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In Pelteobagrus fulvidraco, interleukin-22 reduces intestinal inflammation brought on by hypoxia by modifying pro- and anti-inflammatory factors.

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ABSTRACT

Although the pathophysiology of intestinal inflammation is still unknown, it is a frequent problem in intensive aquaculture. In higher vertebrates, interleukin 22 (IL-22) is known to be a crucial regulator of cellular homeostasis during inflammation; however, less is known about its functions in fish. In order to examine the role of IL-22 in preserving intestinal homeostasis, this study created hypoxia-induced models in the intestinal tissues and primary intestinal epithelial cells of yellow catfish. The gill and gut had the highest amounts of Pelteobagrus fulvidraco IL-22 (Pf_IL-22), which was found to be widely expressed in mucosal tissues. In addition to triggering hypoxia-inducible factor (HIF) signaling and significantly upregulating IL-22 production, hypoxia caused severe intestine damage that was manifested by loosening of the lamina propria and widespread vacuolization. At 24 hours after hypoxia, IL-22 levels rose, indicating a potential role in early immunological responses. Additionally, in primary intestinal epithelial cells, recombinant Pf_IL-22 stimulated transcription of pro-inflammatory mediators such as IL-1β and tumor necrosis factor α (TNF-α), suggesting a dual regulatory role in balancing inflammation and protection. HIF-1α knockdown, dual-luciferase assays, and electrophoretic mobility-shift tests all supported the mechanistic findings that HIF-1α directly interacts with a hypoxia response element in the IL-22 promoter to drive transcription. Pf_IL-22's significance in influencing downstream immune responses was demonstrated by the considerable suppression of Th17 cell development pathways caused by its silencing. These results demonstrate that the HIF-1α/IL-22 axis is a crucial regulatory route influencing immune responses and reducing intestinal inflammation, offering a foundation for the creation of immunotherapies that target IL-22 and aquaculture breeding methods that are selective.

Keywords: Teleost; HIF-1α/IL-22 axis; hypoxia; intestinal inflammation; interleukin-22

OVERVIEW

In fish raised in high-density aquaculture, intestinal inflammation is a significant pathological problem that frequently leads to physiological dysfunction and significant financial losses. High death rates result from affected persons' usual symptoms, which include lethargy, loss of appetite, abdominal distention with hemorrhagic exudates, and empty intestines (Serna-Duque & Esteban, 2020). The majority of fish illnesses appear to be caused by mucosal damage or infection, especially in the intestinal epithelium (Cao et al., 2025), which is constantly subjected to various microbial, viral, and chemical assaults (Rombout et al., 2011; Stosik et al., 2023). As a result, a vital barrier and a crucial part of fish immunity is the gut mucosa. The lamina propria (LP) beneath intestinal epithelial cells

(IECs) is rich in leukocytes, including innate immune cells like neutrophils and macrophages as well as adaptive immune cells dominated by T lymphocytes, despite the fact that teleosts lack organized intestinal-associated lymphoid structures similar to mammalian Peyer patches (Komatsu et al., 2009; Yu et al., 2020). role in allergic and chronic inflammation within the intestine (Park et al., 2005). These cells secrete a repertoire of cytokines with diverse functions, contributing to the regulation of epithelial barrier integrity and maintenance of intestinal immune homeostasis (Leonardi et al., 2022; Maloy & Kullberg, 2008). Dysregulated Th17-mediated responses are frequently associated with pathological enteritis. For example, dextran sulfate sodium salt (DSS)-induced enteritis in grouper (*Epinephelus coioides*) involves marked up-regulation of pro- inflammatory mediators that trigger intestinal mucosal inflammation (Yu et al., 2024). Similarly, soybean meal- induced enteritis in hybrid yellow catfish (*Pelteobagrus fulvidraco* ♀×*P. vachelli* ♂) is characterized by extensive infiltration of inflammatory cells and granulocytes, with significant induction of *IL-1β* expression and a concomitant reduction in *IL-10* expression (Zhang et al., 2025b). Intestinal inflammation induced by *Aeromonas hydrophila* infection in juvenile blunt-snout bream (*Megalobrama amblycephala*) exhibits symptoms comparable to those observed in carp fed soybean meal (Xia et al., 2024). Collectively, these findings underscore the critical involvement of interleukins in regulating intestinal immune dynamics and highlight their central role in mediating the balance between protective immunity and pathological inflammation in fish enteritis.

Th17 cells function as critical mediators of mucosal inflammation by secreting interleukin 22 (IL-22), a multifunctional cytokine with central roles in immune regulation and tissue protection. In mammals, IL-22 signaling activates transcriptional programs associated with anti-inflammatory, mitogenic, proliferative, and anti-apoptotic responses, thereby supporting tissue and organ growth, remodeling, and repair while strengthening epithelial defense mechanisms against pathogen invasion (Keir et al., 2020). In teleost fish, IL-22 is predominantly expressed in mucosal tissues, particularly in the intestine and gills, and its production is strongly induced by pro-inflammatory stimuli such as lipopolysaccharides (LPS) and cytokines (Huo et al., 2019; Watanabe et al., 2023). In rainbow trout (*Oncorhynchus mykiss*), IL-22-producing cells localize to gill filaments and interbranchial lymphoid tissues, where they accumulate during *Aeromonas salmonicida* infection (Hu et al., 2019). The AHR/IL-22/STAT3 signaling axis has also emerged as a pivotal regulator of intestinal mucosal immunity, with IL-22 reported to regulate the expression of antimicrobial peptides (e.g., Reg-3γ) (Zheng et al., 2008) and tight junction proteins (e.g., Claudin) (Wang et al., 2017), thereby maintaining epithelial barrier integrity and microbiota homeostasis. In mandarin fish (*Siniperca chuatsi*), intestinal IL-22 up-regulates the expression of antimicrobial peptides such as hepcidin and liver-enriched antimicrobial peptide 2 (LEAP2) (Huo et al., 2021). In So-iny mullet (*Liza haematocheila*), intraperitoneal injection of recombinant IL-22 significantly boosts β-defensin expression in the intestine, improving survival during *Streptococcus dysgalactiae* infection (Qi et al., 2015). Despite these advances, knowledge regarding IL-22-mediated immune mechanisms in fish, particularly under environmental stressors such as hypoxia, remains limited.

The intestinal epithelium normally functions under a state of physiological hypoxia, a condition essential for maintaining epithelial metabolism and regulating intestinal mucosal barrier integrity. During enteritis, however, oxygen tension within IECs decreases sharply, creating a hypoxic microenvironment accompanied by excessive production of reactive oxygen species (ROS). These ROS regulate hypoxia-inducible factor 1α (HIF-1α) through multiple signaling pathways, triggering mucosal barrier-related regulatory mechanisms (Chen et al., 2023a). HIF-1α has been shown to drive the expression of pro-inflammatory cytokines via various routes, thereby contributing to the development of intestinal inflammation (Shi et al., 2023). In rheumatoid arthritis (RA), IL-33 stimulates synovial fibroblasts to secrete HIF-1α, establishing a HIF- 1α/IL-33 feedback loop that exacerbates inflammatory responses (Hu et al., 2013). In teleosts, butyrate activates the STAT3/HIF-1α/IL-22 signaling cascade in turbot (*Scophthalmus maximus*) intestines, inducing autophagy and enhancing macrophage-mediated pathogen clearance (Zhang et al., 2023). However, the direct role of HIF-1α in regulating inflammatory factor expression to sustain intestinal homeostasis in fish remains insufficiently characterized.

Yellow catfish (*P. fulvidraco*) represents a commercially valuable freshwater species, yet intensive aquaculture practices often lead to localized hypoxia, increased pathogen exposure, and recurrent intestinal inflammation (Zhou et al., 2021). In mammals, IL-22 is recognized as a central regulator of mucosal immunity and inflammatory responses; however, its bioactivity in teleosts is not well understood. Given that the intestine serves as a primary target organ for hypoxia-induced stress responses, exploring the expression patterns and regulatory mechanisms of IL-22 in the intestine of yellow catfish under hypoxic stress is critical for the prevention and treatment of intestinal inflammation. This study examined the expression dynamics of *P. fulvidraco* IL-22 (*Pf_IL-22*) in intestinal tissues and primary IECs under hypoxic stress, evaluated the bioactivity of recombinant *Pf*_IL-22 (r*Pf*_IL-22) in modulating mRNA expression of inflammation-related genes, and characterized the interaction between HIF-1α and IL-22. The findings of this study uncover a previously unrecognized role of the HIF-1α/IL-22 axis in hypoxia-driven intestinal inflammation and provide mechanistic insights into mucosal immune

regulation and potential strategies for mitigating inflammatory disease in teleost aquaculture.

MATERIALS AND METHODS

Fish and cell lines

Healthy yellow catfish (10.2±1.4 g) were obtained from the Nanjing Institute of Fisheries Science (Jiangsu, China). Fish were temporarily cultured in a recirculating water culture system in the lab at a temperature of 25.0±1.0°C and pH of 7.3±0.1 and fed twice daily (0900h and 1700h). The fish were given a two week acclimation period before the commencement of treatment. All animal experiments were conducted in accordance with the Institutional Animal and Use Committee of Nanjing Normal University and China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Human embryonic kidney cells (HEK-293T) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sangon Biotech, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 37°C in a humidified incubator provided with 5% CO_2.

