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Ferroptosis related genes in retinopathy of prematurity: Screening for potential pharmacological targets

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Purpose: To elucidate the molecular mechanisms of retinopathy of prematurity (ROP) and identify potential drug targets for its treatment based on bioinformatics analysis.
Methods: We obtained gene expression profiles from data sets GSE123945 and GSE135844 in the Gene Expression Omnibus database. Differential expression analysis was performed on GSE135844 to identify differentially expressed genes (DEGs). Ferroptosis-related genes were retrieved from FerrDb, and Venn diagrams were used to identify overlapping genes between DEGs and ferroptosis-related genes. Functional enrichment analyses, including Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, and gene set enrichment analysis, were conducted to explore the biological roles of these genes. Protein-protein interaction networks were constructed using the STRING database, and hub genes were identified using Cytoscape. Potential drug targets were screened using the Drug-Gene Interaction Database.
Results: We identified 23 DEGs associated with ferroptosis in ROP. These genes were significantly enriched in biological processes such as angiogenesis, vasculature development, and wound healing. Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that the PI3K-Akt signaling pathway plays a central role in ROP pathogenesis. Gene set enrichment analysis identified the defense response as the most significantly enriched gene set. Hub genes, including *HMOX1, ALB*, and *JUN*, were identified through protein-protein interaction network analysis. Potential drugs targeting these hub genes, such as stannsoporfin, recombinant neurotrophic factors, zinc chloride, and irisolidone, were identified using the Drug-Gene Interaction Database.
Conclusions: Our study demonstrates the utility of bioinformatics approaches in identifying ferroptosis-related genes and potential drug targets for ROP. The findings provide a foundation for further experimental validation and the development of novel therapeutic strategies for ROP.

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the retina that primarily affects preterm infants. First described by Dr. Stewart Clifford in 1942 as retrolental fibroplasia, ROP remains a leading cause of childhood blindness worldwide. The incidence of ROP is closely associated with preterm birth and low birthweight, with approximately 32,200 infants having visual impairment or blindness due to ROP in 2010 alone [1].

ROP is characterized by abnormal retinal vascular development, which occurs in two phases. The first phase involves the suppression of normal vascular growth due to hyperoxia and the loss of maternal-derived growth factors. The second phase is marked by hypoxia-induced pathological angiogenesis, driven by elevated levels of vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1) [2]. While anti-VEGF therapies have shown efficacy in treating ROP, concerns about their long-term neurodevelopmental effects have prompted the search for alternative therapeutic targets [3].

Ferroptosis, a recently discovered form of regulated cell death, is characterized by iron-dependent lipid peroxidation and has been implicated in various diseases, including cancer, neurodegenerative disorders, and ischemic injuries [4]. Emerging evidence suggests that ferroptosis may play a role in the pathological angiogenesis observed in ROP. Iron metabolism dysregulation and oxidative stress are key features of both ferroptosis and ROP, making ferroptosis-related genes potential therapeutic targets for this condition [5]. Therefore, targeting ferroptosis may offer a promising therapeutic approach for ROP.

In this study, we employed bioinformatics approaches to identify ferroptosis-related differentially expressed genes (DEGs) in ROP and explore their functional roles. By

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integrating gene expression data from the Gene Expression Omnibus (GEO) database with functional enrichment and network analyses, we aimed to uncover the molecular mechanisms underlying ROP and identify potential drug targets for its treatment.

The oxygen induced retinopathy (OIR) mouse model recapitulates key features of human ROP including pathological neovascularization and vasobliteration. However, important interspecies differences, such as variations in retinal development and vascular response, must be acknowledged. Despite these limitations in developmental timing and systemic context, the OIR model remains the most widely validated experimental system for studying pathological angiogenesis in retinopathy, particularly for investigating cellular responses to hypoxia reoxygenation injury. This establishes a framework for our subsequent investigation of ferroptosis mechanisms in retinal vascular disease [6,7].

METHODS

Source of microarray data: We downloaded the GSE135844 and GSE123945 data sets from the GEO database. GSE135844 investigates progenitor cell binding in normalizing retinal vascular development by enhancing pericyte nesting in an oxygen-induced retinopathy (OIR) model [8]. The data set, generated using the GPL8321 [Mouse430A_2] Affymetrix Mouse Genome 430A 2.0 Array platform, includes retinal samples from OIR model mice (n = 3) and normal mice (n = 3). The GSE123945 dataset investigates the role of epithelial membrane protein 2 in promoting VEGF signaling to enhance pathological neovascular growth in retinopathy of prematurity. This dataset was generated using high throughput RNA sequencing of mouse retinal tissues and includes samples from hyperoxia mice (n = 4) and normoxia mice (n = 4).

DEG identification: DEGs between OIR and control samples were identified using the "limma" package in R. Genes with |log2 FC| >0.585 and an adjusted *p* value <0.05 were considered differentially expressed. Volcano plots and heatmaps were generated using the R package "heatmap" for visualization.

