A&B) Efficiency of NCOA4 siRNA silencing was detected by qRT-PCR and Western blotting 72 h after transfection. (C) Positive cells of EDU staining of cells at 12 h. (D) The mRNA expression of genes related to cell proliferation after NCOA4 knockdown. (E) The ROS of siNCOA4-treated DPSCs for 4 h in hypoxia. (F) The colocalization of LC3B with NCOA4 and FTH1 were detected by immunofluorescence at 24 h $*p < 0.05$, $**p < 0.001$ *vs.* siScr.

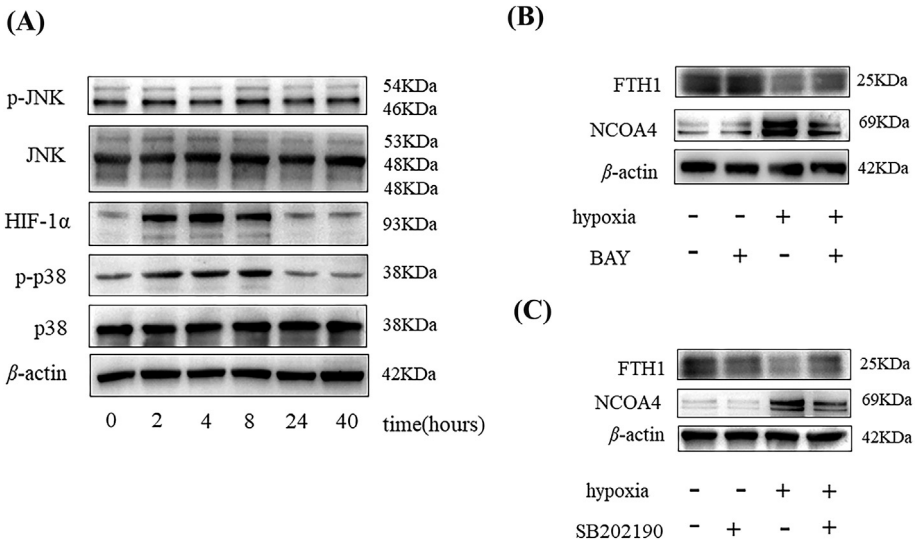

Fig. 4. HIF-1α and p38 pathway was involved in the ferritinophagy in hypoxia-treated DPSCs. (A) Expression of HIF-1α, p38, p-p38, JNK, and *p*-JNK at different time points. (B&C) After the treatment of BAY 87−2243(10 nM) or SB202190(10 mM), the expression of FTH1 and NCOA4 were tested by western blotting.

was downregulated in the cytosol. Protein level of LC3B-II was increased, whereas the level of p62 protein was decreased, indicating elevation in the autophagy after hypoxia treatment (Fig. 1C and D). Immunofluorescence staining of DPSCs showed that both NCOA4 and LC3B expression were promoted by hypoxia incubation, and colocalization of NCOA4 with LC3B and ferritin with LC3B were detected (Fig. 1E and F).

3.2. Increased proliferation, but not ferroptosis, in DPSCs under hypoxia

We further explored whether NCOA4-mediated ferritinophagy promoted cell death in DPSCs under hypoxia. Transcription of Acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), two molecules promoting integration of polyunsaturated fatty acids (PUFAs) into the cell membrane, was reduced in hypoxia-treated DPSCs (Fig. 2A); protein expression of ACSL4 and LPCAT3 was not altered at 24 h, but decreased at 48 h (Fig. 2B). GPX4 expression was elevated in DPSCs after hypoxia treatment at 48 h (Fig. 2B). MDA, a marker of lipid peroxidation, was decreased in hypoxia-treated DPSCs with no changes in the GSH level (Fig. 2C and D). Treatment of hypoxia triggered minor significant cell death at 24 h. DFO, an iron chelator, and Fer-1 (ferrostatin-1), a lipophilic antioxidant, failed to inhibit cell death in DPSCs; they even increased the LDH release from serum-deprived cells under hypoxia (Fig. 2E). Therefore, the present data do not support the onset of ferroptosis in hypoxia-treated DPSCs.