Primary IEC isolation

Fish were anesthetized with eugenol (1:10 000) (purity 99%, Shanghai Reagent Corp, China) after fasting for 24 h prior to sampling. Intestinal tissues were longitudinally excised an function in intestinal allergies and chronic inflammation (Park et al., 2005). These cells release a variety of cytokines that serve a variety of purposes, including preserving intestinal immunological homeostasis and controlling the integrity of the epithelial barrier (Leonardi et al., 2022; Maloy & Kullberg, 2008). Pathological enteritis is often linked to dysregulated Th17-mediated responses. For instance, pro-inflammatory mediators that cause intestinal mucosal inflammation are markedly up-regulated in grouper (Epinephelus coioides) enteritis caused by dextran sulfate sodium salt (DSS) (Yu et al., 2024). Similar to this, soybean meal-induced enteritis in hybrid yellow catfish (Pelteobagrus fulvidraco ♀×P. vachelli ♂) is marked by granulocyte and inflammatory cell infiltration, a marked increase in IL-1β expression, and a concurrent decrease in IL-10 expression (Zhang et al., 2025b). The symptoms of Aeromonas hydrophila-induced intestinal inflammation in juvenile blunt-snout bream (Megalobrama amblycephala) are similar to those seen in carp fed soybean meal (Xia et al., 2024). When taken as a whole, our results demonstrate the vital function that interleukins play in controlling intestinal immune dynamics and in mediating the equilibrium between pathogenic inflammation and protective immunity in fish enteritis. Th17 cells secrete interleukin 22 (IL-22), a multifunctional cytokine that plays crucial functions in tissue protection and immune control, making them important mediators of mucosal inflammation. Mammals' tissue and organ growth, remodeling, and repair are supported by IL-22 signaling, which also strengthens epithelial defense mechanisms against pathogen invasion by activating transcriptional programs linked to anti-inflammatory, mitogenic, proliferative, and anti-apoptotic responses (Keir et al., 2020). Pro-inflammatory stimuli such lipopolysaccharides (LPS) and cytokines greatly promote the production of IL-22 in teleost fish, which is mostly expressed in mucosal tissues, especially in the intestine and gills (Huo et al., 2019; Watanabe et al., 2023). During an Aeromonas salmonicida infection, IL-22-producing cells in rainbow trout (Oncorhynchus mykiss) relocate to gill filaments and interbranchial lymphoid tissues, where they concentrate (Hu et al., 2019). Intestinal mucosal immunity has also been found to be significantly regulated by the AHR/IL-22/STAT3 signaling axis. It has been reported that IL-22 controls the expression of tight junction proteins (like Claudin) and antimicrobial peptides (like Reg-3γ) (Zheng et al., 2008), preserving the integrity of the epithelium barrier and microbiota homeostasis. Intestinal IL-22 increases the expression of antimicrobial peptides such hepcidin and liver-enriched antimicrobial peptide 2 (LEAP2) in mandarin fish (Siniperca chuatsi) (Huo et al., 2021). Recombinant IL-22 administered intraperitoneally to So-iny mullet (Liza haematocheila) improves survival during Streptococcus dysgalactiae infection by dramatically increasing intestinal β-defensin expression (Qi et al., 2015). Despite these developments, little is known about IL-22-mediated immune pathways in fish, especially in the presence of environmental stresses like hypoxia.

Physiological hypoxia, which is necessary for preserving epithelial metabolism and controlling the integrity of the intestinal mucosal barrier, is the typical operating environment for the intestinal epithelium. However, oxygen tension in IECs drastically drops during enteritis, resulting in a hypoxic microenvironment and an overabundance of reactive oxygen species (ROS). These ROS stimulate regulatory mechanisms associated to the mucosal barrier by controlling hypoxia-inducible factor 1α (HIF-1α) through a variety of signaling

pathways (Chen et al., 2023a). It has been demonstrated that HIF-1α promotes the production of pro-inflammatory cytokines through a variety of pathways, which helps to cause intestinal inflammation (Shi et al., 2023). In RA, IL-33 triggers synovial fibroblasts to release HIF-1α, creating a feedback loop between HIF-1α and IL-33 that intensifies inflammatory reactions (Hu et al., 2013). Butyrate stimulates autophagy and improves macrophage-mediated pathogen clearance in teleosts by activating the STAT3/HIF-1α/IL-22 signaling cascade in the intestines of turbots (Scophthalmus maximus) (Zhang et al., 2023). Nevertheless, little is known about how HIF-1α directly controls the production of inflammatory factors to maintain intestinal homeostasis in fish. According to Zhou et al. (2021), intense aquaculture operations frequently result in localized hypoxia, increased pathogen exposure, and recurrent intestinal inflammation, despite the fact that yellow catfish (P. fulvidraco) is a commercially significant freshwater species. Although IL-22 is known to be a key modulator of inflammatory responses and mucosal immunity in mammals, little is known about its bioactivity in teleosts. Investigating the expression patterns and regulation mechanisms of IL-22 in the gut of yellow catfish under hypoxic stress is essential for the prevention and treatment of intestinal inflammation since the intestine is a main target organ for hypoxia-induced stress responses. This study assessed the bioactivity of recombinant Pf_IL-22 (rPf_IL-22) in modifying the mRNA expression of genes linked to inflammation, characterized the interaction between HIF-1α and IL-22, and investigated the expression dynamics of P. fulvidraco IL-22 (Pf_IL-22) in intestinal tissues and primary IECs under hypoxic stress. In addition to offering mechanistic insights into mucosal immune modulation and possible techniques for alleviating inflammatory illness in teleost aquaculture, the study's findings reveal a hitherto unknown involvement of the HIF-1α/IL-22 axis in hypoxia-driven intestinal inflammation.

SUPPLIES AND TECHNIQUES

Cell lines and fish

We purchased healthy yellow catfish (10.2±1.4 g) from the Nanjing Institute of Fisheries Science in Jiangsu, China. Fish were fed twice a day at 0900 and 1700 hours in a recirculating water culture system in the lab at a temperature of 25.0±1.0°C and a pH of 7.3±0.1. Before starting the treatment, the fish were allowed to acclimate for two weeks. The China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training, as well as the Institutional Animal and Use Committee of Nanjing Normal University, were followed in all animal experiments. Dulbecco's Modified Eagle Medium (DMEM, Sangon Biotech, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) was used to cultivate human embryonic kidney cells (HEK-293T) at 37°C in a humidified incubator with 5% CO2.

IEC isolation in primary

Following a 24-hour fast before sampling, fish were put to sleep with eugenol (1:10,000) (purity 99%, Shanghai Reagent Corp, China). The intestinal tissues were removed longitudinally, and

washed several times with phosphate-buffered saline (PBS) that contains streptomycin and penicillin (Beyotime, China). The tissues were cut into 1 mm3 pieces, digested in a T25 cell culture flask with 0.03% collagenase type Ⅰ and Ⅳ (Servicebio, China), and then incubated at 28°C in a shaker.

35 minutes at 100 r/min. To stop digestion, an equivalent volume of stopping solution (DMEM with 10% FBS and 1% penicillin-streptomycin) was added. To allow for sedimentation, the mixture was pipetted for three minutes and then allowed to stand for one minute. After being aspirated out of the flask, the supernatant was run through a 100 μm cell strainer (Biologix, China). The pellet was repeatedly rinsed with PBS/penicillin-streptomycin after the cell solution was centrifuged for five minutes at 1,000 rpm at 28°C. The isolated IECs were cultivated using DMEM that contained 18% FBS (Gibco, USA), 2% yellow catfish serum, 10 ng/mL epidermal growth factor (Sigma-Aldrich, USA), 1× ITS Media Supplement (Gibco, USA), 1× non-essential amino acid (Servicebio, China), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Beyotime, China). The cells were seeded into collagen I-coated 12-well plates (Sigma-Aldrich, USA) at a density of 1.0×105 cells/mL. The cultures were kept at 28°C in a humidified

environment with 5% CO2. Identification of the IL-22 sequence and phylogenetic analysis Gene-specific primers (Supplementary Table S1) created from transcriptome data were used to determine the open reading frame (ORF) of the IL-22 gene in yellow catfish (Zhang et al., 2025a). The 2× Phanta Max Master Mix (Vazyme, China) was used for polymerase chain reaction (PCR) amplification, and the resultant products were cloned into a pMD-19T Vector (Takara, Japan). For sequencing, recombinant bacterial cultures were sent to TSINGKE Biotech Co., Ltd. (China). DNAMAN v.8.0 was used to construct and analyze the IL-22 nucleotide sequence. Using the Simple Molecular Architecture Research Tool (SMART v.7.0) (http://smart.embl-heidelberg.de/), the functional domain architecture of P. fulvidraco IL-22 (Pf_IL-22) was predicted based on the deduced amino acid sequence. ExPASy (https://web.expasy.org/compute_pi/) was used to determine the theoretical isoelectric point and molecular weight, and the ExPASy translate server (https://web.expasy.org/translate/) was used to translate amino acids. IL-22 amino acid sequences were aligned many times using ClustalW in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The SignalP v5.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptides. In order to acquire bootstrap values, a phylogenetic tree was built using the neighbor-joining method with 10,000 iterations.

Gene expression changes in vivo and in vitro when exposed to acute hypoxia

To create three parallel experimental units, 90 yellow catfish were divided equally among three aquariums with aquaculture water-recycling biofilters, with 30 fish each tank. Supplementary Figure S1 shows the complete experimental setup and sampling procedure. Three fish were randomly chosen from each aquarium to collect baseline samples before to the experiment, and three fish were randomly selected from each of the three tanks at each subsequent time point. These fish were dissected, and their intestinal tissues were evenly combined to create a single composite sample for each tank. Three combined samples in all were made and assigned to the hypoxic 0-hour (0 h) group. To elicit consequently, the water intake and aeration system were turned off, and a clear film was placed over the water's surface. In order to lower dissolved oxygen (DO) levels to 0.85±0.05 mg/L—a concentration established by earlier research—nitrogen gas was added (Zhang et al., 2025a). The oxygen pump was adjusted, and nitrogen gas was added as necessary, to keep DO levels at the desired concentration, which was checked every 30 minutes. Using the same sampling and pooling techniques as the 0 h group, fish were sampled at 3, 6, 12, 24, 48, and 72 hours after treatment. While the remaining tissues were preserved in 4% paraformaldehyde (PFA) for histological examinations, all intestinal samples were promptly snap-frozen in liquid nitrogen and kept at -80°C for RNA extraction. Nine healthy fish were employed for the Pf_IL-22 tissue distribution profile, with three individuals combined into each composite sample. The liver, kidney, spleen, brain, heart, hindgut, gills, muscle, blood, and skin were among the ten tissues and organs that were gathered.

Following previously outlined procedures, primary IECs were separated and planted at a density of 1 mL per well into 12-well plates. Before treatment, the cells were cultured for 48 hours. Dimethyl sulfoxide (DMSO) (Solarbio, China) was used as the control in the HIF-1α overexpression test, and 1 mmol/L dimethyloxallyl glycine (DMOG) (Aladdin, China) was added to the culture medium. After 12 hours of treatment, cells were taken out to evaluate the effectiveness of gene overexpression.

Histological analysis

Intestinal tissues were dehydrated using a graded methanol series (30% to 100%) after being fixed for 24 hours. They were then washed with ethanol and xylene, embedded in paraffin, and cut into 5-μm slices. The sections were examined and photographed using a Nikon Ti-E-A1R light microscope (Tokyo, Japan) following staining with hematoxylin and eosin (H&E). TRIzol reagent (Invitrogen, USA) was used to extract total RNA from intestinal tissues in accordance with the manufacturer's instructions for the reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR). Agarose gel electrophoresis was used to assess RNA quality, and a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) was used to measure RNA concentration. As directed by the manufacturer, Hifair® III 1st Strand cDNA

Synthesis SuperMix for qPCR (gDNA digester plus) (Yeasen, China) was used to create first-strand cDNA from 500 ng of total RNA each sample.

The internal reference for normalizing gene expression was the yellow catfish β-actin gene. Supplementary Table S1 contains a list of primer sequences used for RT-qPCR. Ten microliters of Hieff® qPCR SYBR Green Master Mix (No Rox) (Yeasen, China), two microliters of cDNA (10-fold dilution of template), 0.4 microliters of each forward and reverse primer (10 μmol/L), and 7.2 microliters of distilled water were used for the triplicate RT-qPCR. Using the following software, amplification was performed on a LightCycler 480 device (Roche Diagnostics, Switzerland): 5 minutes at 95°C, 40 cycles of amplification at 95°C for 10 seconds and 60°C for 30 seconds, and melting curve analysis at 95°C for 15 seconds to verify specificity. Using the 2-△△Ct technique, relative gene expression levels were determined.