Ferroptosis-related genes and Venn analysis: Ferroptosis-related genes were retrieved from FerrDb, a comprehensive database of ferroptosis regulators and markers. A Venn diagram was constructed to identify overlapping genes between the ferroptosis-related genes and the DEGs identified from GSE135844.

Gene ontology, Kyoto encyclopedia of genes and genomes, and gene set enrichment analysis: Gene Ontology (GO) functional enrichment analyses, including molecular function (MF), biological process (BP), and cellular component (CC) [9], were performed using the "clusterProfiler" package in R [10]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted to identify signaling pathways associated with the ferroptosis-related DEGs. Gene set enrichment analysis (GSEA) was performed to compare gene set differences between the OIR and control groups, using the Molecular Signatures Database (MSigDB) as the background gene set. A *p* value <0.05 was considered statistically significant.

GO enrichment analysis identifies functional annotations (biological processes; molecular functions; cellular components) among DEGs. The KEGG pathway analysis reveals significantly enriched metabolic, signaling, and cellular pathways. Additionally, GSEA display the enrichment of gene sets across a transcriptome ranked according to phenotype correlation.

Protein-protein interaction network construction and hub gene identification: Protein-protein interaction (PPI) networks were constructed using the STRING database [11]. The network was visualized and analyzed using Cytoscape software, and hub genes were identified based on their connectivity and centrality measures.

Screening for potential pharmacological targets: The Drug-Gene Interaction Database (DGIdb) was used to identify drugs or molecular compounds interacting with the hub genes. The drug-gene interaction network was visualized using Cytoscape. In order to further investigate the reliability of the relationship, we calculated the percentage correlation with both the gene and drug and with each alone. Gene relevance was quantified using log2FC, where a 1.5 fold change (log2FC=0.585) translated to 50% relevance, scaling linearly with expression magnitude [12].

RESULTS

Identification of DEGs: Analysis of the GSE135844 data set identified 720 DEGs (495 upregulated and 225 downregulated) between OIR model mice and normal controls. Volcano plots and a heatmap illustrating the DEGs are shown in Figure 1A,B, respectively.

Ferroptosis-related genes and Venn analysis: A total of 223 ferroptosis-related genes were retrieved from FerrDb. Intersection with the DEGs identified 23 overlapping genes, including *ACOT1, ALB, ATF3, BNIP3, CAPG, CD44, CHAC1, DRD4, ENPP2, HIC1, HMOX1, IFNA5, JUN, LCN2, NNMT, NOX4, NUPR1, RRM2, SAT1, TGFB1, TLR4, WWTR1*, and *ZFP36* (Figure 2A). Among these, 20 genes were downregulated, and 3 were upregulated. Normalized expression levels