Microscopic examination of DPSCs revealed an increase in the number of cells under hypoxia at 24 h compared to normoxia control (21% O_2). The increased cell proliferation under hypoxia at 24 h was confirmed by CCK-8 assay, CFSE staining and EDU assay (Fig. 2F–H).

Table 1
Primers designed for qRT-PCR validation.

Gene	Primer
ACTIN	**F** GTGGGGCGCCCCAGGCACCA
	R CGGTTGGCCTTGGGGTTCAGGGGGG
NCOA4	**F** GAGGTGTAGTGATGCACGGAG
	R GACGGCTTATGCAACTGTGAA
TFR1	**F** ACCATTGTCATATACCCGGTTCA
	R CAATAGCCCAAGTAGCCAATCAT
Ferritin (FTH1)	**F** CCCCCATTTGTGTGACTTCAT
	R GCCCGAGGCTTAGCTTTCATT
SLC40A1	**F** CTACTTGGGGAGATCGGATGT
	R CTGGGCCACTTTAAGTCTAGC
VEGF	**F** TGCTTCTGAGTTGCCCAGGA
	R TGGTTTCAATGGTGTGAGGACATAG
FGF2	**F** AGAAGAGCGACCCTCACATCA
	R CGGTTAGCACACACTCCTTTG
FGFR2	**F** GGAAAGTGTGGTCCCATCTGA
	R TCCAGGTGGTACGTGTGATTG
ACSL4	**F** CCTGAGGGGCTTGAAATTCAC
	R GTTGGTCTACTTGGAGGAACG
LPCAT3	**F** GGAGCTGAGCCTTAACAAGTT
	RCAAAGCAAAGGGGTAACCCAG
GPX4	**F** CTGCTCTGTGGGGCTCTG
	R ATGTCCTTGGCGGAAAACTC

3.3. NCOA4-mediated ferritinophagy promotes proliferation of DPSCs in hypoxia

Knockdown of NCOA4 was applied to further verify the role of ferritinophagy in the cell proliferation. Based on the efficiency of four sequences of siNCOA4, we applied siNCOA4 (3) to subsequent experiments (Fig. 3A and B). Knockdown of NCOA4 distinctly decreased the proliferation of DPSCs under hypoxia (Fig. 3C) with decreased transcription of levels of VEGF, FAF2 and FAFR2 ($p < 0.001$) (Fig. 3D). However, knockdown of NCOA4 failed to decrease ROS generation in DPSCs in hypoxia (Fig. 3E). Following knockdown of NCOA4, less colocalization of LC3-GFP with NCOA4 was observed in DPSCs in hypoxia, indicating reduced ferrtinophagy (Fig. 3F).

3.4. HIF-1α and p38 MAPK-dependent regulation of NCOA4

Apoptosis signal-regulating kinase 1 (ASK1)-p38 mitogen-activated protein kinase (MAPK) was activated in cold stress-treated cancer cells to trigger ferroptosis [11]. Moreover, c-Jun N-terminal kinase (JNK) activation has been observed in the hypoxia-treated macrophages [28]. We next explored the activation of MAPK and HIF-1α pathway in DPSCs. Hypoxia treatment in DPSCs raised the level of HIF-1α at 4 h. Hypoxia treatment markedly

increased the phosphorylation p38, while the total amount of p38 was not altered. However, both the total and phosphorylated JNK were not influenced by hypoxia (Fig. 4A). The expression of NCOA4 in hypoxia-treated DPSCs was significantly reduced after pretreatment with the inhibitors of HIF-1α, BAY 87–2243, and p38 MAPK inhibitor, SB202190. On the contrary, protein levels of FTH1 were increased after inhibition of HIF-1α and p38 MAPK (Fig. 4B and C).

