SLC6A14 facilitates epithelial cell ferroptosis via the C/EBPβ-PAK6 axis in ulcerative colitis

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Abstract

Emerging evidence suggests that ferroptosis is involved in the pathogenesis of ulcerative colitis (UC). However, the key regulator of this process remains uncertain. In this study, we aimed to explore the roles of solute carrier (SLC) family 6 member 14 (SLC6A14) in regulating ferroptosis in UC. The expression of SLC6A14 was significantly increased and positively associated with that of prostaglandin-endoperoxide synthase 2 (PTGS2) in tissue samples from patients with UC. Moreover, a series of in vitro and in vivo experiments showed that SLC6A14 knockdown markedly suppressed ferroptosis. RNA sequencing revealed that SLC6A14 inhibited the expression of P21 (RAC1) activated kinase 6 (PAK6) and that PAK6 knockdown abolished the effects of SLC6A14 on RAS-selective lethal 3 (RSL3) -induced ferroptosis in Caco-2 cells. Furthermore, chromatin immunoprecipitation (ChIP) and Western blot analysis demonstrated that SLC6A14 negatively regulated PAK6 expression in a CCAAT enhancer binding protein beta (C/EBPβ)-dependent manner. Collectively, these findings indicate that SLC6A14 facilitates ferroptosis in UC by promoting C/EBPβ expression and binding activity to inhibit PAK6 expression, suggesting that targeting SLC6A14-C/EBPβ-PAK6 axis-mediated ferroptosis may be a promising therapeutic alternative for UC.

Introduction

Ulcerative colitis (UC) is a relapsing and remitting inflammatory bowel disease (IBD) and is characterized by colonic mucosal inflammation resulting in continuous ulceration and bloody diarrhoea[1]. Although the complete pathogenesis of UC remains unclear, it is universally acknowledged that dysfunction of intestinal epithelial cells (IECs) and defects in the epithelial barrier play an essential role in chronic inflammation[2]. Excessive IEC death destroys host-microorganism homoeostasis, mucosal immune regulation, nutrient circulation, and intestinal barrier integrity and eventually causes recurring protracted colitis. Accordingly, exploring the cause and mechanism of IEC death may facilitate the development of effective treatment strategies for UC patients.

Ferroptosis is a new form of regulated cell death (RCD) that is mainly based on uncontrollable iron-dependent accumulation of lethal lipid reactive oxygen species (ROS) and is usually manifested overtly as a shrunken cell morphology and cell detachment[3]. Generally, suppression of glutathione peroxides 4 (GPX4) expression, downregulation of cystine/glutamate antiporter system (System Xc^-) activity, and accumulation of lipid ROS are the three main factors resulting in the induction of ferroptosis[4]. Emerging evidence indicates that ferroptosis is closely associated with the pathogenesis of multiple diseases, including IBD[5, 6]. For instance, impaired GPX4 activity and signs of lipid peroxidation were found in small IECs of Crohn's disease (CD) patients. Moreover, dietary ω-6 polyunsaturated fatty acids (PUFAs), especially arachidonic acid (AA), trigger inflammatory responses and cytokine production in GPX4-deficient IECs by ferroptotic mechanisms[5]. Ferroptosis was observed in IECs from UC patients and mice with colitis, and activation of the ferroptosis signalling pathway was found to be involved in NF-κB axis-mediated endoplasmic reticulum (ER) stress signalling[6]. In addition, numerous studies have shown that

inhibition of ferroptosis can markedly alleviate murine experimental colitis [7-9]. Therefore, understanding the molecular basis of ferroptosis is crucial for improving targeted ferroptosis-based treatment options.

Solute carrier (SLC) transporter family proteins, such as SLC7A11, SLC3A2, and SLC25A28, have been reported to be important regulators of ferroptosis[10-12]. SLC7A11 and the chaperone SLC3A2, two basal subunits of System Xc-, efficiently protect cells against ferroptosis via biosynthesis of glutathione (GSH) [10, 11]. Moreover, SLC25A28 interacts with mitochondrial p53, leading to accumulation of redox-active iron and activation of the electron transfer chain (ETC) as well as ferroptosis induction[12]. In the present research, we focused on SLC6A14, a member of the SLC transporter family, which is upregulated in various colonic diseases, including UC[13]. Whether and how SLC6A14 modulates IEC ferroptosis in UC was investigated in both humans and mice. We demonstrated that SLC6A14 was highly expressed in UC patients, animal models of experimental colitis, and cell models of ferroptotic death. Furthermore, SLC6A14 upregulation contributed to iron overload and GSH consumption, which eventually resulted in lipid peroxidation and ferroptosis. Mechanistically, SLC6A14 positively regulated the multifunctional transcription factor CCAAT enhancer binding protein β (C/EBPβ) to inhibit P21 (RAC1) activated kinase 6 (PAK6) transcription, which played a major role in ferroptosis by activating ERK signalling. In summary, our results indicate that SLC6A14 is a novel regulator of ferroptosis that promotes UC progression through the C/EBPβ-PAK6-ERK axis, thereby providing key insights into UC pathogenesis.

Materials And Methods

Microarray data and differential expression analysis

The microarray gene expression dataset GSE134025 from the Gene Expression Omnibus (GEO) database is based on the GPL20115 platform[6]. Differential expression analysis was performed with R software (package: 'Limma'). Differentially expressed genes (DEGs) were identified by the following criteria: (1) $|\log_2$ (fold change)| > 1 and (2) adjusted P value < 0.05. When DEGs were duplicated, the most significant genes were retained. The heatmap and volcano plot were generated with R software (packages: 'pheatmap', 'ggplot2', and 'ggrepel').