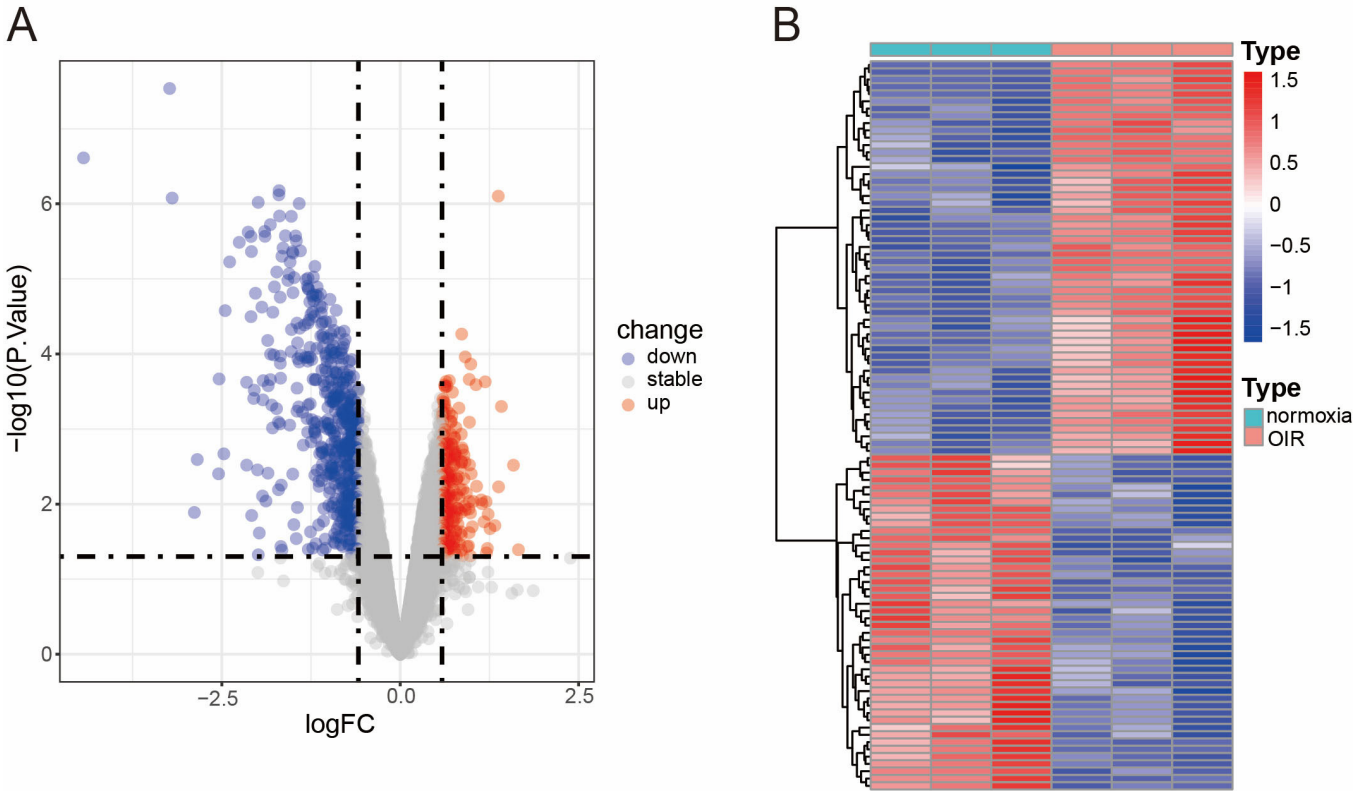

Figure 1. Identification of differentially expressed genes (DEGs). **A**: Differential expression gene analysis was performed on the retinopathy of prematurity (ROP) dataset GSE135844. Blue nodes represented genes downregulated in ROP, red nodes represented upregulated genes, and gray nodes indicated genes with no significant difference compared with controls. **B**: The heatmap illustrated the expression patterns of the identified DEGs. The oxygen-induced retinopathy (OIR) model was commonly used to simulate the pathological process of ROP in experimental animals.

and correlations of these genes are displayed in a heatmap (Figure 2B) and correlation plots (Figure 2C).

Functional enrichment of the ferroptosis-related DEGs by GO, KEGG, and GSEA analysis: The GO enrichment analysis of the ferroptosis-related DEGs includes three parts: biological process, cell component, and molecular function. GO enrichment analysis revealed that the ferroptosis-related DEGs are involved in the cellular response to extracellular stimuli, metal ion homeostasis, and apoptotic signaling pathways (Figure 3A). KEGG pathway analysis indicated their involvement in ferroptosis, chemical carcinogenesis, and the PI3K-Akt signaling pathway (Figure 3B). GSEA identified the defense response as the most significantly enriched gene set (Figure 3C).

PPI network construction and hub gene analysis: A PPI network of ferroptosis-related DEGs was constructed using STRING, consisting of 23 nodes and 34 edges (Figure 4A). The network was condensed into a coexpression network consisting of 8 nodes and 23 edges using the "MCODE" function in Cytoscape (Figure 4B). The eight hub genes identified were HMOX1, ALB, JUN, TLR4, TGFB1, CD44, NOX4, and LCN2. Among these, *HMOX1, ALB*, and *JUN* emerged as the central hub genes (Table 1).

Drug-gene network: Potential drugs targeting ROP were identified using DGIdb. The drug gene interaction network was visualized using Cytoscape (Figure 5). Then, we selected the top ten drugs with the highest interaction scores (Table 2). Among them, the HMOX1 stannsoporfin received the highest score (5.8) and correlation percentage (72%). Scores ≥4 and >60% represent high confidence candidates warranting immediate validation, while scores <2 or <40% may require target optimization.

Hub gene expression in two data sets: Eight hub genes showed significant differential expression in the transcriptome profiling of animal models of retinopathy of prematurity. The visualization showed how these genes were significantly differentially expressed in the datasets GSE135844 and GSE123945 (Figure 6).