4. Discussion

Autophagy is vital for cell proliferation, differentiation, and survival. Previously, ferritinophagy is mainly implicated in the pathogenesis of cancers and pathological states including sepsis and pulmonary diseases [12,15,18,22]. However, recent advances demonstrated that NCOA4-mediated ferritinophagy plays a physiological role by increasing iron availability. NCOA4 facilitates iron mobilization from the liver after blood loss to prevent anemia, and HIF regulates NCOA4 expression in the process [29]; in addition, NCOA4-knockout mice show microcytosis and mild anemia, since NCOA4-mediated ferritinophagy in macrophages favors iron release for erythropoiesis to compensate iron deficiency [30,31]. Moreover, ferritin degradation in the lysosome promotes labile iron pool, and increases ROS generation in angiotensin–II–stimulated human umbilical vein endothelial cells [32]. Our present study further demonstrated that NCOA4-mediated ferritinophagy fueled the proliferation of DPSCs by release of free iron into the cytosol.

Reactive oxygen species (ROS) intricately orchestrate quiescence and self-renewal, cell proliferation/differentiation, and apoptosis via a dose-dependent manner [33]. Intracellular H_2O_2 can be spontaneously catalyzed by ferrous ion (Fe^{2+}) to the highly reactive hydroxyl radical (•OH) (Fenton reaction). The NCOA4-mediated ferritinophagy is critically important in the onset of ferroptosis [34]. In our present study no obvious cell death was observed after hypoxia, a result similar to other DPSCs' studies [19,25,26]. Furthermore, features of ferroptosis, such as upregulation of ACSL4 and LPCAT3, depletion of GSH, exhaust of GPX4, and lipid peroxidation, was not observed, indicating no occurrence of ferroptosis. These results suggest a physiological role of ferritinophagy in the proliferation of MSCs by fueling cell growth and proliferation with iron nutrients. Indeed, ferritinophagy may not definitely induce cell death in macrophages, since ferritinophagy in macrophages favor iron release for erythropoiesis [31].

How host cells upregulate NCOA4 and activate ferritinophagy is less clear. Hypoxia induces autophagy of bone marrow-derived mesenchymal stem cells via activation of ERK1/2 [35]. It has been demonstrated that (miR)-6862–5p and c-Jun N-terminal kinase (JNK) has been implicated in the inhibited NCOA4 expression in hypoxia-treated macrophages [28]; in contrast, p38 signaling pathway has been involved in the increased NCOA4 transcription in hypoxia-treated DPSCs. These results indicated that different cells may intricately regulate their iron metabolism to address the stressful conditions to promote survival or to induce cell death.

Although the NCOA4-mediated ferritinophagy plays a positive role in the proliferating DPSCs, the potential detrimental effect of ferritinophagy in MSCs-based regeneration medicine cannot be ignored. Since MSCs experience a long period of low-tension oxygen and nutrients deprivation until competent angiogenesis functions properly [20], ferritinophagy may still promote ROS generation and autophagic cell death. In addition, the regulation of ferritinophagy by the iron metabolic network should be further explored. Moreover, profiling polyunsaturated fatty acid contents, such as arachidonic acid (AA), adrenic acid (AdA), AA-phosphatidylethanolamines (PE), and AdA-PE, by liquid chromatography mass spectrometry (LC-MS) can further help us understand how hypoxia treatment affects cell proliferation and death.

In conclusion, MSCs may fulfil its high demand for iron during proliferation by both TFR-mediated iron uptake and NCOA4-mediated ferritinophagy, and ferritinophagy in hypoxic state with ample serum supplement may provide iron for dividing cells rather than induce ferroptosis. Our present work demonstrated that NCOA4-mediated ferritinophagy plays a physiological role in hypoxia-enhanced proliferation of DPSCs.Table 1

Author contributions

A. Yang contributed to data acquisition, analysis and drafted the manuscript; L. Wang and K. Jiang contributed to data acquisition; and H. Li and L. Lei conceived of and designed the experiments and revised the manuscript.

Ethical approval

All experiments were reviewed and approved by the Ethics Committee of Nanjing Stomatological Hospital, Medical School of Nanjing University.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.03.075.

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