Human colon tissues

Paraffin-embedded colon specimens from 89 UC patients and 5 healthy individuals were obtained from the First Affiliated Hospital of Soochow University (Jiangsu, China). The clinical data of each patient are provided in Supplementary Table 1. The diagnosis of UC was confirmed histopathologically by H&E staining, and the Mayo score, Ulcerative Colitis Endoscopic Index of Severity (UCEIS), haemoglobin (Hb) level, C-reactive protein (CRP) level, and erythrocyte sedimentation rate (ESR) were available[14] (Supplementary Tables 1, 4-6). Ethical approval (reference number: 2021-325) was obtained from the Ethics Committee of the First Affiliated Hospital of Soochow University, and informed consent was obtained from all participants in this research.

Establishment of experimental colitis models

Six- to- eight-week-old male C57/BL/6 mice (21.45 ± 1.12 g) and BALB/c mice (21.46 ± 1.25 g) were obtained from the Laboratory Animal Center of Soochow University. Animals were raised in compliance with regulations, and experiments were approved by the Ethics Committee of Soochow University (reference number: SUDA20210918A01).

Dextran sulfate sodium (DSS, MeilunBio, Dalian, China)-induced acute colitis was established as previously reported[15]. Briefly, control mice were provided sterile ddH_2O, and others were provided sterile ddH2O containing 3% DSS for 8 days. A total of 20 C57/BL/6 mice were randomly divided into 3 groups: (1) Control (n = 6), (2) DSS + phosphate-buffered saline (PBS) (n = 7), and (3) DSS + α-methyl-DL-tryptophan (α-MT) (n = 7). Mice in the DSS + PBS group were intraperitoneally injected with 100 μl of PBS, while mice in the DSS + α-MT group were intraperitoneally injected with 100 μl of α-MT (Sigma–Aldrich, Bellefonte, USA, 20 mg/ml, dissolved in sterile saline) once a day for 7 days (Fig. S3A). Weight loss, diarrhoea, and bleeding were monitored daily. The grade of DSS-induced intestinal inflammation was assessed using the disease activity index (DAI) as previously described (Supplementary Table 7)[16]. Eight days after induction of colitis, DSS-challenged mice were sacrificed.

To establish oxazolone (OXZ; Sigma–Aldrich)-induced colitis, BALB/c mice were randomly divided into 3 groups: (1) Control (n = 6), (2) OXZ + PBS (n = 8), and (3) OXZ + α-MT (n = 7). OXZ-induced acute colitis was induced according to previous protocols[15]. Briefly, cutaneous sensitization to OXZ was induced in mice by application of 200 μl of 3% OXZ (dissolved in ethanol) for 2 consecutive days. Five days later, 150 μl 1% OXZ (dissolved in 50% ethanol) was carefully delivered into the colonic lumen by insertion of a catheter to a distance of 4 cm from the anus. Then, the mice were maintained in a vertical position for 30 s. Control mice were intrarectally administered 50% ethanol. Mice in the OXZ + PBS group and DSS + α-MT group were intraperitoneally injected with PBS and α-MT, respectively, once a day for 4 days (Fig. S3B). Weight loss, diarrhoea, bleeding, and DAI were recorded. Mice were finally killed on Day 3 after colitis induction.

Cell culture and treatment

Caco-2 cells obtained from ATCC (Manassas, Virginia, USA) were cultured as an adherent epithelial monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 20% foetal bovine serum (FBS) and 1% penicillin–streptomycin–amphotericin solution in a 37 ℃ humidified incubator (Thermo Fisher Scientific, Waltham, USA) with 5% CO_2.

To establish a cell model of ferroptosis, Caco-2 cells were treated with RSL3 (MeilunBio, 15 μM) with or without ferrostatin-1 (Fer-1; Macklin, 4 μM) for 24 h. To explore the effect of SLC6A14 on Caco-2 cell ferroptosis, cells were treated with α-MT (2.5 mM) for 48 h and then treated with RSL3 (15 μM) for 24 h.

Cell transfection

SLC6A14-, PAK6-, and C/EBPβ-specific small interfering RNAs (siRNAs; RiboBio, Guangzhou, China) were transfected into Caco-2 cells for 48-72 h using Lipofectamine 2000 (Thermo Fisher Scientific) according

to the manufacturer's instructions.

RNA sequencing and bioinformatic analysis

Total RNA was isolated from RSL3-induced Caco-2 cells transfected with si-SLC6A14-NC, si-SLC6A14-1, or si-SLC6A14-2 for poly(A) enrichment. mRNA transcripts with poly(A) tails were fragmented, reverse-transcribed into double-stranded cDNA, and inserted into a single-stranded circular DNA backbone. The qualified DNAs were sequenced on the DNBSEQ platform (Beijing Genomics Institute, Shenzhen, China). mRNA expression was obtained after quality control, filtering, and read alignment.

DEGs were identified by the following criteria: (1) $|\log_2 (\text{fold change})| > 0.585$ and (2) adjusted P value < 0.05. The heatmap and volcano plot were generated with R software (packages: 'pheatmap', 'ggplot2', and 'ggrepel'). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with R software (packages: 'clusterProfiler', 'org.Hs.eg.db', 'pathview', and 'topGO').

Haematoxylin–eosin staining and histological scoring

For histopathological analysis, haematoxylin and eosin (H&E) staining (Beyotime, Shanghai, China) was performed according to the manufacturer's protocols. Briefly, colon tissues of mice and humans were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. The paraffin-embedded colon tissues were sliced into 5 μm sections and stained sequentially with haematoxylin to visualize nuclei and eosin to visualize the cytoplasm. H&E staining was evaluated with the tissue damage index (TDI) as previously described (Supplementary Table 8)[17].

Immunohistochemistry

Immunohistochemical (IHC) staining was conducted as previously mentioned with a 3,3'-diaminobenzidine (DAB) Detection Kit (Gene Tech, Shanghai, China). Briefly, paraffin-embedded sections of colon tissue were incubated with the indicated primary antibodies overnight at 4 ℃ and then with the corresponding HRP-conjugated goat anti-mouse/rabbit IgG secondary antibody for 1 h at 37 ℃. IHC reactions were visualized by DAB staining (brown) and haematoxylin counterstaining (purple). The IHC sections were scored blindly by two experienced pathologists and additionally analysed with ImageJ (IHC Profile) with the H-score semiquantitative scoring system (Supplementary Table 9)[18]. The antibodies used for IHC analysis are shown in Supplementary Table 11.