Pf_IL-22 recombinant expression (rPf_IL-22) and the production of polyclonal antibodies

The Pf_IL-22 gene fragment that did not include the anticipated signal peptide was cloned into the pET-32a vector for bacterial

expression. PEG-20000 (Solarbio, China) was used to concentrate the refolded recombinant Pf_IL-22 protein, which was then kept at -80°C. The Bradford protein assay (Jiancheng Bioengineering, China) was used to measure the amount of protein present. Rabbits were immunized with rPf_IL-22 three times at intervals of 14 days to produce rabbit polyclonal antibodies against rPf_IL-22. Seven days following the last boost, serum samples were taken from the immunized animals, and the enzyme-linked immunosorbent assay (ELISA) was used to measure the antibody titers. After being collected, the serum was filtered and kept for further use.

Western blot examination

A commercial extraction kit (Jiangsu KeyGEN BioTECH, China) was used to extract the total protein from intestinal tissues. Protein samples underwent separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after being combined with 5× loading buffer (Beyotime, China) and heated for 10 minutes. Following resolution, the proteins were placed onto membranes made of polyvinylidene fluoride (PVDF) (Millipore, USA). Blocking buffer (10% (w/v) skim milk in TBST buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% Tween 20, and pH) was then used to block the membranes.

7.6)) at room temperature for two hours, and then three TBST washes (10 minutes each). Primary antibodies, such as rabbit anti-rPf_IL-22 (1:5 000) and mouse β-actin (1:5 000; TransGen Biotech, China), were incubated on membranes for 12 hours at 4°C. Membranes were incubated with goat anti-rabbit IgG (1:5 000; TransGen Biotech, China) or goat anti-mouse IgG (1:5 000; TransGen Biotech, China) secondary antibodies for two hours at room temperature following three more TBST washes (10 minutes each). A high-sensitivity chemiluminescence substrate kit (Tanon, China) was used to observe protein bands after three final TBST washes (10 min each).

rPf_IL-22's alteration of gene expression in IECs Prior to treatment, primary IECs were cultured for 48 hours after being seeded into 12-well plates at a density of 1 mL per well. After that, cells were treated for 12 hours to either rPf_IL-22 at a concentration of 5 ng/mL or EB-1 buffer (control) at a concentration of 20 ng/mL. Total RNA was extracted as previously described after cells were removed after treatment. The expression levels of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-10, IL-21, IL-34, IL-17A/F1, TGF-β1, STAT3, and TNF-α, that may be sensitive to IL-22 were evaluated using RT-qPCR. Supplementary Table S1 contains a list of the primers used for RT-qPCR.

Dual-luciferase reporter assays and transfections

To create a pGL3-HRE reporter construct, a 2 600 bp segment upstream of the ATG start codon of Pf_IL-22—which is thought to be a crucial area of the Pf_IL-22 promoter—was amplified and cloned in the pGL3 basic vector. To facilitate HIF-1α overexpression, a pcDNA-HIF-1α transcription factor plasmid was created. A QuickMutationTM Site-Directed Mutagenesis Kit (Beyotime, China) was used to create a mutant reporter plasmid (pGL3-HRE-Mut), which damaged the expected HIF-1α-binding site. HEK293T cells were seeded into 24-well plates at a density of 1.5×105 cells/well prior to transient transfection, and they were grown for 24 hours until they reached 70%–80% convergence. Following the manufacturer's instructions, plasmids were transiently transfected into HEK293T cells using LipofectamineTM 3000 (Invitrogen, USA). Reporter plasmids were co-transfected in equimolar quantities utilizing Opti-MEM (Invitrogen, USA) as the transfection medium and 20 ng of pRL-Thymidine Kinase (pRL-TK) plasmid as an internal control. Cells were stimulated with or without 1 mM DMOG for 30 minutes at 48 hours after transfection. They were then harvested and put through dual-luciferase tests using the Dual-Luciferase Reporter Assay System (Promega, USA). The ratio of fireflies to Renilla luciferase was used to calculate relative luciferase activity. The control reporter, pGL3-Basic, was used to standardize the results. Every experiment was conducted in duplicate and measured at least three times.

The binding of HIF-1α to the anticipated hypoxia response elements (HREs) inside the yellow catfish IL-22 promoter was assessed using the electrophoretic mobility-shift assay (EMSA). In summary, a Chemiluminescent EMSA Kit (Beyotime, China) was used to create biotin-labeled and unlabeled double-stranded DNA probes with the predicted HRE as well as biotin-labeled probes with altered HIF-1α-

binding sites. A Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN, China) was used to recover nuclear proteins from the intestinal tissues of yellow catfish. For 20 minutes, reaction mixtures comprising 1× binding buffer, 100 nmol/L biotin-labeled probes, and 10 μg of nuclear protein were incubated at room temperature. Before adding labeled probes to the nuclear extracts for competitive binding experiments, a 100-fold molar excess of unlabeled or mutant probe was added. Protein-DNA complexes were electrophoresed at 100 V for 90 minutes on 4% polyacrylamide gels in 0.5× TBE, and then they were transferred to nylon membranes. The chemiluminescent EMSA kit was used to identify DNA that had been biotin-labeled. Supplementary Table S1 contains a list of all the oligonucleotide sequences utilized for EMSA.

Using small interfering RNA (siRNA) and previously outlined procedures, RNA interference (RNAi) in vivo Pf_HIF-1α knockdown was carried out (Zhang et al., 2024). Using the GenScript online tool (https://www.genscript. com/tools/sirna-target-finder), four potential siRNAs that target Pf_HIF-1α were created. Plasmids pPf_HIF-1αsi-1, pPf_HIF-1αsi-2, pPf_HIF-1αsi-3, and pPf_HIF-1αsi-4 were created by inserting the relevant sequences into the siRNA expression vector pRNAT-CMV3.1 (GenScript, USA) after they were amplified by PCR. To measure interference efficiency, groups of five yellow catfish (n=5; 9.50±1.44 g) were intramuscularly injected with either the siRNA plasmid (20 μg/fish) or PBS (control). As previously mentioned, RT-qPCR was used to determine the intestinal expression level of Pf_HIF-1α 48 hours after injection. The plasmid that had the highest knockdown efficiency was chosen and given the name pPf_HIF-1αsi. Supplementary Table S1 lists the primer sequences that were employed.

Yellow catfish were randomly assigned to one of two groups (n=20) for Pf_HIF-1α knockdown, and pPf_HIF-1αsi or pPf_HIF-1αsiC (control) were injected intramuscularly. As explained in Section 2.4, fish were exposed to acute hypoxia 48 hours after injection. The interfering plasmid was given again every 48 hours for the duration of the 96-hour hypoxia exposure. Following 48 hours of acute hypoxia, intestinal IL-22 expression levels were measured using RT-qPCR. The midgut tissues of both wild-type and IL-22 knockdown fish were collected, prepared for H&E staining, and histologically analyzed under normoxia and following 96 hours of hypoxia. To assess structural changes, the height of the intestinal villus was measured.

Analysis of IL-22 knockdown transcriptome under hypoxic stress

The identical technique used to create pPf_IL-22si was also used to create the pPf_IL-22si interference plasmid. Yellow catfish were randomly allocated to two groups (n=20) for Pf_IL-22 knockdown, and they received intramuscular injections of either pPf_IL-22si or pPf_IL-22siC (control). Intestinal tissues were obtained for transcriptome analysis after 48 hours of normal culture and another 48 hours of acute hypoxia exposure (three pooled samples per group, each comprising of tissues from two fish). Following the extraction of total RNA, Novogene Co., Ltd. (China) created and sequenced six cDNA libraries using the Illumina NovaSeq 6000 platform (USA). The methods outlined in our earlier study (Zhang et al., 2025a) were followed for quality control and data processing.

Analysis of statistics

SPSS v.26.0 was used for all statistical analyses. ImageJ software was used to process confocal pictures and quantify the intensity of the Western blot bands. The mean±standard deviation (SD) of three separate experiments, each with three technical replicates, is used to display the data. One-way analysis of variance (ANOVA) and the least significant difference (LSD) post-hoc test were used to assess group differences. At $P<0.05$ and $P<0.01$, respectively, significance and strong significance were deemed acceptable.

OUTCOMES

Pf_IL-22 cDNA and protein sequence characterization

Pf_IL-22's ORF covered 546 bp and encoded an 181 amino acid peptide with an isoelectric point of 5.15 and a projected molecular weight of 44 966.97 Da (Figure 1A). A 26-amino acid potential signal peptide

was discovered. The Pf_IL-22 protein had two N-glycosylation sites and an IL-22 domain (29–175 amino acids (aa)), according to structural analysis (Figure 1B). Four highly conserved cysteine residues creating two intramolecular disulfide bonds—a trait common to teleosts—were discovered by multiple sequence alignment (Figure 1C). Strong conservation of the genomic architecture surrounding IL-22 was shown by synteny analysis, which also revealed substantial similarities with the zebrafish genome, including the inclusion of downstream IFN-γ rel genes particular to fish (Figure 1D). Notably, rap1b and mdm1 genes are always found upstream of Pf_IL-22, while IL-26 and IFN-γ are found downstream, demonstrating that this conserved syntenic arrangement is present in humans and other species. Pf_IL-22 clustered closely with its orthologs from P. vachelli and Ictalurus punctatus, according to phylogenetic reconstruction using the neighbor-joining method. This formed a separate clade that is sister to the lineage that contains the orthologs of Ctenopharyngodon idella and Danio rerio IL-22 (Figure 1E). The evolutionary relatedness of these teleost species is demonstrated by their phylogenetic topology, which also shows how highly conserved the IL-22 gene sequences are among vertebrates.

rPf_IL-22 production and purification

The yellow catfish genome database was used as the basis for bioinformatic analysis that confirmed the nucleotide sequence of the Pf_IL-22 ORF. The pET32a-IL-22 prokaryotic expression plasmid was then created to enable Escherichia coli to express recombinant rPf_IL-22. SDS: According to PAGE and western blot analysis, rPf_IL-22 has a molecular weight of about 35.5 kDa (Supplementary Figure S2), which is in line with what sequence structure analysis predicted.

