

Post-Transcriptional Regulation of Necroptosis Gene *EIF2AK2* in Peripheral Blood of Bronchopulmonary Dysplasia

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Background: Bronchopulmonary dysplasia (BPD) remains the most frequent chronic lung disease among infants, characterized by multifactorial pathogenesis. Necroptosis represents a caspase-independent mode of programmed cell death, and its deregulation is associated with lung diseases, but the mechanisms of necroptosis in BPD are still unclear. This work was conducted to unveil the post-transcriptional regulatory mechanisms of necroptosis in BPD.

Methods: We sequenced messenger RNA (mRNA) (BPD, $n = 4$; controls, $n = 4$) and microRNA (miRNA) (BPD, $n = 5$; controls, $n = 5$) profiles in peripheral blood specimens. The GSE125873 dataset comprising mRNA profiles from 11 BPD individuals and 10 controls was obtained. The circular RNA (circRNA)-miRNA-mRNA networks and a necroptosis-based subnetwork were constructed on the basis of the circBank and starBase databases. The diagnostic efficacy of eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*) was assessed using receiver operator characteristic curve (ROC). *EIF2AK2*-relevant molecules were analyzed with weighted correlation network analysis (WGCNA), followed by molecular classification through consensus clustering method. *In vitro* experiments were conducted for validating the main findings.

Results: Aberrantly expressed mRNAs and miRNAs were determined in peripheral blood of BPD relative to controls. Specifically, hsa_circ_0075945/hsa_circ_0038897/hsa_circ_0038898/hsa_circ_0041143 were found to mediate hsa-miR-143-3p, while hsa_circ_0075924/hsa_circ_0075925/hsa_circ_0033776/hsa_circ_0089761 were associated with hsa-miR-589-5p, both of which post-transcriptionally regulated *EIF2AK2* expression. *EIF2AK2* can effectively diagnose BPD (area under the curve (AUC) = 0.938). Thirty-one *EIF2AK2*-related genes were selected, which classified BPD into two molecular subtypes. *EIF2AK2* knockdown alleviated apoptosis of hyperoxia-induced BEAS-2B cells ($p < 0.0001$).

Conclusions: Collectively, necroptosis gene *EIF2AK2* acts as a molecular determinant of BPD, and our findings uncover the contribution of post-transcriptional regulation to its aberrant expression.

Keywords: Bronchopulmonary dysplasia; necroptosis; *EIF2AK2*; diagnosis; molecular classification

Introduction

Bronchopulmonary dysplasia (BPD) remains the most frequent chronic lung disease among infants, which affects >45% of infants born before 29 weeks of gestational age [1]. Moreover, it is the leading cause of death in children under the age of five [2]. The lungs of infants with BPD exhibit the typical features of decreased alveolar formation and disturbed matrix remodeling, resulting in structural changes that may persist beyond adolescence [3]. Its etiology is multifactorial, including preterm birth, mechanical ventilation, oxygen exposure, and inflammatory reactions, contributing to its progression [4]. The diagnosis of BPD is associated with subsequent aberrant growth, neurodevelopment, respiratory function, etc. [5]. Although notable

progress has been made in neonatal management, the incidence of BPD is static or even increasing, but potent prevention or treatment protocols are still lacking [6]. Hence, selecting reliable molecules that affect BPD may result in promising therapeutics.

Necroptosis represents a caspase-independent mode of programmed cell death, which is manifested as a morphological trait of necrosis. Usually, necroptosis is driven by receptor interacting protein kinase 1 (*RIPK1*)-*RIPK3*-mixed lineage kinase domain-like protein (*MLKL*) signaling cascade [7]. Deregulation of necroptosis has been implicated in BPD [8]. For instance, oxidative stress-induced necroptosis activation is associated with the pathogenesis of hyperoxic acute lung damage [9]. The knockdown of *RIPK3* siRNA reduced *RIPK3* expression and necrotic cell

death [10]. In a study on the alleviation of experimental BPD by the exosome-related factor tumour necrosis factor-stimulated gene 6 (*TSG-6*) secreted by early pregnancy MSCs, *TSG-6* was an essential protective factor in the treatment [11]. The comprehensive understanding of necroptosis mechanisms may offer novel therapeutic strategies and targets for the effective management of BPD.