Cell viability assay

Cell viability was evaluated with a Cell Counting Kit-8 (CCK-8; NCM, Suzhou, China) according to the manufacturer's instructions. Briefly, (3-5)×10^3 Caco-2 cells were seeded in a 96-well plate with 100 μl of DMEM containing 20% FBS and incubated for 48 h. After the indicated treatments, 10 μl of CCK-8 reagent was added to each well and incubated at 37 ℃ for 3 h. The absorbance at 450 nm (A450) was measured using a microplate photometer (Biotek, Vermont, USA).

Total iron content assay

The total iron content in Caco-2 cells and mouse colon tissue was measured with an Iron Content Assay Kit (Solarbio, Beijing, China) according to the manufacturer's protocols. Briefly, Caco-2 cells and the medium were centrifuged at 3000 × g for 15 min at room temperature. The precipitates were resuspended in 170 μl of iron extraction reagent and homogenized by sonication (ice bath; 20% power; ultrasonication 3 s, interval 3 s, 10 repeats). For analysis of mouse colon tissue, 100 mg of colon tissue was homogenized. The supernatant of each sample was obtained by centrifugation at 4 ℃ and 4000 × g for 10 min. The protein concentration (Cpr) in the supernatant was measured for normalization with a BCA protein assay (Beyotime). Next, the abovementioned supernatant (144 μl; ddH_2O for the blank wells and 0.125 μmol/ml standard solution for the standard wells) was mixed with iron assay reagents (reagent one: 72 μl; reagent two: 144 μl) and then heated at 100 ℃ for 1 h. The inorganic phase was obtained after mixing with trichloromethane and centrifugation at 10000 rpm for 10 min at room temperature. The absorbance at 520 nm (A520) was measured. Thus, . The relative iron content was determined by normalization to the controls.

Reduced glutathione assay

The content of reduced GSH in Caco-2 cells and mouse colon tissue was measured with a Reduced GSH Assay Kit (Njjcbio, Nanjing, China) in accordance with the instructions. Briefly, the cell and colon samples were homogenized in 60 μl of RIPA lysis buffer and centrifuged at 4 °C and 8000 × g for 10 min. The protein concentration (Cpr) in the supernatant was measured for normalization. Then, the supernatant (30 μl) was mixed with Reagent One Application Buffer (120 μl) and centrifuged at 4 ℃ and 4000 rpm for 10 min. The absorbance at 420 nm (A420) was measured, and the GSH concentration (CGSH) was calculated according to the standard concentration curve. Thus, . The relative GSH content was determined by normalization to the controls.

Malondialdehyde assay

The malondialdehyde (MDA) content in Caco-2 cells and mouse colon tissues was assessed with an MDA Assay Kit (Beyotime) based on the manufacturer's instructions. In brief, cell and colon samples were homogenized in 140 μl of RIPA lysis buffer and centrifuged at 4 °C and 12000 × g for 10 min. The protein concentration (Cpr) in the supernatant was measured for normalization. The supernatant (120 μl) was mixed with 240 μl of MDA assay buffer, incubated at 100 ℃ for 1 h and then centrifuged at 25 ℃ and 1000 ×g for 10 min. The absorbance at 532 nm (A532) was measured. Then, the MDA concentration (μmol/mg prot) was calculated according to the standard concentration curve, and the relative MDA content was determined by normalization to the controls.

Immunofluorescence staining

A total of (3-5)×10^3 Caco-2 cells were seeded on coverslips in a glass bottom dish (φ = 20 mm, NEST, Shenzhen, China) in 1 ml of DMEM containing 20% FBS and incubated for 48 h. After the indicated

treatments, the cells were fixed with 4% PFA for 15 min at room temperature and then permeabilized by using 0.2% Triton X-100. The cells were incubated with a rabbit polyclonal anti-human/mouse prostaglandin-endoperoxide synthase 2 (PTGS2) antibody (Proteintech, Wuhan, China) overnight at 4 ℃ after blocking with 2% bovine serum albumin (BSA) for 1 h. Then, the cells were incubated with the corresponding Cy3-labelled goat anti-rabbit secondary antibody for 1 h at 25 ℃ and finally mounted in the confocal glass bottom dish with antifade medium containing 4',6-diamidino-2-phenylindole (DAPI; Beyotime). The targeted cells were observed with a laser scanning confocal microscope (Zeiss, Oberkochen, Germany). Relative PTGS2 expression was calculated by dividing the number of $PTGS2^+$ cells (with red staining in the cytoplasm) by the number of $DAPI^+$ cells (with blue staining in the nucleus) followed by normalization to the controls.

C11 $BODIPY^{581/591}$ fluorescence assay

A total of (3-5)×10^3 Caco-2 cells were seeded on coverslips in a glass bottom dish and incubated for 48 h. After the indicated treatments, the cells were incubated with C11 $BODIPY^{581/591}$ dye (ABclonal, Wuhan, China, 10 μM) at 37 ℃ for 1 h. The targeted cells were observed with a laser scanning confocal microscope after excess dye was removed by wiping with PBS. Quantitative analysis of stained regions was performed with ImageJ (NIH, MD, USA). C11-$BODIPY^{581/591}$ levels were calculated as the ratio of the area with lipid oxidation (green) to the area without lipid oxidation (red).

Promoter prediction analysis

The promoter sequences of PAK6 were obtained from the GRCh38/hg38 human assembly via the University of California Santa Cruz (UCSC) Genome Browser (https://genome.ucsc.edu/cgi-bin/hgGateway). Then, the potential transcription factors and their binding sites were predicted with the TRANSFAC, ChIP-Atlas, and CIS-BP databases. The intersection of these three datasets was visualized with an interactive Venn diagram viewer, jvenn[19]. The predicted binding sites were visualized with the WebLogo program (http://weblogo.berkeley.edu/logo.cgi).