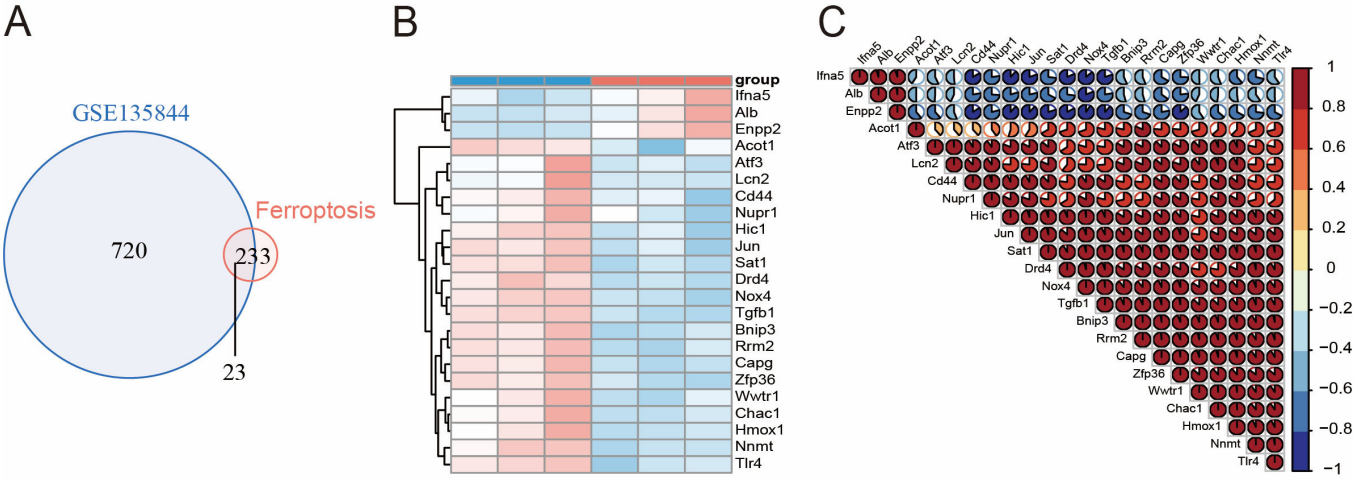

Figure 2. Ferroptosis-related genes and Venn analysis. **A**: A Venn diagram was constructed to show the intersection between differentially expressed genes (DEGs) from dataset GSE135844 and known ferroptosis-related genes. The left count (720 genes) represented DEGs that are specific to the GSE135844 dataset. The middle count (23 genes) indicated the number of DEGs that are associated with ferroptosis. The right count (233 genes) represented genes that are uniquely associated with ferroptosis from the reference gene set. **B**: A heatmap displayed the expression patterns of the 23 ferroptosis-related DEGs identified in the Venn analysis. **C**: A correlation analysis illustrated the relationships among the 23 ferroptosis-related DEGs.

DISCUSSION

ROP is a complex vascular disorder affecting the retina, characterized by the abnormal proliferation of retinal blood vessels. In severe cases, ROP can lead to complete retinal detachment behind the lens in premature infants, resulting in irreversible vision loss. The development of ROP is influenced by a multitude of risk factors, including exposure to high oxygen levels, below-normal gestational age, low birthweight, reduced postpartum levels of IGF-1, and elevated blood glucose levels. These factors are often associated with preterm birth and the neonatal environment. Additionally, emerging evidence suggests that maternal risk factors, such as preeclampsia and maternal iron-deficiency anemia, may

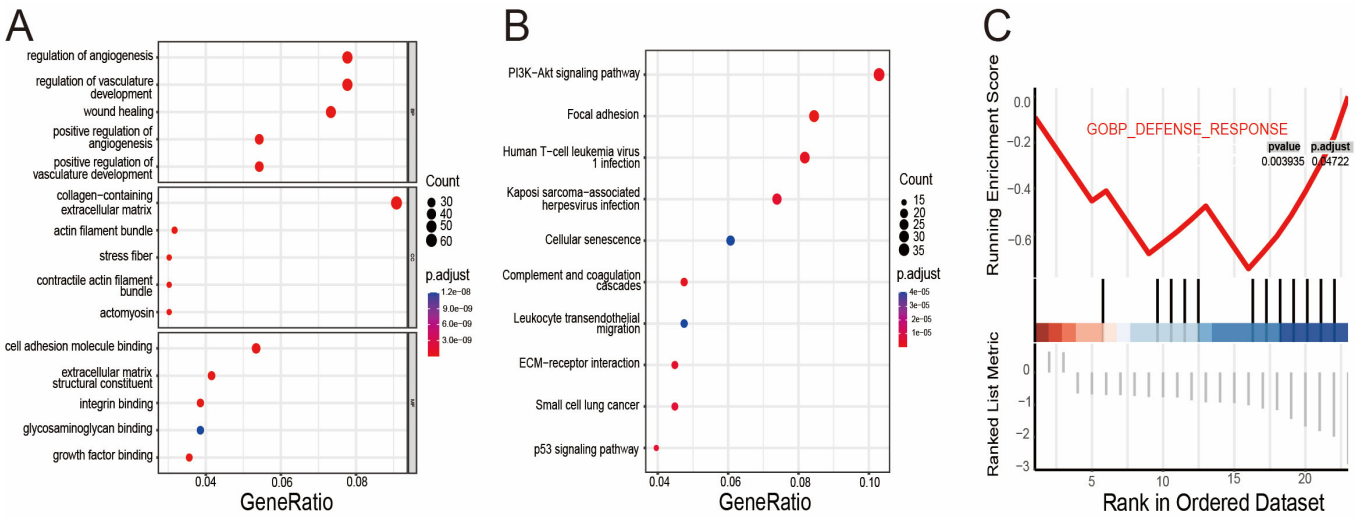

Figure 3. Functional enrichment analysis of ferroptosis-related DEGs. **A**: Gene Ontology (GO) enrichment analysis was performed on the ferroptosis-related differentially expressed genes (DEGs), with results summarized in bubble plots representing three functional categories: biological process (BP), cellular component (CC), and molecular function (MF). **B**: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the ferroptosis-related DEGs was displayed in the form of a bubble plot. **C**: Gene Set Enrichment Analysis (GSEA) was conducted to identify the most significantly enriched gene sets in the GSE135844 dataset.