Building a model of intestinal hypoxia in yellow catfish

To assess the physiological and molecular reactions to acute oxygen deprivation, a yellow catfish intestinal hypoxia model was developed. When compared to the control group, histopathological analysis showed that 72 hours of acute hypoxia exposure caused notable structural changes in midgut tissues. Significant single-layer columnar cell disarray, lamina propria loosening, increased vacuolation, decreased goblet cell density, and widespread villus erosion were all observed in the intestinal epithelium (Supplementary Figure S3A). Under hypoxic stress, the HIF system was robustly activated, according to RT-qPCR study of hypoxia-responsive genes. HIF-1α expression rose dramatically and peaked at 24 hours before progressively decreasing ($P<0.05$) (Supplementary Figure S3B). On the other hand, FIH expression rose at first but thereafter decreased (Supplementary Figure S3C). Together with its function as an HIF-1α inhibitor, the decrease in FIH at 24 hours suggests increased HIF-1α activity during this period. At the same time, in hypoxic conditions, VHL continued to be strongly expressed ($P<0.05$) (Supplementary Figure S3D). In the early phases of hypoxia (0–12 h), HIF-2α expression varied little (Supplementary Figure S3E). However, it rose dramatically at 24 h and peaked at 48 h ($P<0.05$). All of these results point to the intestinal HIF signaling cascade in yellow catfish being substantially activated by acute hypoxia stress, which triggers inflammatory reactions and transcriptional reprogramming.

Pf_IL-22 and cytokine gene expression dynamics under hypoxic stress Pf_IL-22 transcript distribution was measured in ten different yellow catfish tissues. All investigated tissues showed Pf_IL-22 mRNA expression, according to RT-qPCR analysis (Supplementary Figure S4), but the gill, hindgut, and skin—mucosal regions linked to immune defense—had noticeably greater levels, indicating a potentially important role for Pf_IL-22 in mucosal immunity. On the other hand, the liver, heart, blood, kidney, and head showed comparatively low transcript abundance.

After 72 hours of acute hypoxia, the temporal expression profiles of Pf_IL-22 and associated cytokines were measured in intestinal tissues to examine responses to hypoxic stress (Figure 2). The levels of Pf_IL-22 increased somewhat during the first six hours of hypoxia, then sharply at 24 hours ($P<0.05$), reaching around 17 times higher than control levels, before gradually falling to baseline by 72 hours ($P<0.05$). IL-22RA, IL-22BP, IL-10, IL-17A/F1, TGF-β1, STAT3, and AHR all showed similar expression patterns, indicating coordinated transcriptional regulation among these cytokines. On the other hand, six hours after hypoxia, IL-1β showed persistently elevated expression ($P<0.05$).

In light of these transcriptional patterns, HIF-1α expression was examined in further detail to evaluate possible regulatory relationships with IL-22. Both HIF-1α and IL-22 transcripts were markedly up-regulated in acute hypoxia. A link between the protein-level expression of IL-22 and HIF-1α was

confirmed by subsequent analysis (Figure 3). Intestinal HIF-1α expression first rose in hypoxia, according to Western blotting.

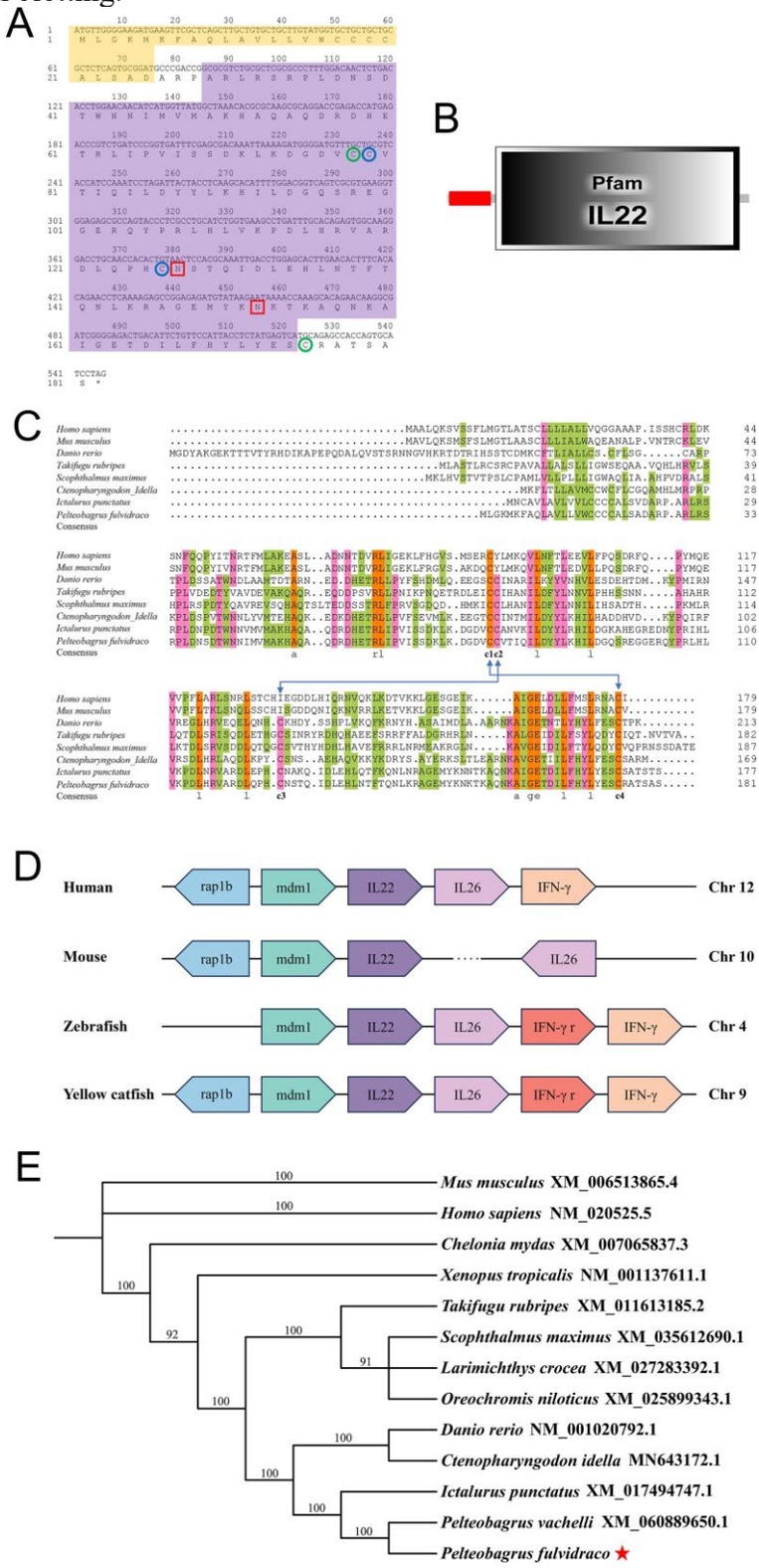

Figure 1 Characteristics of *Pf*_IL-22

A: ORF and deduced amino acid sequence of *Pf*_IL-22. Yellow box represents putative transmembrane region; purple region denotes IL-10 family domain; green and blue circles indicate cysteine residues; red square highlights N-glycosylation site. B: Protein structure of *Pf*_IL-22. Red box

indicates transmembrane region. C: Multiple sequence alignment of teleost IL-22 amino acid sequences. Orange background indicates identical amino acid residues; green background indicates conserved amino acid residues; dot represents alignment gaps; C-C denotes disulfide bond. D: Synteny analysis of IL-22 across four species. Same color boxes represent orthologous genes between species; arrowhead represents direction of genes. E: Bayesian phylogenetic tree of IL-22 constructed using sequences from *P. fulvidraco* and other vertebrates, with node values representing bootstrap support; MCMC=200 000 generations.

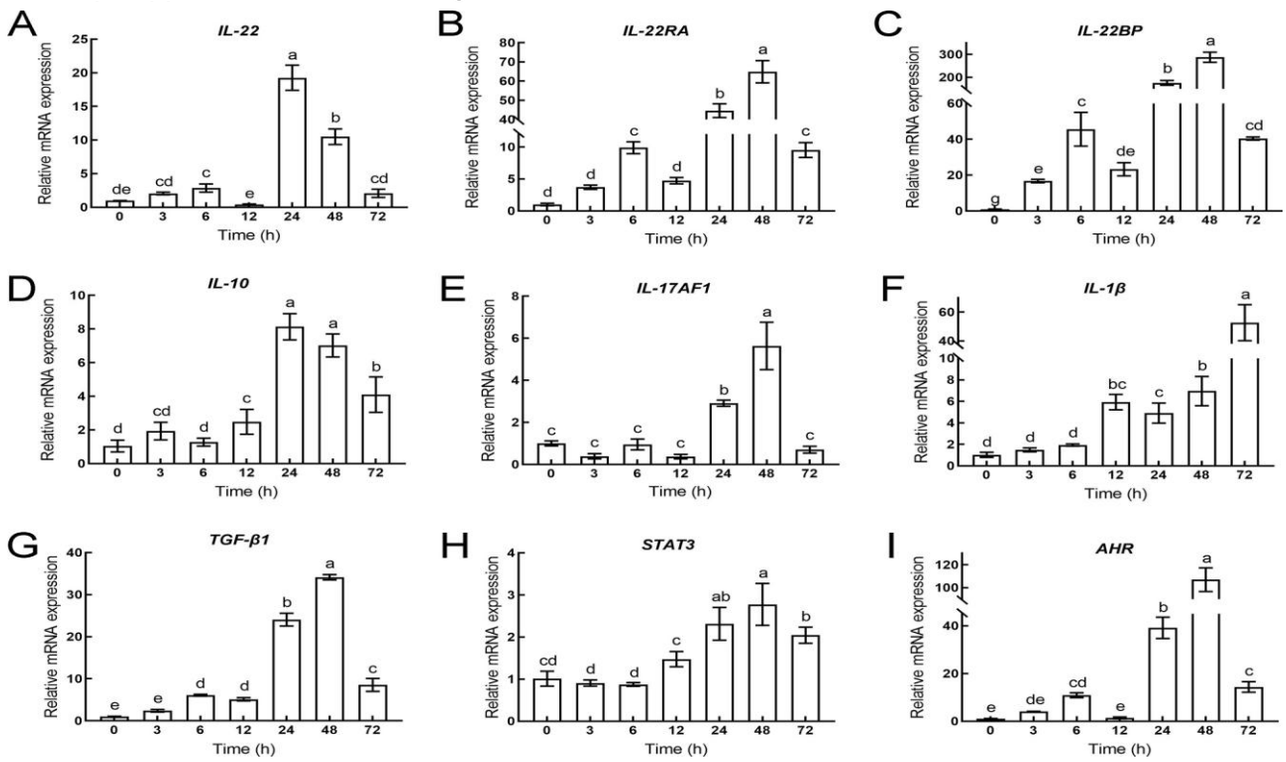

Figure 2 Temporal mRNA expression patterns of *Pf_IL-22* and related cytokines in the intestine of *P. fulvidraco* under hypoxic stress

Results are presented as mean±SD (n=3). Different lowercase letters indicate statistically significant differences (P<0.05).