Dual-luciferase reporter assay

The relative luciferase activity of the C/EBPβ promoter was determined with a Dual-Luciferase Reporter Assay System (Promega, Wisconsin, USA) following the instruction manuals. In brief, Caco-2 cells were cotransfected with the C/EBPβ luciferase reporter plasmid (pGM C/EBPβ-Lu; Genomeditech, Shanghai, China) and SLC6A14-specific siRNAs with Lipofectamine 2000. After 48 h, the cells were lysed with passive lysis buffer (PLB), and firefly and Renilla luciferase activities were measured with a luminometer (Biotek). The relative luciferase activity was calculated by dividing the firefly luciferase activity by the Renilla luciferase activity and was normalized to si-SLC6A14-NC.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using an EZ-ChIP Kit (Sigma–Aldrich) according to the manuals, as previously reported. Briefly, RSL3-induced Caco-2 cells were cross-linked with 37% formaldehyde for 10 min. The cross-linked chromatin was sheared with the appropriate sonication protocol (ice bath; 10% power; ultrasonication 5 s, interval 10 s, 16-18 repeats) into fragments between 200 bp and 1000 bp in length, which were immunoprecipitated with a rabbit polyclonal anti-human/mouse C/EBPβ antibody (Proteintech). Normal mouse IgG was used for the negative control, while an anti-RNA polymerase antibody was used for the positive control. After the complexes were subjected to reverse cross-linking, the purified short hairpin DNA was detected by qPCR and DNA agarose gel electrophoresis (AGE) using primers specific for the PAK6 promoter region designed with Primer Premier 6 (Premier, Canada). The primers are listed in Supplementary Table 10.

Protein extraction and Western blot analysis

Caco-2 cells and mouse colon tissues (100 mg) were lysed with RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail. The total protein concentration was measured with a BCA protein assay kit. Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a 0.45 μm polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% BSA for 1.5 h and incubated with the indicated primary antibodies overnight at 4 ℃. The following day, the membrane was incubated with the corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the membrane was visualized with ECL reagents in a ChemiDocTM MP Imaging System (Bio–Rad, CA, USA). The antibodies used in the Western blot analysis are listed in Supplementary Table 11.

Total RNA isolation and qRT–PCR assays

Total RNA from Caco-2 cells or mouse colon tissue (100 mg) was extracted with TRIzol reagent (Vazyme, Nanjing, China) according to the manufacturer's protocol. For analysis of individual genes, cDNA was synthesized from 1.0 μg of total RNA with RTIII Super Mix with dsDNase (Monad, Guangdong, China) in a 20 μl volume system with the following thermal cycling conditions: 37 °C for 2 min, 55 °C for 5 min, 50 °C for 15 min, and 85 °C for 5 min. qPCR was performed with a CFX96 TouchTM Real-Time PCR System (Bio–Rad) with ChemoHS qPCR Mix (Monad). The PCR conditions were as follows: 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. GAPDH was used as the control. PCR was performed with three technical replicates. The primers used for qRT–PCR are listed in Supplementary Table 10.

Statistical analysis

All statistical data were analysed with GraphPad Prism 8 (La Jolla, CA, USA). The results with normal distribution are presented as the means ± standard error of means (SEMs) and the non-normal results are presented as medians ± interquartile ranges (IQRs). An unpaired t test was used to analyse normal data with equal standard deviation (SD), Welch's t test was used to analyse normal data without equal SD, and Mann Whitney u test was used to analyse non-normal data in two groups. Analysis of variance (ANOVA)

was performed to analyse normal data with equal SD, Welch's ANOVA analysis was used to analyse normal data without equal SD, and Kruskal-Wallis test was used to analyse non-normal data in multiple groups. Pearson correlation analysis was performed to analyse correlations between numerical normal data, while Spearman correlation analysis was performed in non-normal data in two groups. R^2 represents the coefficient of determination, and r represents the Pearson/ Spearman correlation coefficient. $P < 0.05$ was considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). All experiments in this study were performed in triplicate.

Results

SLC6A14 was overexpressed in UC tissue specimens and associated with ferroptosis in mice with experimental colitis

To identify the possible critical factors mediating ferroptosis in UC, we retrieved the microarray expression dataset GSE134025 from the GEO database and screened the DEGs (adjusted P value < 0.05 and $|\log_2$ (fold change)| > 1). In total, 26 genes were upregulated and 8 genes were downregulated in the tissues of UC patients compared with healthy individuals (Fig. 1A, Fig. S1A, Supplementary Table 2). Given that SLC proteins, such as SLC7A11 and SLC25A28, are critical to ferroptosis regulation[10, 12], SLC6A14, among the 26 upregulated genes, attracted our attention. Then, IHC analysis was conducted to validate SLC6A14 expression. SLC6A14 protein expression was markedly elevated in 89 tissues of patients with UC compared with 5 tissues of healthy individuals (Fig. 1B, Fig. S1B, Supplementary Table 1). In addition, in UC patients, SLC6A14 expression had a positive relationship with the Mayo score and UCEIS (Fig. 1C-D), two objective evaluative instruments of clinical and endoscopic activity in UC.

To analyse the correlation between SLC6A14 and ferroptosis, we next examined the expression of PTGS2, a well-known biomarker of lipid peroxidation[20], in the tissues of UC patients. The PTGS2 protein level was increased in UC tissues and positively correlated with the Mayo score and UCEIS in UC patients (Fig. 1B, Fig. S1C-D). Importantly, there was a positive correlation between the expression of SLC6A14 and PTGS2 (Fig. 1E).

To further evaluate the influence of SLC6A14 on ferroptosis in UC, DSS-induced and OXZ-induced colitis were established in mice. The results of the Western blot and IHC analyses revealed that the SLC6A14 level was apparently higher in colon tissues of colitic mice than in control mice (Fig. 1F-G, Fig. S1E). Moreover, the SLC6A14 expression level was positively associated with PTGS2 level in the colons of mice with DSS-induced or OXZ-induced colitis (Fig. 1G). In addition, the protein level of SLC6A14 was markedly increased in Caco-2 cells after treatment with RSL3 (Fig. 1H, Fig. S1F).