Lung biopsies pose a significant risk of inducing pulmonary hypertension, so whole blood RNA analysis offers an alternative to liquid biopsies [12]. Rhodes *et al.* [12] derived a whole blood transcriptomic profile linked with disease severity and risk for poor clinical outcomes in individuals with pulmonary arterial hypertension. Similarly, Moll *et al.* [13] developed a blood-based transcriptomic risk score for predicting chronic obstructive pulmonary disease.

Non-coding RNAs, such as microRNAs (miRNAs) have been broadly explored in BPD. For instance, exosomal miR-876-3p can predict and protect against severe BPD in extremely premature infants [14]. The down-regulation of hyperoxia-induced miR-342-5p exacerbates neonatal BPD by modulating *Spre3*, a Raf1 regulator [15]. The correlation between down-regulated microRNA-451 and up-regulation of MIF appears to alleviate the cardio-pulmonary phenotype in BPD [16]. Circular RNAs (circRNAs) are single-stranded non-coding RNAs that covalently form a closed circular structure [17]. CircRNAs usually exhibit tissue-restricted and developmental stage-specific expression patterns [18]. Based upon the subcellular locality, circRNAs exert multifarious cellular functions, particularly through their role as miRNAs [19]. Several circRNAs have been proven to participate in physiological and pathophysiological processes of BPD. For example, circRNA circabcc4 has been found to facilitate BPD progression by up-regulating *PLA2G6* expression via sequestering miR-663a [20]. Nonetheless, so far, no study has demonstrated the implications of circRNAs on necroptosis during BPD. Therefore, this study aims to investigate the post-transcriptional regulatory mechanisms of necroptosis in peripheral blood of BPD.

Materials and Methods

Sample Collection

Peripheral blood specimens were collected from 9 infants with BPD and 9 age-matched healthy controls at the Eighth Affiliated Hospital of Guangxi Medical University. The blood specimens were preserved in anticoagulant tubes containing a combination of potassium and sodium EDTA. Then, 0.1 mL blood sample was separated into RNase-free microcentrifuge tubes and mixed with 0.3 mL RNeasy Lysis Buffer (R0901, Sigma-Aldrich, St Louis, MO, USA). These samples were further stored at -80°C before sequencing. This study was approved by the Ethics Committee of the Eighth Affiliated Hospital of Guangxi Med-

ical University (GYYXLL-20210601-18). The written informed consent was obtained from each patient in accordance with institutional guidelines. Inclusion criteria for all infants included in the study were (1) gestational age <31 wk, (2) no major congenital malformations, and (3) requirement of respiratory support at the time of enrollment. Inclusion criteria for healthy infants included in the study were gestational age >37 wk and in health. Human subjects, human materials or human data were conducted in accordance with the principles outlined in the Declaration of Helsinki.

mRNA and MiRNA Sequencing

After conventional RNA extraction from peripheral blood and library preparation, messenger RNA (mRNA) (BPD, $n = 4$; controls, $n = 4$) and microRNA (miRNA) (BPD, $n = 5$; controls, $n = 5$) profiles were sequenced using Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA, USA). The sequencing services were provided by Wuhan Kangce Technology Co., Ltd, with the sequenced data shared on GEO (<https://www.ncbi.nlm.nih.gov/bioproject/?term=gse219156>). Specifically, The Qiagen PreAnalytiX PAXgene Blood miRNA Kit (763134, Qiagen, Dusseldorf, Germany) was used to extract total RNA from PAXgene™ Blood RNA tubes. The extraction protocol was performed following the specific operating protocol of the company using the Qiagen QIAcube Extraction Robot. RNA samples with RIN >7 and concentration ≥ 25 $\mu\text{g}/\mu\text{L}$ were sequenced. The Illumina TruSeq Stranded Total RNA with Ribo-Zero Globin Kit (RS-122-2501, Illumina, Inc., San Diego, CA, USA) was used for globin reduction and cDNA library preparation of total RNA. Library quality control (QC) was performed by pico-green quantitation, size analysis on an Agilent Bioanalyzer or TapeStation 2200 (Agilent, Palo Alto, CA, USA), and Quantitative polymerase chain reaction (qPCR) quantitation against a standard curve. A HiSeq 2500 flow cell was used to generate 75 bp paired end reads. To generate the optimal number of clusters per lane of the flow cell, libraries were loaded at an empirically determined concentration. The average depth of sequencing of the samples was 20 million reads.