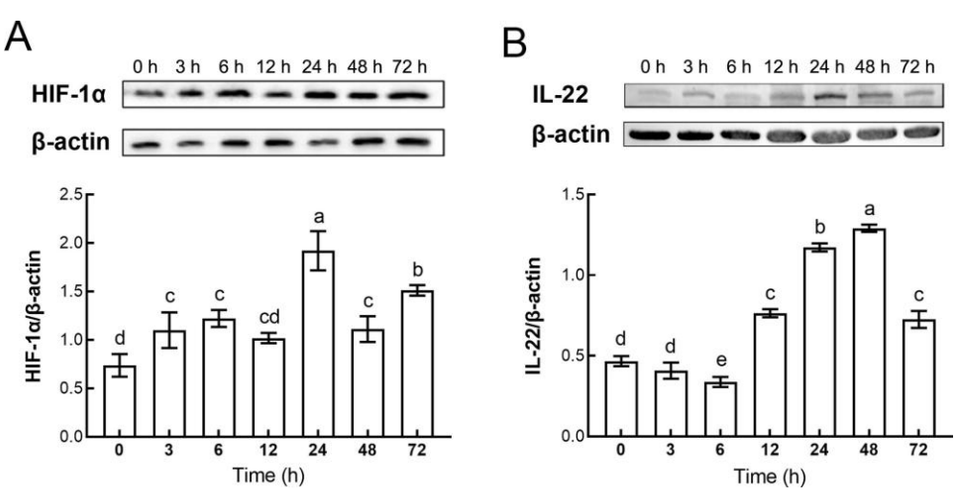

Figure 3 Western blot analysis of HIF-1α and IL-22 under hypoxic stress

Data are presented as mean±SD (n=3). Different lowercase letters indicate statistically significant differences (P<0.05).

peaking at 24 h (P<0.05) and declining thereafter. IL-22 protein expression exhibited a parallel temporal profile, aligning closely with HIF-1α dynamics across all measured time points. These concordant expression patterns suggest a potential mechanistic link between HIF-1α activation and IL-22 induction during hypoxic adaptation in yellow catfish.

Expression patterns of cytokine genes in IECs induced by r*Pf*_IL-22

The immunomodulatory effects of r*Pf*_IL-22 on inflammatoryresponses were evaluated in primary IECs derived from yellow catfish. As shown in Figure 4, exposure to 5 ng/mL r*Pf*_IL-22 inhibited the expression of most inflammatory cytokines, except *IL-34*, which exhibited a marked up-regulation. At 20 ng/mL, r*Pf*_IL-22 strongly induced *IL-1β*, *IL-6*, *IL-8*, *IL-17A/F1*, *IL-34*, and *TNF-α* expression, while significantly reducing *IL- 10*, *IL-21*, *TGF-β1*, and *STAT3* levels, indicating a concentration-dependent bidirectional regulatory effect on cytokine production.

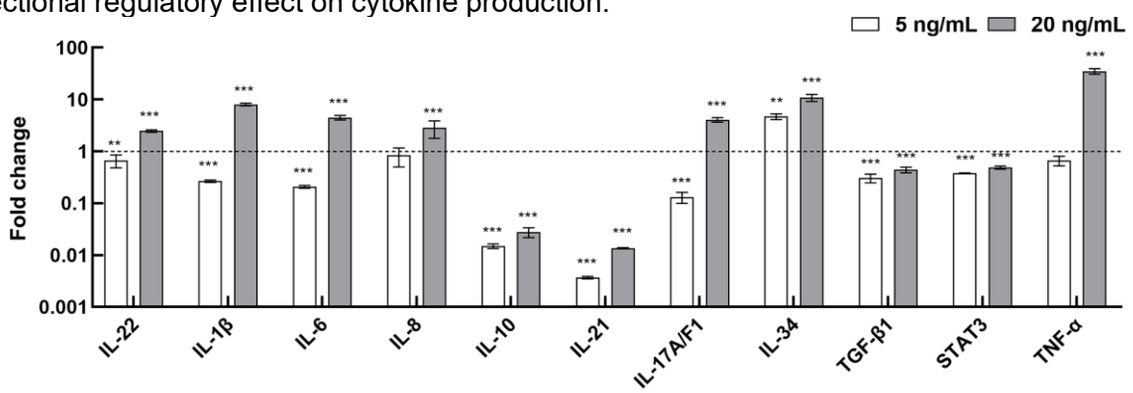

Figure 4 Effects of bacteria-derived r*Pf*_IL-22 on gene expression in primary intestinal epithelial cells (IECs)

Primary IECs were stimulated with r*Pf*_IL-22 for 12 h, and mRNA expression of cytokines was quantified by RT-qPCR. Data are presented as mean±SD (n=3). "*" indicates statistically significant differences (**: P<0.01; ***: P<0.001).

Expression analysis of *Pf*_IL-22 and cytokine genes in IECs following DMOG treatment

To investigate the regulation of IL-22 under hypoxia-mimicking conditions, IECs were treated with 1 mM DMOG, a HIF prolyl hydroxylase inhibitor and potent activator of HIF signaling, for 12 h (Figure 5A). Compared with DMSO-treated controls, DMOG significantly enhanced HIF-1α expression (P<0.05) (Figure 5B). This activation was accompanied by a two-fold increase in IL-22 transcript levels, suggesting a regulatory link between HIF-1α and IL-22. Furthermore, DMOG treatment markedly up-regulated *IL-22BP*, *IL-22RA1*, *IL-10*, *IL-1β*, *TGF- β1*, and *STAT3* expression in IECs (P<0.05) (Figure 5C). In contrast, *IL-17A/F1* and *AHR* expression showed a non- significant increase (P>0.05). These findings are consistent with *in vivo* observations, demonstrating that HIF-1α activation promotes IL-22 up-regulation and modulates downstream cytokine networks.

HIF-1α triggers transcriptional activation of *Pf*_IL-22 under hypoxic stress

To further investigate the regulatory role of HIF-1α on IL-22 gene expression, a 2 600 bp promoter sequence upstream of the IL-22 gene was initially cloned and analyzed. A putative HRE was identified between −1 940 to −1 947 bp (Figure 5D), suggesting a potential role for HIF-1α in modulating *Pf_IL-22* expression through direct HRE binding. Functional validation was performed using a dual-luciferase reporter system combined with site-directed mutagenesis. Three constructs were generated, including pGL3-HRE, pGL3-HRE-Mut, and pcDNA-HIF-1α expression vector. As shown in Figure 5E, co- transfection of pGL3-HRE with pcDNA-HIF-1α into HEK293T cells induced a significant increase in luciferase activity compared with the control group (pGL3-HRE+pcDNA-3.1). Treatment with DMOG further amplified this induction (P<0.05), indicating that DMOG enhances HIF-1α binding and transcriptional activation of the IL-22 promoter. In contrast to the pGL3-HRE+pcDNA-HIF-1α group, pGL3-HRE-

Mut+pcDNA-HIF-1α showed a significant reduction in luciferase activity (P<0.05), comparable to that observed in the pGL3-HRE-Mut+pcDNA-3.1 group (P>0.05). Following DMOG stimulation, luciferase activity in the pGL3-HRE- Mut+pcDNA-HIF-1α group was unchanged, showing no significant difference from baseline (P>0.05). These findings suggest that HIF-1α regulates IL-22 transcription through direct interaction with the HRE in its promoter region.

To confirm HIF-1α binding specificity, an electrophoretic mobility shift assay (EMSA) was performed using biotin-labeled probes corresponding to the predicted HRE within the IL-22 promoter (Figure 5F). Nucleoproteins extracted from yellow catfish intestinal tissue formed distinct DNA-protein complexes with wild-type probes, demonstrating direct interaction between HIF-1α and the intact HRE. This binding was competitively disrupted by excess unlabeled wild-type probes but remained unaffected by unlabeled mutant probes, confirming sequence specificity. Furthermore, acute hypoxia exposure enhanced the formation of HIF-1α–HRE complexes, supporting a hypoxia-responsive mechanism of transcriptional regulation.

To further validate the role of HIF-1α in regulating *Pf*_IL-22 expression *in vivo*, HIF-1α knockdown was performed in *P. fulvidraco* using the p*Pf*_HIF-1αsi-interference construct. RT-qPCR confirmed significant suppression of HIF-1α expression in intestinal tissue following treatment (Figure 6A). At 48 h post-administration, p*Pf*_HIF-1αsi-treated fish exhibited significantly reduced *Pf*_IL-22 expression compared with control fish (Figure 6B), establishing HIF-1α as a critical driver of hypoxia-induced P*f*_IL-22 transcription.

Effects of *Pf_IL-22* knockdown on intestinal immune responses under hypoxic stress

To evaluate the functional role of *Pf*_IL-22 in regulating intestinal immune responses of *P. fulvidraco*, *Pf_IL-22* RNAi and transcriptomic analysis were carried out. The silencing efficiency of *Pf*_IL-22 was confirmed (Supplementary Figure S5). Principal component analysis (PCA) revealed a distinct separation between the interference and control groups (Figure 7A), with the first principal component (PC1) accounting for 72.5% of the total variance. This separation verified the successful induction of hypoxic stress and demonstrated a distinct transcriptional response upon *Pf_IL- 22* knockdown. Differential expression analysis identified 214 up-regulated genes and 297 down-regulated genes in the interference group compared with controls (Figure 7B). KEGG pathway enrichment analysis of these differentially expressed genes (DEGs) identified significant enrichment of immune- related pathways such as microRNAs in cancer, cytokine- cytokine receptor interaction, and Th17 cell differentiation, and cell cycle-related pathways such as cell cycle and DNA replication (Figure 7C). Immunogenetic interaction network mapping yielded similar results (Figure 7D), with the DEGs primarily involved in pathways such as cytokine-cytokine receptor interaction, Th17 cell differentiation, antigen processing and presentation, viral protein interaction with cytokines and cytokine receptors, and microRNAs in cancer.