SLC6A14 inhibition inhibits ferroptosis in colonic epithelial cells

To investigate the function of SLC6A14 in ferroptosis, three nonoverlapping siRNAs targeting SLC6A14 (si-SLC6A14-1, si-SLC6A14-2, and si-SLC6A14-3) were used to knock down the mRNA and protein expression of SLC6A14 in Caco-2 cells. Among the three siRNAs, si-SLC6A14-1 and si-SLC6A14-2 had the

greatest inhibitory effect on SLC6A14 expression (Fig. 2A, Fig. S2A-B). Therefore, we chose si-SLC6A14-1 and si-SLC6A14-2 for further experiments. As shown in Fig. S2C-D, depletion of SLC6A14 increased the viability and inhibited the ferroptotic death of Caco-2 cells treated with RSL3. In addition, knockdown of SLC6A14 reduced the expression of PTGS2, FTL, and FTH (ferroptosis-related genes; Fig. 2F, Fig. S2E-F) and decreased the total cellular iron content and GSH consumption as well as the MDA level in RSL3-treated Caco-2 cells (Fig. 2B-D). Furthermore, we observed decreased lipid peroxidation, as determined by C11-BODIPY$^{581/591}$ staining (Fig. 2E), in Caco-2 cells after cotreatment with SLC6A14 siRNA and RSL3. These results were further confirmed by treatment with the chemical SLC6A14 inhibitor α-MT (Fig. 2G-K, Fig. S3G-J).

SLC6A14 suppression ameliorates inflammation and ferroptosis in experimental colitis

To further investigate whether SLC6A14 modulates ferroptosis in vivo, α-MT was administered to mice with DSS-induced and OXZ-induced colitis (Fig. S3A-B). As shown in Fig. 3, compared with those in the DSS group, mice in the DSS + α-MT group exhibited higher body weights, lower DAI scores, and longer colon lengths (Fig. 3A-C, Fig. S3C). Additionally, α-MT treatment markedly alleviated macroscopic colon inflammation and histological damage (Fig.3G, Fig.S3D). A similar result was found in mice with OXZ-induced colitis (Fig. 3H-J, 3N, Fig. S3E-F).

Next, we explored the effect of ferroptosis in colitic mice by analysing ferroptosis-related indices. DSS-challenged mice administered α-MT showed a lower iron load and MDA level and exhibited a higher level of GSH (Fig. 3D-F). The results of IHC analysis of the mouse colon also indicated that treatment with α-MT reduced the protein expression of PTGS2 in DSS-challenged mice (Fig. 3G). Furthermore, a decreased iron load, reduced MDA and PTGS2 levels, and an increased GSH level were observed in OXZ-challenged mice treated with α-MT (Fig. 3K-N).

SLC6A14 regulates PAK6 expression

To clarify the possible mechanisms underlying the role of SLC6A14 in intestinal epithelial cell ferroptosis, RNA sequencing (RNA-seq) was performed to profile the transcriptome changes in SLC6A14-knockdown Caco-2 cells after RSL3 treatment. In summary, 14 upregulated and 3 downregulated genes (adjusted P value < 0.05 and $|\log_2$ (fold change)| > 0.585) were identified in SLC6A14-knockdown Caco-2 cells compared to control cells (Fig. 4A, Fig. S4A-B, Supplementary Table 3). To validate the results of the RNA-seq analysis, several genes identified as dysregulated in the RNA-seq data were analysed by qRT–PCR. Consistent with the RNA-seq results, the expression of PAK6, HABP4, CDKN1A, and ACAD10 was increased but the expression of DACT3 was decreased in SLC6A14-knockdown Caco-2 cells (Fig. S4C). Among these DEGs, PAK6, a member of the type II PAK family, was obviously upregulated in ferroptotic SLC6A14-knockdown cells and is involved in the regulation of mitochondrial activity and ROS production[21, 22]. Hence, we hypothesized that PAK6 may participate in SLC6A14-mediated ferroptosis. IHC analysis showed that the expression of PAK6 in UC tissue samples was slightly upregulated compared with that in the samples of healthy individuals (Fig. 4B-D). Although the expression levels of

both SLC6A14 and PAK6 were increased in UC tissue specimens, we observed an inverse correlation between SLC6A14 and PAK6 protein levels in our cohort (Fig. 4E). Additionally, α-MT administration evidently elevated the protein expression of PAK6 in the colons of mice with either DSS-induced or OXZ-induced colitis (Fig. 4F-G, Fig. S4D-G). Moreover, PAK6 protein expression was significantly upregulated in RSL3-treated Caco-2 cells after treatment with SLC6A14 siRNA or α-MT (Fig. 4H, Fig. S4H-I). Collectively, our results suggested that SLC6A14 negatively modulates PAK6 expression in UC.

PAK6 is involved in SLC6A14-mediated ferroptosis

To assess whether SLC6A14-mediated ferroptosis is PAK6 dependent, we synthesized PAK6 siRNA-2 and PAK6 siRNA-3, which markedly decreased the protein level of PAK6 in Caco-2 cells after RSL3 treatment (Fig. 5A, Fig. S5A). Upon siRNA-mediated silencing of PAK6 in Caco-2 cells, RSL3-induced ferroptosis was significantly increased (Fig. S5C), and cell viability was apparently decreased (Fig. S5B). In addition, knockdown of PAK6 obviously elevated the iron content, GSH consumption, MDA level, and lipid peroxidation, as well as PTGS2 expression (Fig. 5B-F). These results suggested that PAK6 plays an important inhibitory role in Caco-2 cell ferroptosis induced by RSL3. Importantly, we observed that silencing of PAK6 abolished the α-MT-induced increase in RSL3-induced ferroptosis in Caco-2 cells and the decrease in cell viability (Fig. S5B-C). Additionally, PAK6 knockdown reversed the effects of α-MT on the iron content, GSH consumption, MDA level, lipid peroxidation, and PTGS2 expression in Caco-2 cells after RSL3 treatment (Fig. 5B-F). These data showed that SLC6A14 mediates RSL3-induced ferroptosis in Caco-2 cells via PAK6.