Acquisition of BPD Public Datasets

This study utilizes data from the Gene Expression Omnibus, specifically the GSE125873 dataset (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125873>) which include RNA-seq profiling of peripheral blood specimens from 11 BPD individuals and 10 controls on the GPL16791 platform [21].

Differential Expression Analysis

mRNAs with aberrant expression were identified in BPD relative to controls both in RNA-seq and the GSE125873 datasets. The screening threshold values were set as $p < 0.05$ and $|\log_2 \text{fold change (FC)}| > 0.585$. This analysis was performed by using edgeR package (version

4.0.15, The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) [22]. The shared mRNAs were subsequently selected for further analysis. Using the same threshold values, aberrantly expressed miRNAs in BPD versus controls were screened. These findings were visualized in volcano and heatmap diagrams.

CircRNA-MiRNA-mRNA Network Construction

CircRNAs targeting aberrantly expressed miRNAs were inferred through the circBank (<http://www.circbank.cn/>) that is a comprehensive database for human circRNAs [23]. In addition, interaction pairs between aberrantly expressed miRNAs and circRNAs were retrieved from the starBase database (<http://starbase.sysu.edu.cn/>) [24]. The shared circRNA-miRNA pairs were acquired. After integrating circRNA-miRNA and miRNA-mRNA pairs, circRNA-miRNA-mRNA networks were constructed and visualized using Cytoscape software (version 3.7.2, Institute for Systems Biology, Seattle, WA, USA) [25].

Functional Annotation Analysis

The enrichment analysis on Gene Ontology (GO) was performed with clusterProfiler package (version 0.4.6, Baylor College of Medicine, Houston, TX, USA) [26]. GO comprises biological process, molecular function, and cellular component. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted. The significance of enrichment was determined by a false discovery rate (FDR) < 0.05.

Gene Set Enrichment Analysis (GSEA)

Using GSEA (version 0.4.6, Baylor College of Medicine, Houston, TX, USA) [27], the differences in GO and KEGG pathways between groups were estimated in accordance with the reference gene set from the Molecular Signature Database [28]. |NES| > 1 and FDR < 0.05 was utilized as the enrichment cutoff value.

Weighted Correlation Network Analysis (WGCNA)

WGCNA (version 1.72-5, University of California, Los Angeles, CA, USA) was carried out based upon the top 5000 genes of standard deviation among samples [29]. Sample dendrogram was drawn, and outlier samples were discarded. Gene relationships were determined through Pearson's test and subsequently, a similarity matrix was constructed. The optimal soft thresholding value was selected by considering scale independence along with mean connectivity. The similarity matrix was converted into the adjacency matrix. The construction of a hierarchical clustering dendrogram was performed for detecting modules. The modules were then merged using the dynamic tree cutting method. Pearson's test was employed for computing the connections of merged modules with BPD and eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*).

Gene significance across modules was compared. The module presenting the strongest association with *EIF2AK2* was determined. Genes in this module with coefficient > 0.6 were regarded as *EIF2AK2*-relevant genes.

Protein-Protein Interaction (PPI) Analysis

EIF2AK2-relevant genes were uploaded to the STRING online database (<https://string-db.org/>, version 1.5) [30]. Protein interactions were predicted, and a PPI network was downloaded.

Consensus Clustering Analysis

Consensus clustering analysis was carried out for estimating the number of molecular subtypes across the merged mRNA dataset in accordance with the transcriptome profiling of *EIF2AK2*-relevant genes using ConsensusClusterPlus package (version 1.66.0, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) [31]. The consensus matrix was constructed for determining the optimal number of clusters.

Cell Culture and Administration

Human lung epithelial cells BEAS-2B were authenticated using the current STR typing technique of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Cells have been STR identified and the mycoplasma detection results are negative. BEAS-2B (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; SH30022.01B, Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS; SH30071.03, Hyclone, Logan, UT, USA) and penicillin and streptomycin (100 mg/mL) at 37 °C with 5% CO₂. To simulate BPD, BEAS-2B cells were exposed to hyperoxia (85% O₂, 5% CO₂) for 6 h, with normoxia as a control. Hyperoxia-induced BEAS-2B cells were transfected with knockdown control siRNA (si-NC) using Lipo3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) for 48 h. Transfected with siRNA specifically knockdown *EIF2AK2* expression (*EIF2AK2* siRNA)#1: 5'-GACAGTTTAAACAGTTCTTCG-3', *EIF2AK2* siRNA#2: 5'-GAAGGTGAAGGTAGATCAAAG-3', *EIF2AK2* siRNA#3: 5'-GGAATTACATAGGCCTTATCA-3', and si-NC: 5'-TTCTCCGAACGTGTCACGT-3'.