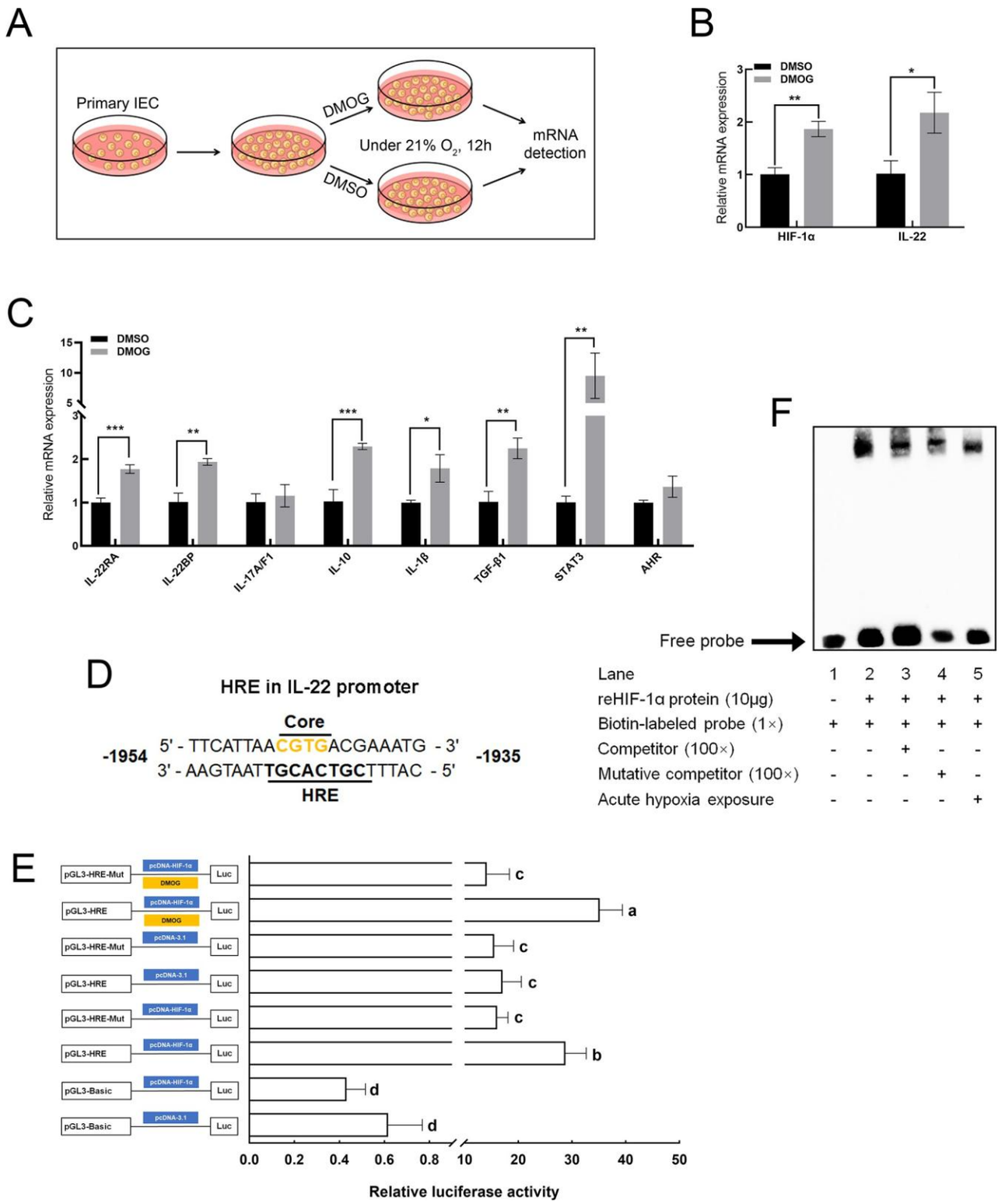

Figure 5 HIF-1α induces IL-22 production by binding to the hypoxia response element (HRE) in the IL-22 promoter region

A: Primary intestinal epithelial cells (IECs) were stimulated with DMOG or DMSO (control) for 12 h under normoxic conditions. B: Relative mRNA levels of HIF-1α and IL-22 in primary IECs. C: mRNA expression patterns of representative pro- and anti-inflammatory genes in IECs. RT-qPCR data are presented as mean±SD (*n*=3). "*" indicates statistically significant differences (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$). D: HRE binding sequence located between −1 940 bp and −1 947 bp of the *Pf*_IL-22 promoter region in yellow catfish. E: Site-directed mutagenesis analysis of HIF-1α binding sites in pGl3-*Pf*_IL-22 vectors. F: Electrophoretic mobility-shift assay (EMSA) of putative HRE-binding sequences.

5′-biotin-labeled double-stranded oligomers were incubated with nuclear proteins. For competition and mutant competition assays, a 100-fold excess of competitor or mutant competitor oligomers was added. Dual-luciferase reporter assay data are presented as mean±SD (*n*=6). Different lowercase letters indicate statistically significant differences (*P*<0.05).

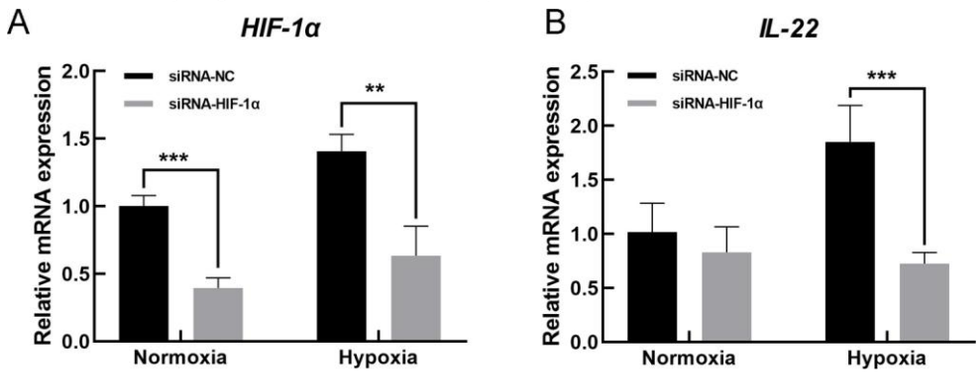

Figure 6 Effect of HIF-1α knockdown on hypoxic stress

Yellow catfish were pre-administered with p*Pf*_HIF-1αsi or p*Pf*_HIF-1αsiC (control) for 48 h, followed by 24 h exposure to hypoxia. Relative mRNA levels of *HIF-1α* and *IL-22* under normoxic and hypoxic conditions were detected in the intestine. Data are presented as mean±SD (*n*=3). "*" indicates statistically significant differences (**: *P*<0.01; ***: *P*<0.001).

Validation of selected genes confirmed a significant reduction in *Pf_IL-22* expression in the interference group, verifying the efficiency of RNAi (Figure 7E). HIF-1α expression remained unchanged, supporting earlier results that IL-22 does not influence HIF-1α regulation through negative feedback mechanisms. Similarly, STAT3 expression also showed no significant difference, contrasting with previous *in vivo* and *in vitro* findings. Further analysis of key genes in the Th17 cell differentiation pathway demonstrated significant down-regulation of nearly all genes except STAT3 (Figure 7F). Histopathological assessment revealed that IL-22 knockdown significantly reduced hypoxia-induced intestinal inflammation damage (Supplementary Figure S6), as evidenced by improved villus morphology and significantly increased villus length relative to wild-type individuals.

To validate the reliability of the transcriptomic data, RT-qPCR was performed on 12 DEGs associated with the Th17 pathway. The expression patterns obtained were highly consistent with transcriptomic trends (Supplementary Figure S7), demonstrating the accuracy and reliability of the sequencing results.

DISCUSSION

IL-22 plays a pivotal role in preserving intestinal immune homeostasis by strengthening epithelial barrier integrity and coordinating host defenses against pathogenic invasions (Wolk et al., 2004). Understanding the molecular regulation and functional roles of IL-22 in teleosts is imperative for advancing strategies to mitigate intestinal inflammation and immune dysfunction under environmental and physiological stress. Although IL-22 has been recognized as an important mediator in fish mucosal immunity, the regulatory mechanisms governing its expression under hypoxic stress remain poorly defined. This study comprehensively characterized IL-22 in yellow catfish based on its structural features, evolutionary relationships, tissue distribution, and expression profiles, and further assessed its regulatory relationship with HIF-1α in modulating intestinal inflammation. These findings expand the understanding of IL-22-mediated immune regulation in teleosts and highlight the HIF-1α/IL-22 axis as a potential modulator of intestinal inflammatory responses under hypoxia.

*Pf*_IL-22 exhibits conserved structural and functional features

The identified *Pf_IL-22* ORF, encoding a 181 aa protein with a predicted molecular weight of 20.7 kDa, exceeds the size reported for IL-22 homologs in blunt-snout bream (*M. amblycephala*) (168 aa) and grass carp (*C. idella*) (169 aa) (Wang et al., 2024; Yang et al., 2020b). Structural analysis revealed a predominantly hydrophilic profile, which may enhance solubility and facilitate interactions with other intracellular components (Bär et al., 2013). Four conserved cysteine residues were identified, with Cys78, Cys126, and Cys175 broadly conserved among vertebrates, while Cys79 represents a teleost-specific feature potentially linked to functional divergence. Comparative sequence analysis demonstrated high amino acid identity between *Pf*_IL-22 and other fish IL-22 orthologs, suggesting strong evolutionary conservation across teleosts.

Synteny mapping further revealed strong genomic conservation in the region surrounding *Pf_IL-22*, likely driven by evolutionary selective pressures (Marchi et al., 2021).

Notably, *IFN-γ rel*, located downstream of *IL-22*, represents a unique interferon class found exclusively in teleosts with critical roles in antiviral defense and immune regulation (Ruan et al., 2017). The conserved genomic arrangement of IL-22, IL-26, and adjacent interferons suggests potential functional coordination in immune signaling. Supporting this, IL-22 has been reported to mitigate IFN-γ-mediated pulmonary inflammation (Pennino et al., 2013), implying a broader regulatory interplay among these cytokines. These findings indicate that IL-22 may act in concert with interferons to modulate immune responses in yellow catfish, highlighting a potentially important but unexplored regulatory network. Phylogenetic analysis revealed that *Pf_IL-22* shares strong sequence identity and evolutionary conservation with IL-22 orthologs from other teleosts, confirming accurate gene identification and supporting the likelihood of conserved functional roles across species.

Tissue distribution analysis revealed that *Pf*_IL-22 expression was highest in the gills, followed by the midgut, skin, and other mucosal tissues, suggesting an important role in mucosal immune defense. In teleosts, mucosal immune tissues enriched with B cells, T cells, and immunoglobulins are essential for pathogen clearance and maintenance of mucosal homeostasis (Yu et al., 2020). Previous studies on yellow catfish have also reported high IL-22 expression in the fin and other mucosal tissues, including the gills and midgut, consistent with these findings (Jiang et al., 2018) Given this distribution pattern, our study focused on the immunological functions of *Pf*_IL-22 within the intestinal environment.