SLC6A14 inhibits PAK expression via C/EBPβ

We next sought to investigate how SLC6A14 controls the expression of PAK6. Predictions by the UCSC Genome Browser, TRANSFAC, ChIP-Atlas, and CIS-BP databases identified three possible binding sites of C/EBPβ in the PAK6 promoter (Fig. 6A-B). To confirm this prediction, we performed a ChIP assay in Caco-2 cells induced by RSL3. The ChIP results indicated that compared with the DNA purified with control IgG, the DNA purified with the anti-C/EBPβ antibody was significantly enriched in the three predicted sequences in the PAK6 promoter (Fig. 6C). Furthermore, transfection with C/EBPβ siRNA-1 and C/EBPβ siRNA-2 obviously reduced C/EBPβ protein expression in Caco-2 cells (Fig. 6D) and significantly elevated in PAK6 expression in Caco-2 cells induced by RSL3 (Fig. 6E). These results indicated that as a transcription factor, C/EBPβ can directly bind to the promoter of PAK6. To elucidate the importance of SLC6A14 in relation to C/EBPβ, we first detected by a luciferase reporter assay whether the activity of C/EBPβ (as determined by the C/EBPβ DNA-binding activity) is enhanced by SLC6A14. As shown in Fig. 6F, SLC6A14 silencing significantly decreased C/EBPβ activity in Caco-2 cells. Moreover, Western blot analysis showed that SLC6A14 knockdown greatly decreased C/EBPβ expression and increased PAK6 expression in Caco-2 cells after RSL3 induction (Fig. 6G).

Discussion

Recently, ferroptosis has been recognized as a key factor in the occurrence and development of UC[6]. Hence, accumulating evidence has indicated the roles and underlying molecular mechanisms of ferroptosis in UC. Inhibition of ferroptosis by ferrostatin-1, liproxstatin-1, or deferiprone was found to effectively ameliorate DSS-induced UC by negatively regulating the Nrf2/HO-1 signalling pathway[9]. Natural active ingredients from plants, such as curculigoside and astragalus polysaccharide, were found to obviously ameliorate DSS-induced UC through suppression of IEC ferroptosis[23] [7]. However, the key protein involved in this process remains obscure. Herein, we analysed the GSE134025 dataset to identify DEGs between UC patients and healthy individuals and found that SLC6A14, which shares analogous structures with other SLC transport proteins and is functionally coupled to SLC1A5, SLC7A5, and SLC7A11, was one of the upregulated DEGs in UC [24, 25]. Thus, in the current study, we concentrated on the potential impact and molecular mechanisms of SLC6A14 in mediating IEC ferroptosis in UC.

Multiple studies have demonstrated that SLC6A14 is upregulated in various colonic diseases, including UC[13]. Notably, microarray analysis of colon tissues from UC patients and control individuals revealed that SLC6A14 mRNA expression was noticeably increased in UC[26], in line with our analysis of recent microarray expression data[6]. Moreover, SLC6A14 expression was upregulated in human UC specimens in our cohort and was positively correlated with disease activity and endoscopic mucosal injury. Furthermore, we demonstrated that SLC6A14 was elevated in mice with DSS- and OXZ-induced colitis. These results suggest that upregulation of SLC6A14 is closely associated with the progression of UC. More importantly, our study revealed that SLC6A14 was positively correlated with lipid peroxidation in UC patients, and this finding was validated in mice with DSS- and OXZ-induced inflammation and in ferroptotic cells. Additionally, SLC6A14 knockdown by siRNA or inhibition by α-MT dramatically alleviated ferroptosis in colonic epithelial cells and experimental colitis, as evidenced by the regulatory effects on the expression of ferroptosis-related genes, the iron content, GSH consumption, the MDA level, and lipid peroxidation. These results indicate that SLC6A14 plays a crucial role in mediating IEC ferroptosis in UC.

To extensively explore the mechanisms underlying SLC6A14-mediated ferroptosis, RNA-seq analysis was performed using Caco-2 cells cotreated with SLC6A14 siRNAs and RSL3. The results of this RNA-seq analysis indicated that PAK6 was obviously upregulated in ferroptotic SLC6A14-knockdown cells. As a member of the type II PAK family, PAK6 has been demonstrated to be associated with mitochondrial function and ROS production[21, 22]. Given that dysregulation of mitochondrial function and ROS elevation contribute to ferroptosis[27-29], we speculated that PAK6 is involved in regulating IEC ferroptosis in UC. Herein, we observed that silencing PAK6 significantly enhanced ferroptosis in Caco-2 cells after treatment with RSL3. The expression of PAK6 was inversely correlated with SLC6A14 expression in tissue samples of patients with UC. Additionally, suppression of SLC6A14 by α-MT evidently upregulated the protein expression of PAK6 in the colons of mice with either DSS-induced or OXZ-induced colitis. Importantly, PAK6 knockdown abolished the effects of SLC6A14 on RSL3-induced ferroptosis in Caco-2 cells. These results suggested that SLC6A14 promotes IEC ferroptosis in UC via negative regulation of PAK6.