Real Time-PCR

Total RNA was extracted using Trizol extraction kit (15596026, Invitrogen, Carlsbad, CA, USA) followed by cDNA synthesis. Real time-PCR was performed using SYBR Premix Ex Taq (Takara, Osaka, Osaka Prefecture, Japan) on a 7500 Realtime PCR System (Thermo Fisher, Bremen, Germany). The primer sequences included:

EIF2AK2, 5'-TATAAATGCAAAATGGGACAGA-3' (forward), 5'-AGTAGCAAAAGAACCAGAGGAC-3' (reverse);

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward), 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse). Relative expression values were calculated with $2^{-\Delta\Delta C_t}$ method.

Western Blot

Total protein was isolated utilizing RIPA reagent (P0013B, Beyotime, Shanghai, China). Protein was transferred onto polyvinylidene fluoride membrane (IPVH00010, Millipore Corporation, Billerica, MA, USA), and the membrane was blocked with 5% bovine serum albumin. Subsequently, overnight incubation with primary antibody against *EIF2AK2* (1:5000; ab32506; Abcam, Cambridge, UK) or *GAPDH* (1:2500; ab9485; Abcam, Cambridge, MA, USA) was conducted at 4 °C, followed by incubation with secondary antibody (1:5000; ab7090; Abcam, Cambridge, UK) for 1 h at room temperature. ECL reagent (P0018S, Beyotime, Shanghai, China) was used for immunodetection and visualization with Bio-Image Analysis System (ChemiScope 5300 Pro, Syngene, Cambridge, UK). Densitometric analysis for the quantification relative to *GAPDH* was performed using the Image J software (Version 2.3.0, Syngene, Cambridge, UK).

Flow Cytometry

Cell apoptosis was measured using Annexin V-fluorescein isothiocyanate (Annexin V-FITC)/propidium iodide (PI) apoptosis Assay Kit (V13242, Invitrogen, Carlsbad, CA, USA). After centrifugation, the pellets were suspended and dyed with Annexin V-FITC and PI solution for 15 min at room temperature. Stained cells were detected on a flow cytometer (LSRFortessa TM X-20, BD Biosciences, San Jose, NJ, USA) using FlowJo7.6 software (Treestar, Ashland, OR, USA).

Statistical Analysis

Receiver operator characteristic curve (ROC) was plotted for appraising the diagnostic efficacy of *EIF2AK2*. Correlation analysis was performed using Pearson's test. All the analyses were carried out with R packages (<https://www.r-project.org/>, version 3.6.3, University of Auckland, Auckland, New Zealand) or GraphPad Prism software (version 9.0.1, Inc., San Diego, CA, USA). Experimental data from three replicates are presented as the mean $\pm$ standard deviation. Comparisons between two groups were made using paired two-tailed Student's *t*-test, and comparisons among multiple groups were made using one-way ANOVA with Dunnett's post-hoc test. $p < 0.05$ indicated statistical significance.

Results

Aberrantly Expressed mRNAs in Peripheral Blood of BPD

This work conducted RNA-seq on peripheral blood of 4 BPD and 4 control infants. Aberrantly expressed mRNAs in BPD were selected based upon the cutoff of $p < 0.05$ and $|\log_2 FC| > 0.585$. The results pertaining to Aberrantly Expressed mRNAs revealed that 852 mRNAs exhibited the up-regulation in BPD versus controls, and 715 exhibited the down-regulation (Fig. 1A–C; **Supplementary Tables 1,2**). Differential expression analysis was also performed in the GSE125873 dataset. Utilizing the same cutoff, there were 319 up-regulated mRNAs in BPD, and 188 down-regulated mRNAs (Fig. 1D–F; **Supplementary Tables 3,4**). To obtain the reliable aberrantly expressed mRNAs, the aforementioned mRNAs in the two datasets were shared. Finally, fourteen up-regulated mRNAs in BPD were identified, comprising of *IFIT3*, *OTOF*, *IFIT2*, *CPSF1P1*, *IL1R2*, *UBXN10*, *PHLDB2*, *MYO18B*, *ITPRIPL1*, *SAMD9*, *EIF2AK2*, *PLIN4*, *IFIT5*, and *EXOC6B* (Fig. 1G). Additionally, three mRNAs (*MTRNR2L1*, *RN7SL396P*, and *SSPO*) exhibited the down-regulation in BPD (Fig. 1H). These aberrantly expressed mRNAs might be associated with BPD pathogenesis.