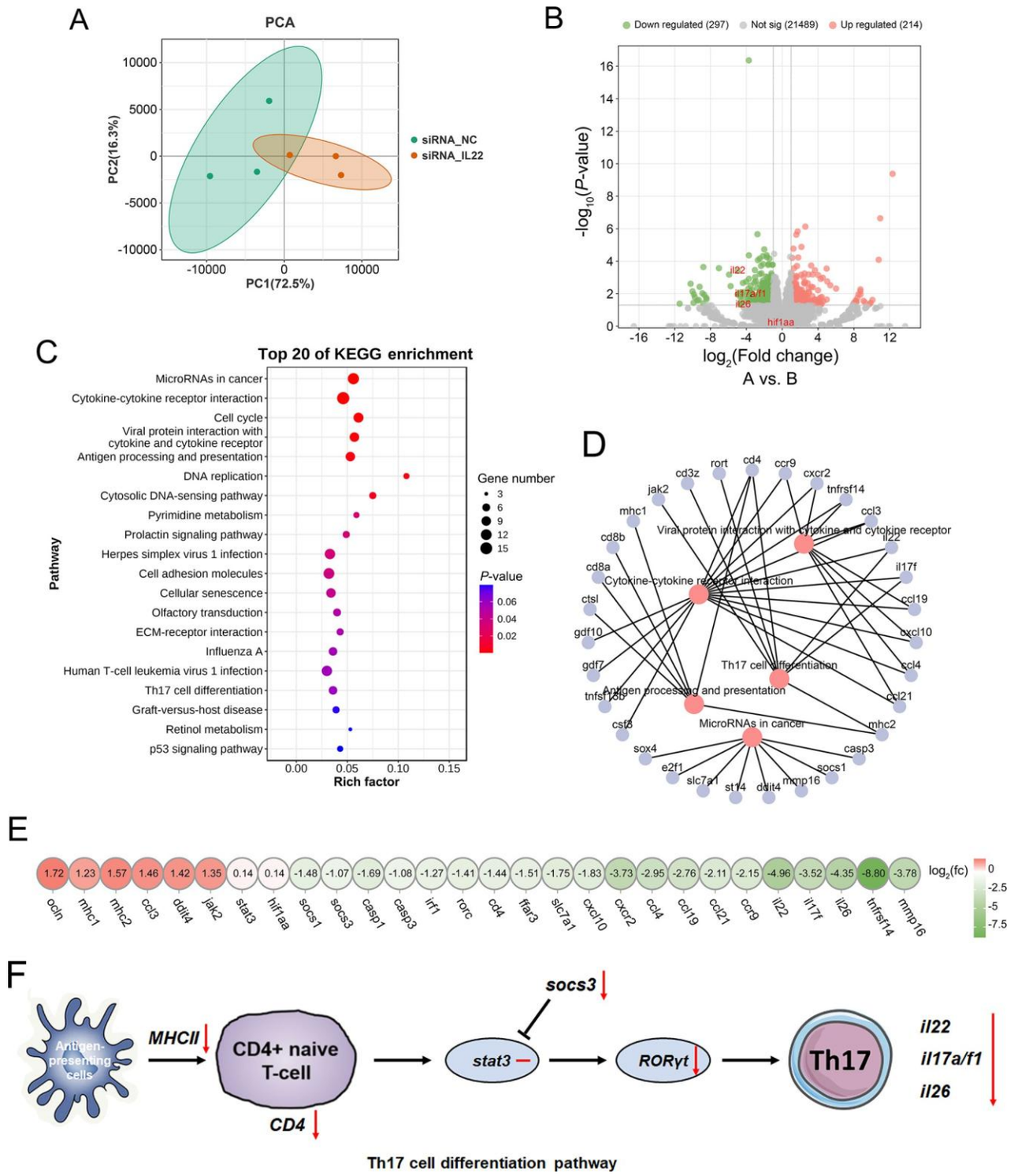

Figure 7 Effect of *Pf*_IL-22 knockdown on hypoxic stress based on transcriptomic analysis

A: Principal component analysis between p*Pf*_IL-22si and p*Pf*_IL-22siC (control). B: Volcano plot depicting DEGs between two groups. Gray dots indicate no significant difference, orange dots represent significantly up-regulated genes, and green dots represent significantly down-regulated genes. Four genes (*il22*, *il26*, *il17a/f1*, and *hif1αα*) are highlighted in red. C: KEGG pathway enrichment analysis of DEGs. D: Network showing interactions between hub genes and enriched pathways. E: Expression changes of immune-related genes after *Pf_IL-22* knockdown. F: Diagram illustrating changes in key Th17 pathway genes following IL-22 interference.

Expression dynamics of *Pf*_IL-22 under hypoxia exposure *in vivo*

Low DO can compromise intestinal structural integrity in fish, triggering inflammation and epithelial barrier dysfunction. IL-22 plays a crucial role in epithelial repair by promoting cellproliferation, regulating antimicrobial peptide production, and maintaining barrier homeostasis (Aujla et al., 2008; Wang et al., 2017). Hypoxic stress has been reported to induce IL-22 transcription (Budda et al., 2016). In this study, RT-qPCR analysis showed that *Pf*_IL-22 mRNA expression peaked a 24 h following hypoxia exposure, while IL-22 protein abundance reached its maximum at 48 h. The temporal lag between transcript and protein accumulation likely reflects post-transcriptional regulatory processes, indicating that mRNA levels may not directly predict protein secretion.

Histopathological analysis showed marked intestinal injury in yellow catfish under hypoxia, characterized by goblet cell swelling, vacuolation, and villous erosion. IL-22 is known to up-regulate genes involved in antimicrobial defense, facilitating secretion of antimicrobial peptides that protect intestinal tissues from microbial invasion (Liang et al., 2006). Therefore, the gradual increase in IL-22 protein levels may contribute to epithelial cell proliferation and restoration of barrier integrity disrupted by hypoxia. However, sustained overproduction of IL-22 has been linked to impaired intestinal homeostasis, with reports showing suppression of IEC proliferation, crypt loss, morphological changes, and reduced Lgr5+ stem cell potency in the small intestine, ultimately limiting stem cell differentiation into mature epithelial lineages (Zha et al., 2019). Thus, the decline in *Pf*_IL-22 expression after peak induction may represent a regulatory mechanism to down-regulate IL-22 and prevent excessive inflammation, suggesting a dual role of *Pf*_IL-22 in regulating protective immune responses and limiting excessive inflammation under hypoxic stress.

IL-22RA1 and IL-22BP exhibited expression profiles that paralleled IL-22, with a general increase over time and transient decreases at 12 h and 72 h post-hypoxia. In this study, the up-regulation of IL-22RA1 corresponded with rising IL-22 protein levels, suggesting enhanced receptor engagement during hypoxic stress. IL-22RA1 functions as the primary receptor for IL-22, forming a heterodimeric complex with IL-10R2 upon ligand binding to activate downstream signaling (Nikoopour et al., 2015). In contrast, IL-22BP acts as an endogenous inhibitor by binding IL-22 and preventing its interaction with IL-22RA1, thereby suppressing downstream responses. Previous studies have shown that IL-22 provides mucosal protection during acute inflammation but becomes detrimental if sustained at high levels during recovery. In IL- 22BP$^{-/-}$ mice, excessive IL-22 availability promotes uncontrolled intestinal epithelial cell proliferation and increases tumorigenic potential (Huber et al., 2012). These findings, together with our observations, suggest that IL-22 in yellow catfish contributes to protection during the peak phase of hypoxic injury, whereas the subsequent rise in IL-22BP expression serves to modulate IL-22 activity, thereby preventing excessive intestinal cell proliferation and reducing the risk of inflammation or tumor development.

IL-22 functions within a complex cytokine network that integrates both pro-inflammatory and anti-inflammatory signals. For instance, IL-1β and IL-17 have been shown to induce IL-22 production in NK-22 cells (Cella et al., 2010), while IL-17A, IL-22, and TNF-α act synergistically to enhance keratinocyte activity (Guilloteau et al., 2010). IL-1β serves as a key pro-inflammatory mediator that activates and regulates immune responses across multiple cells, including T cells, B cells, neutrophils, and endothelial cells. In contrast, IL-10 functions as a critical immunoregulatory cytokine, suppressing inflammatory mediator release by monocytes and macrophages while promoting anti-inflammatory factor production (Wu et al., 2021). IL-17A and IL-17F, key drivers of inflammatory signaling, are essential for pathogen defense and maintenance of intestinal mucosal immunity (Okamura

et al., 2020). In this study, the expression levels of *IL-1β* and *IL-10* increased with prolonged hypoxia, while IL-17A/F1, a homolog of IL-17A and IL-17F, also displayed marked up-regulation. Similar patterns have been reported in coho salmon (*Oncorhynchus kisutch*), where IL-1β and IL-10 expression in the spleen has been shown to increase under low DO conditions (Martínez et al., 2020). Together, these findings suggest that IL-22 may act in concert with other interleukins, amplifying its anti-inflammatory effects through synergistic interactions.

STAT3 and TGF-β1 are critical transcriptional regulators involved in diverse cellular processes, including differentiation, proliferation, apoptosis, and immune modulation. The IL-22 promoter in $CD4^+$ T cells contains binding sites for both STAT3 and AhR (Yeste et al., 2014), implicating these factors in the regulation of IL-22 expression. In turbot (*S. maximus*), butyrate promotes STAT3 phosphorylation in IECs, activating autophagy via the HIF-1α/IL-22 signaling pathway and enhancing host defense against invasive pathogens (Zhang et al., 2023). In this study, *STAT3* and *TGF-β1* exhibited comparable expression patterns under hypoxia, remaining largely unchanged during the early phase of hypoxia but showing marked up-regulation during the mid-phase, with peak levels observed at 48 h. These results suggest that the up-regulation of STAT3 and TGF-β1 may contribute to compensatory responses against hypoxia-induced damage by enhancing robust IL-22 production in intestinal cells. TGF-β has also been reported to promote IL-22 production in Th17 cells via AhR induction and PI3K-mediated signaling. AhR, a ligand-dependent transcription factor, plays a pivotal role in regulating processes such as cell development, inflammation, and immunity, particularly in the gut (Yeste et al., 2014). Supporting its relevance, oral administration of chitooligosaccharides (COS) has been shown to restore AhR/IL-22 signaling, increase MUC2 expression, repair the intestinal mucosal barrier, and alleviate colitis in mice (Wang et al., 2023). Consistently, in *P. fulvidraco* under hypoxic stress, intestinal *AhR* expression exhibited a consistent upward trend, peaking at 48 h, indicating that hypoxia may drive AhR up-regulation to facilitate IL-22 activation, thereby mitigating hypoxia-induced intestinal damage and promoting epithelial barrier repair.

Dual functions of HIF-1α/IL-22 axis in primary IECs under hypoxic stress

DMOG, a prolyl hydroxylase domain inhibitor, mimics hypoxic conditions by preventing HIF-1α degradation and promoting its nuclear accumulation (Chen et al., 2023b). In primary IECs, stimulation with 1 mM DMOG significantly increased *Pf_HIF- 1α* expression, confirming successful activation of the HIF pathway. *Pf*_IL-22 also markedly increased under these conditions. Previous studies have demonstrated that HIF-1α enhances IL-22 transcription in $CD4^+$ T cells (Yang et al., 2020a). In this study, DMOG stimulation promoted HIF-1α nuclear accumulation in primary IECs, which was associated with a marked induction of IL-22 protein. Additionally, DMOG- treated IECs exhibited significant upregulation of *IL-22BP, IL-22RA1, IL-10, IL-1β, TGF-β1,* and *STAT3*, while *IL-17A/F1* and *AHR* showed non-significant upward trends. These results indicate that hypoxia-driven HIF-1α activation contributes to the regulation of IL-22 production and modulates a broader network of innate immune genes in yellow catfish.