Previous studies have indicated that PAK6 can activate multiple signalling pathways and perform its biological functions. For instance, overexpression of PAK6 was found to promote the proliferation, migration, and invasion of cervical cancer cells by activating the Wnt/β-catenin signalling pathway[30]. PAK6 is involved in the modulation of androgen receptor signalling in various types of prostate cancer[31]. Moreover, pharmacological inhibition of PAK6 was found to sensitize therapy-resistant cells to tyrosine kinase inhibitors in chronic myeloid leukaemia by disrupting the RAS/MAPK pathway and mitochondrial activity[22]. In the current study, we identified that the RAS signalling pathway was upregulated in ferroptotic SLC6A14-knockdown cells (Fig. S6A). Moreover, the results of KEGG pathway enrichment analysis (https://www.genome.jp/kegg/) showed the association between PAK6 and the RAS signalling pathway (Fig. S6B). Additionally, PAK6 knockdown significantly increased the protein level of phosphorylated ERK1/2, the key protein in the RAS signalling pathway (Fig. S6C). Given that the RAS signalling pathway plays an important role in the regulation of ferroptosis[32] , we concluded that the RAS-ERK signalling pathway is required for SLC6A14-PAK6 axis-mediated ferroptosis. Since numerous factors and intersecting pathways were related to RAS signalling, we did not verify all involved elements, and there may be other potential pathways involved in this process.

C/EBPβ, an important transcription factor belonging to the leucine zipper family, plays a critical role in several cellular processes, including the inflammatory response[33-36]. C/EBPβ has been demonstrated to modulate inflammation in intestinal epithelial cells[37]. Moreover, upregulation of C/EBPβ induces inflammatory responses resulting in mitochondrial dysfunction and ROS accumulation[38]. Herein, we predicted possible TF binding sites in the PAK6 promoter region and fortunately found binding sites for the most compelling factor, C/EBPβ. Our ChIP assay showed that the DNA precipitated with the anti-C/EBPβ antibody was significantly enriched in PAK6 promoter sequences. Furthermore, C/EBPβ knockdown markedly reduced C/EBPβ protein expression in Caco-2 cells. Importantly, the results of the luciferase reporter assay indicated that SLC6A14 knockdown significantly decreased C/EBPβ activity. In addition, SLC6A14 silencing greatly decreased C/EBPβ expression and increased PAK6 expression in Caco-2 cells after treatment with RSL3. Therefore, our results suggested that SLC6A14 controls the expression of PAK6 via C/EBPβ. However, we have yet to fully understand the specific mechanism by which SLC6A14 activates C/EBPβ. Further exploration is required in future research.

In conclusion, we investigated the function and molecular mechanism of SLC6A14 in mediating IEC ferroptosis in UC. Our findings indicated that SLC6A14 overexpression promotes IEC ferroptosis in UC via the C/EBPβ-PAK6 pathway. Moreover, high SLC6A14 expression was positively associated with ferroptosis in UC tissue samples. Therefore, our results revealed that SLC6A14 is a novel regulator of SLC6A14 via control of the C/EBPβ-PAK6 pathway in UC, suggesting that SLC6A14 is a promising therapeutic target for UC.

Statements And Declarations

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Competing interests The authors have no relevant financial or non-financial interests to disclose.

Author contributions YJC, TGS, and WCC contributed to the study conception and design. Material preparation, data collection and analysis were performed by YJC, WYY, JYW, YQC, JHZ, HYJ, HYW, GBZ, TGS, QHX and SHZ. The first draft of the manuscript was written by YJC, TGS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The microarray datasets analysed during the current study are available in the GEO (GSE134025) repository, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134025.

Ethics approval All animal procedures were performed in line with the principles of the Ethics Committee of Soochow University (reference number: SUDA20210918A01). All studies for human tissue samples complied the Ethics Committee of the First Affiliated Hospital of Soochow University (reference number: 2021-325).

Consent to participate Written informed consent was obtained from the parents.

Consent for publish The authors affirm that human research participants provided informed consent for publication of the images in Figures 1b, 4b and S1b.

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Figures

Figure 1

SLC6A14 expression was increased in UC tissue specimens and correlated with ferroptosis. (A) Heatmap of 34 differentially expressed genes between UC patients (n = 3) and healthy individuals (n = 3) in colonic biopsy samples from the GSE134025 dataset. (B) Representative images of IHC staining of SLC6A14 and PTGS2 in colon tissues from UC patients (n = 89) and healthy individuals (n = 5). Scale bar, 50 μm. IHC staining was semiquantitatively analysed by the H-score. (C-E) The correlations between SLC6A14 expression and the Mayo score (C), the UCEIS (D), and PTGS2 expression (E) in human colonic tissues. (F) Western blot analysis of SLC6A14 in colon tissues from mice with DSS-induced and OXZ-induced colitis. β-Actin was used as the control. (G) Representative images of IHC staining of SLC6A14 and PTGS2 in colon tissues from mice with DSS-induced and OXZ-induced colitis. Scale bar, 50 μm. (H) Western blot analysis of SLC6A14 in the Caco-2 cell model of RSL3-induced ferroptosis. β-Actin was used as the control. The results are presented as the means ± SEMs; ***P < 0.001 vs. Normal.

Figure 2

SLC6A14 inhibition inhibits ferroptosis in Caco-2 cells. (A) Western blot analysis of SLC6A14 in Caco-2 cells after cotreatment with SLC6A14 siRNAs and RSL3. β-Actin was used as the control. (B-D) Iron content (B), GSH content (C), and MDA content (D) in Caco-2 cells after cotreatment with SLC6A14 siRNAs and RSL3. (E) Representative images of C11-BODIPY$^{581/591}$ fluorescence in Caco-2 cells after cotreatment with SLC6A14 siRNAs and RSL3. Scale bar, 50 μm. Relative C11-BODIPY$^{581/591}$ levels were calculated by dividing the area with lipid oxidation (green) by the area without lipid oxidation (red) and then normalizing to the controls. (F) The relative PTGS2 expression in Caco-2 cells after cotreatment with SLC6A14 siRNAs and RSL3 was determined based on the immunofluorescence staining shown in Fig. S3E. (G-I) Iron content (G), GSH content (H), and MDA content (I) in Caco-2 cells after cotreatment with α-MT and RSL3. (J) Representative images of C11-BODIPY$^{581/591}$ fluorescence in Caco-2 cells after cotreatment with α-MT and RSL3. Scale bar, 50 μm. Relative C11-BODIPY$^{581/591}$ levels were calculated by dividing the area with lipid oxidation (green) by the area without lipid oxidation (red) and then normalizing to the controls. (K) The relative PTGS2 expression in Caco-2 cells after cotreatment with α-MT and RSL3 was determined based on the immunofluorescence staining shown in Fig. S3I. The results are presented as the means ± SEMs; *P < 0.05, **P < 0.01, ***P < 0.001 vs. si-SLC6A14-NC + RSL3; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. RSL3.