MiRNAs with Aberrant Expression in Peripheral Blood of BPD

MiRNA expression profiles were sequenced on peripheral blood specimens from 5 BPD and 5 control infants. On the basis of $p < 0.05$ and $|\log_2 FC| > 0.585$, 47 miRNAs displayed the overexpression in BPD relative to controls (Fig. 2A–C). In addition, 127 miRNAs with down-regulation in BPD were selected (Fig. 2A–C; **Supplementary Table 5**).

Establishment of CircRNA-MiRNA-mRNA Networks in Peripheral Blood of BPD

The interaction pairs of circRNAs and miRNAs were inferred using the circBank and starBase databases. Following the intersection process, there were 207 interactions of circRNAs with down-regulated miRNAs (Fig. 2D). In addition, there were 64 relationship pairs between circRNAs and up-regulated miRNAs (Fig. 2E). Afterwards, we constructed a network of 13 circRNAs (*hsa_circ_0058718*, *hsa_circ_0041143*, *hsa_circ_0075924*, *hsa_circ_0003960*, *hsa_circ_0009085*, *hsa_circ_0013827*, *hsa_circ_0075942*, *hsa_circ_0038027*, *hsa_circ_0003812*, *hsa_circ_0075925*, *hsa_circ_0005885*, *hsa_circ_0038449*, and *hsa_circ_0005455*), 25 down-regulated miRNAs (*hsa-miR-1224-5p*, *hsa-miR-143-3p*, *hsa-miR-145-5p*, *hsa-miR-185-5p*, *hsa-miR-216a-5p*, *hsa-miR-3179*, *hsa-miR-512-3p*, *hsa-miR-515-5p*, *hsa-miR-516b-5p*, *hsa-miR-519a-3p*, *hsa-miR-519b-3p*, *hsa-miR-519c-3p*, *hsa-miR-519e-5p*, *hsa-miR-520a-3p*, *hsa-miR-*