Table 1. MCODE plug-in screened genes and their functions.

Gene	Function[#]
HMOX1	HMOX1 and HMOX2 belong to the heme oxygenase family. Heme oxygenase, an essential enzyme in heme catabolism, cleaves heme to form biliverdin, which is subsequently converted to bilirubin by biliverdin reductase, and carbon monoxide, a putative neurotransmitter. Heme oxygenase activity is induced by its substrate heme and by various nonheme substances.
ALB	This gene encodes the most abundant protein in human blood. This protein functions in the regulation of blood plasma colloid osmotic pressure and acts as a carrier protein for a wide range of endogenous molecules including hormones, fatty acids, and metabolites, as well as exogenous drugs. Additionally, this protein exhibits an esterase-like activity with broad substrate specificity. The encoded preproprotein is proteolytically processed to generate the mature protein.
JUN	This gene is the putative transforming gene of avian sarcoma virus 17. It encodes a protein which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression.

[#]Data fromNCBI.

further increase the susceptibility of preterm infants to ROP [13].

From a pathogenic perspective, ROP progresses through two distinct phases. The initial phase is marked by the suppression of normal retinal vascular development due to hyperoxia and the loss of essential circulating nutrients, such as ω-3 polyunsaturated fatty acids, growth factors, and IGF-1. This phase leads to the arrest of retinal blood vessel growth. In the second phase, poorly vascularized retinal tissue becomes hypoxic, triggering a compensatory response characterized by elevated levels of intraocular VEGF, a potent angiogenic stimulator, and increased synthesis of IGF-1. The combination of hypoxia, elevated VEGF, and rising IGF-1 levels creates a conducive environment for pathological angiogenesis, a hallmark of stage 2 ROP. This abnormal vascular growth results in the formation of fragile blood vessels, which are prone to leakage and rupture, ultimately leading to visual impairment [13].

Hyperoxia inhibits VEGF production and suppresses the formation of new blood vessels, whereas hypoxia stimulates VEGF production and promotes neovascularization. However, the overactivation of VEGF receptors during this

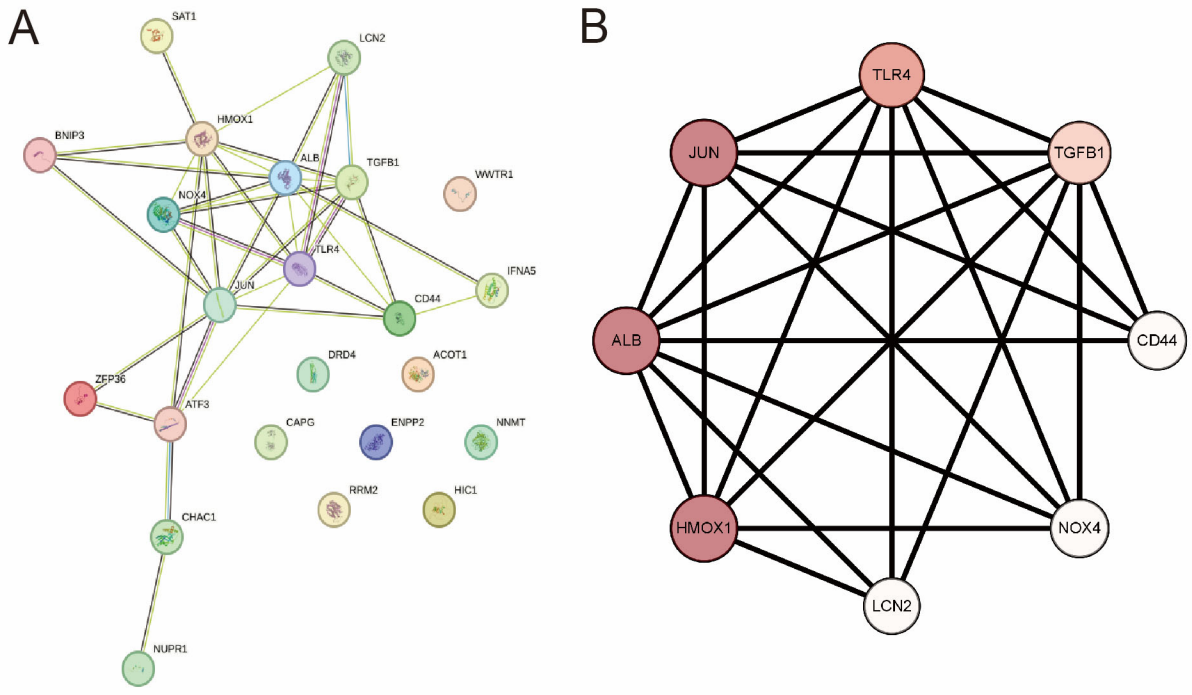

Figure 4. Protein-protein interaction network construction and hub gene analysis. **A**: A protein-protein interaction (PPI) network was constructed using the STRING database. **B**: Hub genes were identified with the MCODE plugin in Cytoscape.