Subsequently, the biological effects of r*Pf*_IL-22 onimmune-related gene expression in primary IECs were assessed. Results indicated that r*Pf*_IL-22 significantly induced the expression of multiple cytokines in IECs. While previous studies in grass carp primary head kidney leukocytes and CIK cells have reported that IL-22 predominantly regulates gene expression in leukocytes with limited influence on non-leukocytes (Yang et al., 2020b), the present findings indicated that IL-22 also exerts a notable regulatory effect on non-leukocyte populations. However, potential leukocyte-specific effects in yellow catfish were not investigated here, warranting further exploration. Moreover, the response to r*Pf*_IL-22 was dose-dependent: low-dose stimulation (5 ng/mL) produced minimal induction and even suppressed certain genes, whereas higher concentrations (20 ng/mL) robustly enhanced cytokine expression.

Previous studies have demonstrated that IL-22 performs dual and context-dependent functions: at moderate levels, it supports intestinal epithelial barrier integrity, while excessive expression can exert detrimental effects, including the promotion

of colorectal carcinogenesis (Budda et al., 2016). In teleosts, IL-22 engages in complex crosstalk with both pro- and anti-inflammatory cytokines, paralleling observations in mammals. For instance, in grass carp (*C. idella*), IL-22 acts synergistically with IL-17A/F1 to enhance antimicrobial peptide production, such as β-defensin, within mucosal tissues, reflecting the cooperative roles of Th17-associated cytokines in mucosal defense (Yang et al., 2020b). Conversely, IL-22 also modulates anti-inflammatory pathways by inducing IL-10 and TGF-β1 expression in IECs, as observed in turbot (*S. maximus*) under bacterial challenge (Zhang et al., 2023). This dual functionality—amplifying pro-inflammatory signals for pathogen clearance and anti-inflammatory signals for tissue repair—appears evolutionarily conserved among teleosts. Thus, defining the optimal IL-22 concentration required to achieve effective immune protection without triggering pathological outcomes is essential. Notably, the present study revealed that IL-22 did not activate cellular responses via STAT3-mediated signaling in yellow catfish. Treatment with r*Pf*_IL-22 did not increase STAT3 expression, and silencing *Pf*_IL-22 similarly had no effect on STAT3 levels. This contrasts with observations in mammals (Yeste et al., 2014) and turbot (Zhang et al., 2023), where STAT3 functions as a central transcription factor downstream of IL-22.

HIF-1α activates IL-22 expression by directly binding to HREs

The hypoxic microenvironment of the gut induces the expression of several HIF-1α target genes in IECs, influencing their metabolism, barrier function, and survival (Fachi et al., 2024). As the regulatory subunit of the HIF-1 complex, HIF-1α modulates gene expression by binding to HREs in target promoters and has been implicated in the regulation of various $CD4^+$ T cell functions (Wang et al., 1995; Zhao et al., 2024). Using dual-luciferase reporter assays and EMSA, this study demonstrated that HIF-1α directly interacts with the IL-22 promoter in yellow catfish, establishing a mechanistic link between hypoxia signaling and IL-22 induction. Evidence from mammals supports this regulatory pathway; in mice, butyrate enhances the binding of HIF-1α to *IL-22* promoter HREs by increasing H3K9 acetylation and inhibiting H3K9 trimethylation (Sun et al., 2017). Similarly, in *S. maximus*, where butyrate activates the HIF-1α/IL-22 axis via type 3 innate lymphoid cells and STAT3-dependent signaling (Zhang et al., 2023).Notably, microbiota-derived short-chain fatty acids (SCFAs), particularly butyrate, play a critical role in sustaining physiological hypoxia in the gut by modulating epithelial oxygen metabolism. Butyrate activates peroxisome proliferator-activated receptor γ (PPARγ), enhancing mitochondrial β-oxidation and promoting oxygen consumption in IECs, which subsequently triggers HIF-1α stabilization and transcriptional activity (Byndloss et al., 2017; Kelly et al., 2015). In $CD4^+$ T cells and ILCs, butyrate treatment elevates HIF-1α abundance, while pharmacological or genetic inhibition of HIF-1α abolishes butyrate-induced IL-22 production, highlighting the critical role of HIF-1α in linking microbial metabolites to IL-22 regulation (Yang et al., 2020a). Hypoxia itself independently promotes IL-22 induction in $CD4^+$ T cells, and butyrate treatment under hypoxic conditions further enhances this response. This study provides the first evidence of a direct interaction between HIF-1α and IL-22 in teleosts. *In vivo* knockdown of HIF-1α significantly affected IL-22 expression under both normoxic and hypoxic conditions, indicating that both physiological and environmental hypoxia robustly activate the HIF-1α/IL-22 axis, playing a crucial role in maintaining intestinal immune homeostasis.

***Pf*_IL-22 exerts immune protection through the Th17 signaling pathway**

IL-22 gene knockdown in *P. fulvidraco* followed by RNA-seq of intestinal tissues under hypoxic stress identified key pathways and genes involved in the regulation of inflammation by IL-22. KEGG pathway enrichment analysis indicated that DEGs were predominantly enriched in immune- and inflammation-related pathways, including cytokine-cytokine receptor interaction, microRNA signaling, and Th17 signaling. The Th17 pathway plays a crucial role

in host defense, tissue repair, and inflammatory disease pathogenesis (Amatya et al., 2017), prompting focused analysis of the role of this pathway in intestinal inflammatory responses. In mammals, Th17- associated cytokines such as IL-17A, IL-17F, and IL-22 contribute critically to mucosal barrier protection (Rutz et al., 2013). *Edwardsiella ictaluri* infection has been shown to reduce Th17 cell activity in the intestine of turbot, driving intestinal inflammation (Yang et al., 2024). IL-22 functions as a conserved marker for Th17 cell activity. In this study, IL-22 knockdown markedly inhibited Th17 signaling under acute hypoxia, with pronounced down-regulation of key genes including *IL-17A/F1* and *RORγt*. These findings indicate that IL-22 plays a pivotal role in sustaining hypoxia-induced intestinal inflammation. Notably, HIF-1α expression remained unchanged compared to controls, suggesting an absence of negative feedback regulation on IL-22 under hypoxia. Furthermore, STAT3 transcript levels were unaffected, likely reflecting its function via phosphorylation rather than transcriptional control, although potential compensatory mechanisms through alternative pathways warrant further investigation.

The gut microbiota, particularly SCFA-producing bacteria, plays a vital role in intestinal health. SCFAs serve as a critical link between the microbiome and intestinal immunity (Rooks & Garrett, 2016), with butyrate functioning via the FFAR3 receptor on ILC3 and $CD4^+$ T cells to induce AhR and HIF-1α expression, enhance accessibility of HIF-1α binding sites in the IL-22 promoter via histone modifications, and amplify IL-22 expression (Yang et al., 2020a). Consistent with this mechanism, IL-22 interference significantly reduced FFAR3 expression, implicating IL-22 in microbiota-driven immune modulation. SCFAs generated via microbial fermentation within the gut microbiota also exert immunomodulatory effects by engaging the FFAR3 receptor and inhibiting histone deacetylase activity, further enhancing IL-22 production by $CD4^+$ T cells and ILCs. Increased IL-22 secretion fortifies the intestinal epithelial barrier, thereby mitigating pathogen translocation and dampening inflammatory signaling pathways. Collectively, these mechanisms contribute to the maintenance of intestinal homeostasis in the host (Yang et al., 2020a). However, further comprehensive studies are needed to test these hypotheses and define the pathways through which the hypoxia-induced HIF-1α/IL-22 axis regulates ILC3 differentiation, maintenance, and migration, as well as its impact on microbiota composition.

CONCLUSION

This study demonstrated the roles of an IL-22 homolog in *P. fulvidraco* in defense against hypoxia. Hypoxia-induced activation of HIF-1α promoted its binding to HREs, leading to up-regulated IL-22 expression, which modulated downstream cytokine networks and mitigated intestinal inflammation. This regulatory process exhibited dual characteristics, with early-

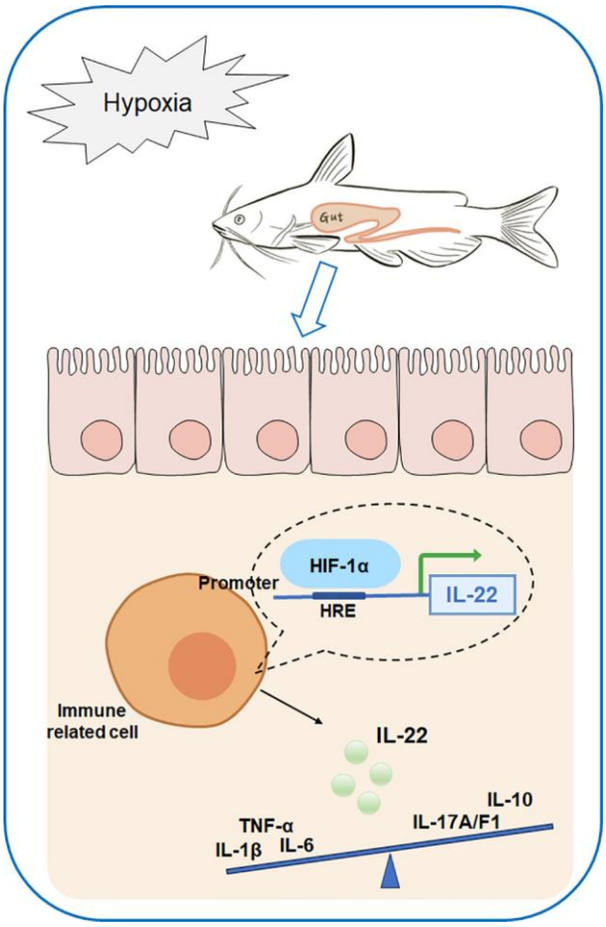

Figure 8 Schematic representation of HIF-1α/IL-22 signaling activation conferring resistance to hypoxia-induced intestinal inflammation in *P. fulvidraco*

Hypoxic stress activates HIF signaling, leading to HIF-1α binding to the HRE of IL-22 and inducing IL-22 production, which subsequently regulates the balance between pro-inflammatory and anti-inflammatory cytokines.stage hypoxia exerting predominantly anti-inflammatory effects. Moreover, the Th17 signaling pathway was identified as a key mediator of IL-22-driven immunomodulation in the intestine. These findings clarify the molecular mechanisms by which the HIF-1α\IL-22 axis orchestrates immune responses during hypoxia, providing new insights into intestinal immune regulation and potential strategies for mitigating hypoxia- induced inflammatory damage in teleosts (Figure 8).

DATA AVAILABILITY

All sequencing data in the study were uploaded to the NCBI database under accession number SUB15212178/PRJNA1243326, National Genomics Data Center under GSA accession number CRA039430 (https://bigd.big.ac.cn/gsa/browse/CRA039430), and Science Data Bank under the link https://doi.org/10.57760/sciencedb.j00139.00191.

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