Figure 3

Inhibition of SLC6A14 ameliorates inflammation and ferroptosis in mice with experimental colitis. (A-C) The body weight (A), disease activity index (DAI) score (B), and photograph of the colon (C) in DSS-challenged mice treated with α-MT. (D-F) Iron content (D), GSH content (E), and MDA content (F) in DSS-challenged mice treated with α-MT. (G) Representative images of H&E staining (upper) and IHC staining (lower) of PTGS2 in colon tissues from DSS-challenged mice treated with α-MT. Scale bar, 50 μm. IHC staining was semiquantitatively analysed by the H-score. (H-J) The body weight (H), DAI score (I), and photograph of the colon (J) in OXZ-challenged mice treated with α-MT. (K-M) Iron content (K), GSH content (L), and MDA content (M) in OXZ-challenged mice treated with α-MT. (N) Representative images of H&E staining (upper) and IHC staining (lower) of PTGS2 in colon tissues from OXZ-challenged mice treated with α-MT. Scale bar, 50 μm. IHC staining was semiquantitatively analysed by the H-score. The results are presented as the means ± SEMs or medians ± IQRs; *P < 0.05, **P < 0.01, ***P < 0.001 vs. DSS-Control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. DSS + α-MT; &P < 0.05, &&P < 0.01, &&&P < 0.001 vs. OXZ-Control; @P < 0.05, @@P < 0.01, @@@P < 0.001 vs. OXZ + α-MT.

Figure 4

SLC6A14 modulates PAK6 expression. (A) Heatmap showing the 17 differentially expressed genes in Caco-2 cells after cotreatment with SLC6A14 siRNAs and RSL3. (B) Representative images of IHC staining of PAK6 in colon tissues from UC patients (n = 89) and healthy individuals (n = 5); scale bar, 50 μm. IHC staining was semiquantitatively analysed by the H-score. (C-E) The correlations between PAK6 expression and the Mayo score (C), UCEIS (D), and SLC6A14 expression (E) in human colonic tissues. (F) Western blot analysis of SLC6A14 expression in colon tissues from mice with DSS-induced and OXZ-induced colitis. β-Actin was used as the control. (G) Representative images of IHC staining of PAK6 in colon tissues from mice with DSS-induced and OXZ-induced colitis. Scale bar, 50 μm. (H) Western blot analysis of SLC6A14 expression in the Caco-2 cell model of RSL3-induced ferroptosis. β-Actin was used as the control. The results are presented as the means ± SEMs. ***P < 0.001 vs. Normal.

Figure 5

PAK6 participates in SLC6A14-mediated ferroptosis. (A) Western blot analysis of PAK6 expression in RSL3-induced Caco-2 cells transfected with PAK6-siRNA. β-Actin was used as the control. (B-D) Iron content (B), GSH content (C), and MDA content (D) in Caco-2 cells after cotreatment with PAK6-siRNA and RSL3. (E) Representative images of C11-BODIPY$^{581/591}$ fluorescence in Caco-2 cells after cotreatment with PAK6-siRNA, α-MT and RSL3. Scale bar, 50 μm. Relative C11-BODIPY$^{581/591}$ levels were calculated by dividing the area with lipid oxidation (green) by the area without lipid oxidation (red) and then normalizing to the controls. (F) Representative images of immunofluorescence staining of PTGS2 in Caco-2 cells after cotreatment with PAK6-siRNA, α-MT and RSL3. Scale bar, 50 μm. The results are

presented as the means ± SEMs. *P < 0.05, **P < 0.01, ***P < 0.001 vs. si-PAK6-NC + RSL3; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. si-PAK6-NC + α-MT + RSL3; &P < 0.05, &&P < 0.01, &&&P < 0.001 vs. RSL3.

Figure 6

SLC6A14 inhibits PAK6 expression via C/EBPβ. (A) The intersection of transcription factors (TFs) predicted by the TRANSFAC, ChIP-Atlas, and CIS-BP databases. (B) Schematic diagram of C/EBPβ binding sites in the PAK6 promoter region. There are three C/EBPβ-binding sites in the 5' region of PAK6: P1 (-841 nt to -831 nt), P2 (-1301 nt to -1288 nt), and P3 (-505 nt to -495 nt). (C) ChIP analysis of C/EBPβ binding to the PAK6 promoter in RSL3-induced Caco-2 cells. Normal mouse IgG was used as the control. (D) Western blot analysis of C/EBPβ expression in Caco-2 cells transfected with C/EBPβ-siRNA. β-Actin was used as the control. The Western blot band densities were quantified using ImageJ software. (E) Western blot analysis of C/EBPβ and PAK6 expression in RSL3-induced Caco-2 cells transfected with C/EBPβ-siRNA. β-Actin was used as the control. The Western blot band densities were quantified using ImageJ software. (F) C/EBPβ-responsive luciferase activity was detected in Caco-2 cells transfected with C/EBPβ-siRNA. (G) Western blot analysis of SLC6A14, PAK6, and C/EBPβ expression in RSL3-induced Caco-2 cells transfected with SLC6A14-siRNA. β-Actin was used as the control. The Western blot band densities were quantified using ImageJ software. The results are presented as the means ± SEMs. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. IgG; &&&P < 0.001 vs. si-C/EBPβ-NC; φφP < 0.01, φφφP < 0.001 vs. si-C/EBPβ-NC + RSL3; %%P < 0.01 vs. si- SLC6A14-NC; ***P < 0.001 vs. si- SLC6A14-NC + RSL3.

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