Table 2. Drug-gene network analysis.

Gene	Drug	Interaction score (0-6) #	Gene relevance to ROP (%) #	Drug relevance to retinopathy (%) #	Combined Correlation (%) #
HMOX1	STANNSOPORFIN	5.800422062	0.78	0.65	0.72
HMOX1	RECOMBINANT NEUROTROPHIC FACTOR	2.320168825	0.78	0.5	0.67
HMOX1	ZINC CHLORIDE	2.320168825	0.78	0.4	0.63
ALB	COCHINCHINENIN C	2.372899934	0.42	0.3	0.36
ALB	CHEMBL:CHEMBL1945287	2.372899934	0.42	0.25	0.35
JUN	NEOCHAMAEJASMIN A	2.269730372	0.68	0.35	0.55
JUN	BRUCEANTIN	2.269730372	0.68	0.3	0.53
JUN	IRISOLIDONE	2.269730372	0.68	0.55	0.62
JUN	CHEMBL:CHEMBL477052	2.269730372	0.68	0.2	0.48
JUN	SERGEOLIDE	2.269730372	0.68	0.15	0.45

Interaction scores for the drug-gene pairs were derived from the DGIdb database and were calculated by integrating drug-target affinity (scored 0-3) with gene-disease association strength (scored 0-3). Scores of ≥4 indicated high-confidence interactions, scores between 2 and 4 suggested moderate evidence, and scores <2 reflected minimal support. Gene relevance (%) was derived from the log2 fold change in the ROP dataset (GSE135844). Drug relevance (%) was based on DrugBank annotations and PubMed-reviewed mechanisms. The Combined Correlation (%) was a weighted score based on target priority (gene contribution: 60%; drug contribution: 40%).

process leads to disorganized vascular growth, with vessels growing on top of each other rather than extending in an orderly manner toward the ora serrata. Anti-VEGF therapy has become a widely used clinical treatment for ROP, significantly improving outcomes and reducing the need for laser therapy [14,15]. While recent studies have shown no increased risk of adverse neurodevelopmental outcomes following anti-VEGF treatment [16], other large-scale

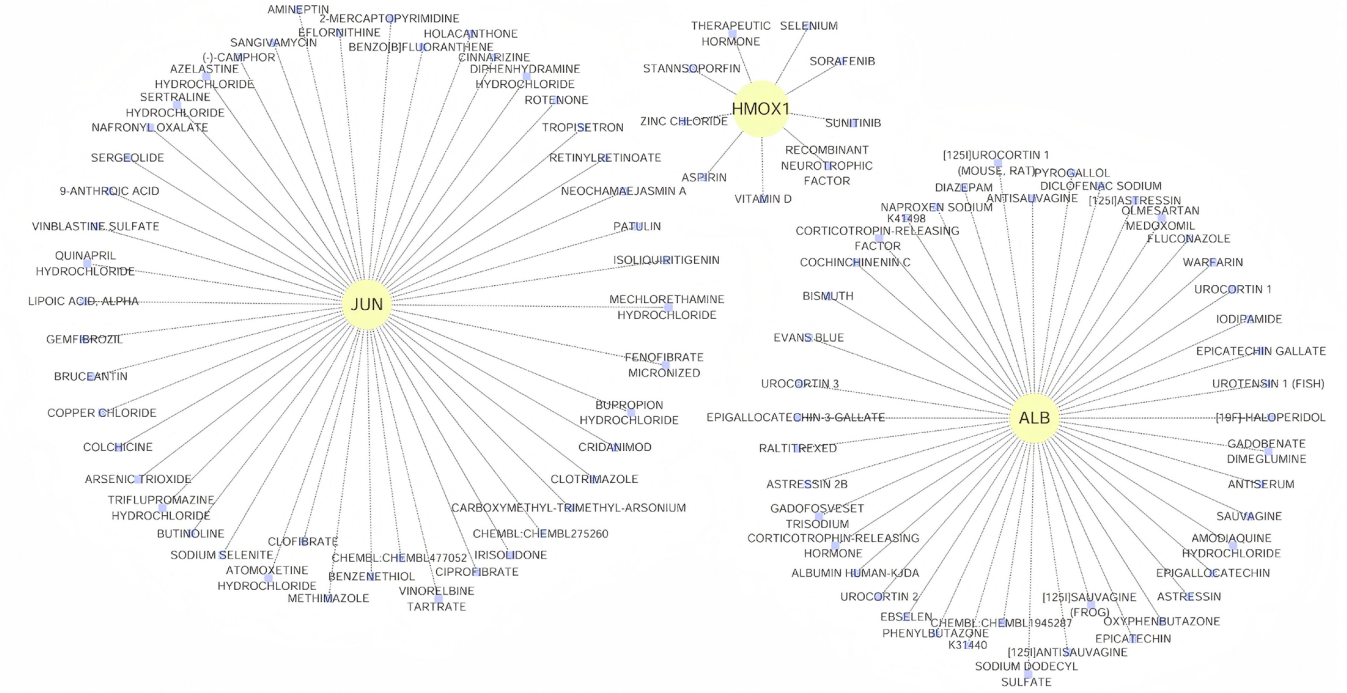

Figure 5. Gene-drug interaction network analysis. A gene-drug interaction network was constructed and visualized using Cytoscape software.

follow-up studies from Canada suggest potential risks of adverse neurodevelopment in infants treated with these drugs [17]. Given these concerns, there is a pressing need to unravel the biological mechanisms underlying ROP and identify alternative therapeutic strategies. In this study, we aimed to shed light on these mechanisms by investigating the role of ferroptosis-related DEGs in ROP.

In our study, we first analyzed the gene expression profiles of retinal tissues from OIR model mice and normal mice to identify ferroptosis-related DEGs associated with ROP. A total of 23 DEGs (3 upregulated and 20 downregulated) were identified, suggesting that ferroptosis-related genes play a significant role in the pathogenesis of ROP. We then explored potential drug targets and performed GO enrichment and KEGG pathway analyses to elucidate the functional roles of these genes. The results revealed that the primary biological processes involving these genes include the regulation of angiogenesis, vasculature development, wound healing, and positive regulation of angiogenesis and vasculature development. These findings suggest that targeting ferroptosis inhibition could offer a novel therapeutic approach for ROP [18].

KEGG pathway analysis further indicated that the ferroptosis-related DEGs are primarily involved in the PI3K-Akt signaling pathway, which plays a critical role in cell survival, growth, and metabolism. Chronic or severe cellular stress caused by abnormal metabolic and biochemical processes can lead to nonapoptotic cell death, including ferroptosis, which is directly influenced by several metabolic pathways that regulate cellular susceptibility to lipid peroxidation [18]. Additionally, GSEA identified the defense response as the most significantly enriched gene set, highlighting the importance of oxidative stress and immune responses in ROP pathogenesis.

To further explore the interactions among these genes, we constructed a PPI network using the STRING database (Figure 4A). Eight hub genes central to ferroptosis in ROP were identified: HMOX1, ALB, JUN, TLR4, TGFB1, CD44, NOX4, and LCN2 (Figure 4B). These hub genes were also differentially expressed in both data sets (GSE123945 and GSE135844; Figure 6). HMOX1 (heme oxygenase 1) is an enzyme that degrades heme into biliverdin, which is subsequently converted to bilirubin. HMOX1 is highly active in the spleen, where it helps maintain cellular homeostasis by degrading senescent red blood cells [19,20]. In the retina, HMOX1 expression increases with photoreceptor degeneration, and its role can be either protective or harmful, depending on its expression levels [21]. Targeting HMOX1-mediated ferroptosis in retinal pigment epithelium may offer a protective strategy against retinal degenerative diseases [22]. As the most abundant protein in blood plasma, ALB (serum albumin) regulates colloid osmotic pressure and serves as a carrier for fatty acids and other compounds [22]. JUN is an angiogenic factor that promotes endothelial cell migration and vascular maturation. It binds to specific enhancer motifs and plays a role in tissue-specific vascular development during neurovascular interactions [23,24]. TLR4 is a pattern recognition receptor that activates immune responses to pathogens. While it plays a protective role in host defense, its overactivation can lead to chronic inflammation. TLR4 is primarily expressed on immune cells and has dual roles in maintaining tolerance and eliminating pathogens [25-28]. TGFB1 is a multifunctional cytokine expressed in nearly all cell types. It plays a critical role in cell growth, differentiation, and immune regulation [29]. As a nonkinase transmembrane glycoprotein, CD44 acts as a receptor for hyaluronic acid and regulates intercellular interactions, cell adhesion, and migration. It is involved in T lymphocyte activation, hematopoiesis,

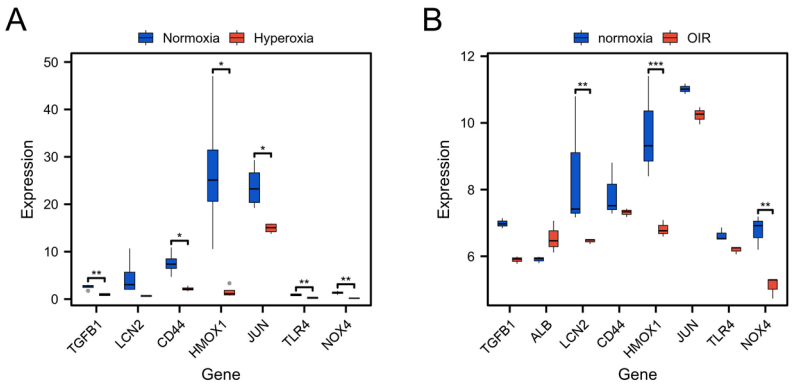

Figure 6. Expression analysis of hub genes in two datasets. **A**: The expression levels of hub genes were analyzed in the GEO dataset GSE123945. **B**: The expression levels of hub genes were analyzed in the GEO dataset GSE135844.

inflammation, and immune responses to bacterial infections, making it a key player in tissue homeostasis [29,30]. NOX4, an enzyme that generates reactive oxygen species, is widely expressed in various tissues. It regulates cell proliferation, migration, and death, and its overactivation can lead to disease development through reactive oxygen species–mediated signaling pathways [31,32]. As a member of the lipid carrier protein family, LCN2 is associated with acute and chronic inflammation and regulates iron homeostasis. It plays a key role in various diseases, including those involving oxidative stress [33-35].

By analyzing potential drug targets for these ferroptosis-related DEGs, we identified several promising candidates for ROP treatment, including stannsoporfin, recombinant neurotrophic factors, zinc chloride, and irisolidone. Stannsoporfin is a synthetic heme analogue used to treat neonatal jaundice. It works by inhibiting the enzyme heme oxygenase, which is responsible for the breakdown of heme into bilirubin. By inhibiting this enzyme, stannsoporfin reduces the production of bilirubin, thereby helping to manage hyperbilirubinemia in newborns [36]. Recombinant neurotrophic factors are proteins produced by genetic engineering that promote the growth, survival, and repair of nerve cells. They are used to protect retinal neurons, promote optic nerve repair, and treat various ophthalmic diseases. For example, brain-derived neurotrophic factor can protect retinal ganglion cells in patients with glaucoma [37]. Although neurotrophic factors have a short half-life in the eye and face delivery challenges, advancements in gene therapy, sustained-release devices, and other new technologies hold promise for providing more effective treatments for ophthalmic diseases in the future. Zinc ions exhibit antioxidant properties and can be used in the prevention or treatment of oxidative stress–related ocular diseases, such as age-related macular degeneration [38]. Irisolidone is a natural compound with diverse biological activities, including anti-inflammatory, antioxidant, neuroprotective, antitumor, and cardiovascular protective effects [39].

Interestingly, in AMD, HMOX1 and LCN2 have also been implicated in oxidative stress and ferroptosis. A study shows that LCN2 mediated ferroptosis contributes to RPE degeneration in AMD, and proposed iron chelators as potential therapies. While their study focused on AMD pathogenesis, the overlap with our identified hub genes suggests shared mechanisms across hypoxic retinopathies [34]. However, ferroptosis centric drug discovery remains underexplored in this disease. Our study bridges this gap by applying a standardized interaction scoring system (DGIdb) to prioritize high confidence drugs for ROP, a method that could be extended to other retinopathies with hypoxic components.

While the OIR model replicates key aspects of pathological angiogenesis, it differs fundamentally from human ROP. Human ROP arises from premature birth and its systemic complications, whereas mice develop their retinal vasculature postnatally without the physiological stresses of prematurity. Besides, OIR--induced neovascularization often regresses spontaneously unlike human ROP, where abnormal vessels persist and progress without intervention [6,7].

CONCLUSION: In conclusion, our study identified eight key genes associated with ROP and highlighted their potential as therapeutic targets. Through drug-gene interaction analysis, we also identified potential pharmacological agents that could expand the treatment options for ROP. However, further research is needed to validate these findings. Limitations of this study include the small sample size and the lack of experimental validation. Future studies should include larger clinical cohorts and experimental validation using techniques such as quantitative reverse transcription polymerase chain reaction and immunohistochemistry. Additionally, animal models should be employed to further investigate the therapeutic potential of the identified drugs. These efforts will provide a more comprehensive understanding of the molecular mechanisms underlying ROP and pave the way for the development of effective treatments.

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Authors' contributions: Ying Li conceived and designed the study. Qiujuan Liao and Suyu Liu collected, analyzed and interpreted the data, drafted the manuscript, while Liqing Wei and Yuli Cai contributed to its manuscript modification. All the authors approved the final version. Foundation: Supported by Hubei Province Health and Family Planning Scientific Research Project (WJ2023M083); Natural Science Fund of Hubei Province (2025AFB830). Conflicts of Interest: The authors report no conflict of interest. Dr. Ying Li (zaiwanyebuwan@whu.edu.cn) and Dr. Yuli Cai (caiyuli@whu.edu.cn) are co-corresponding authors for this paper.

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