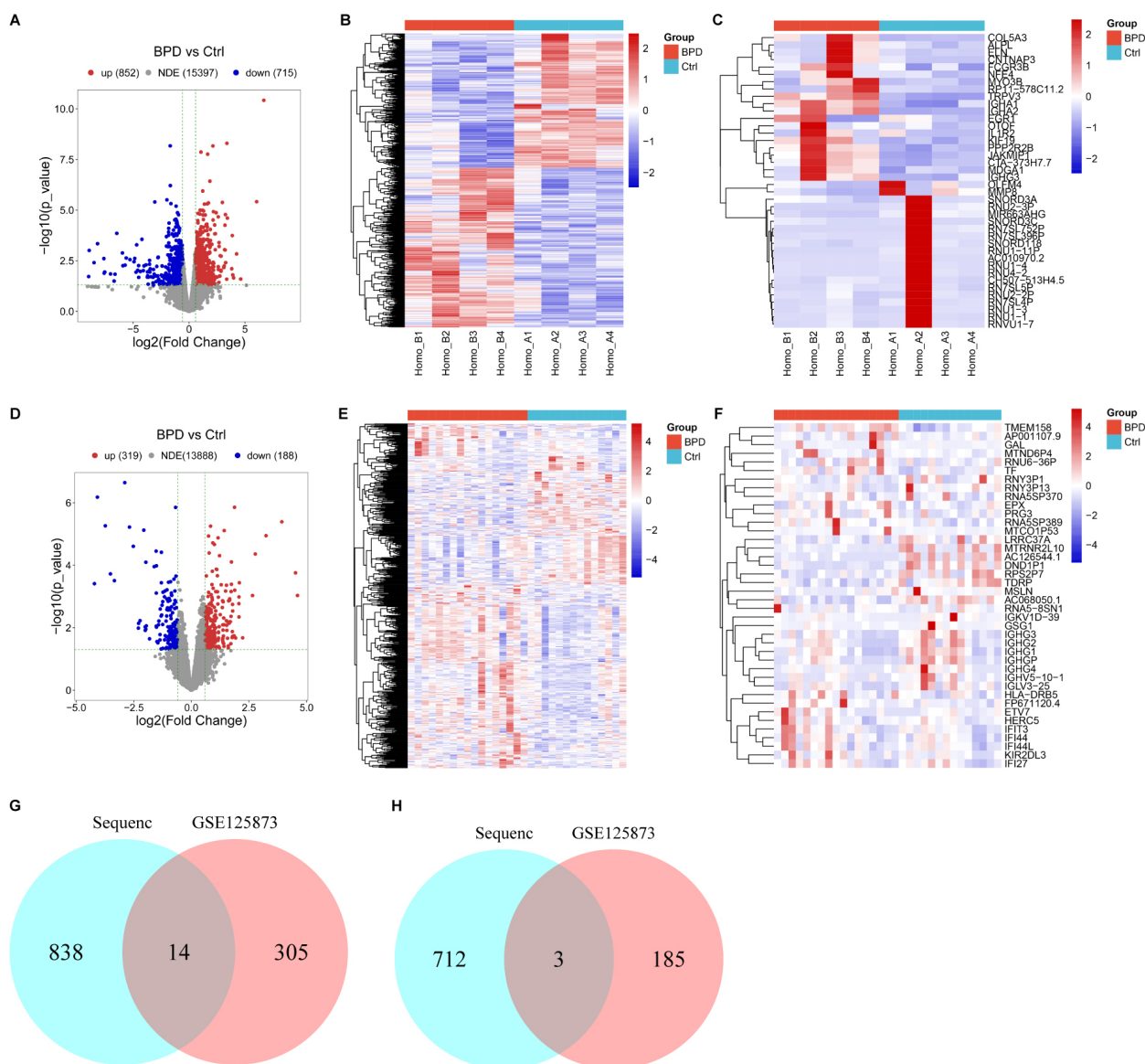


Fig. 1. Aberrantly expressed messenger RNA (mRNAs) in peripheral blood of Bronchopulmonary dysplasia (BPD). (A) Volcano plot illustrating mRNAs with aberrant expression in BPD relative to controls in our RNA-seq dataset. (B) Transcript level of aberrantly expressed mRNAs across BPD and control peripheral blood. (C) The top twenty down- and up-regulated mRNAs in above specimens. (D) Selection of aberrantly expressed mRNAs in BPD versus controls in the GSE125873 dataset. (E) Transcript level of selected mRNAs across BPD and control peripheral blood. (F) The top twenty down- and up-regulated mRNAs. (G) Venn diagram of the shared up-regulated mRNAs between our RNA-seq and the GSE125873 dataset. (H) The shared down-regulated mRNAs between above datasets. Ctrl, Control; NDE, Non-Differentially Expressed.

520a-5p, hsa-miR-520c-3p, hsa-miR-520d-3p, hsa-miR-520d-5p, hsa-miR-520h, hsa-miR-522-3p, hsa-miR-524-5p, hsa-miR-525-5p, hsa-miR-526b-3p, hsa-miR-526b-5p, and hsa-miR-589-5p), and 12 up-regulated mRNAs (*PHLDB2*, *OTOF*, *EXOC6B*, *EIF2AK2*, *IFIT3*, *IFIT5*, *UBXN10*, *IL1R2*, *SAMD9*, *MYO18B*, *IFIT2*, and *PLIN4*) (Fig. 2F). Moreover, a network of 16 circRNAs (hsa_circ_0075921, hsa_circ_0075953, hsa_circ_0085975, hsa_circ_0004036, hsa_circ_0038901, hsa_circ_0075924, hsa_circ_0081678, hsa_circ_0003812, hsa_circ_0006417,

hsa_circ_0007185, hsa_circ_0007780, hsa_circ_0018292, hsa_circ_0018293, hsa_circ_0089761, hsa_circ_0089763, and hsa_circ_0089792), 5 up-regulated miRNAs (hsa-miR-1301-3p, hsa-miR-197-3p, hsa-miR-326, hsa-miR-483-3p, and hsa-miR-642a-5p), and 2 down-regulated mRNAs (*SSPO*, and *MTRNR2L1*) (Fig. 2G).

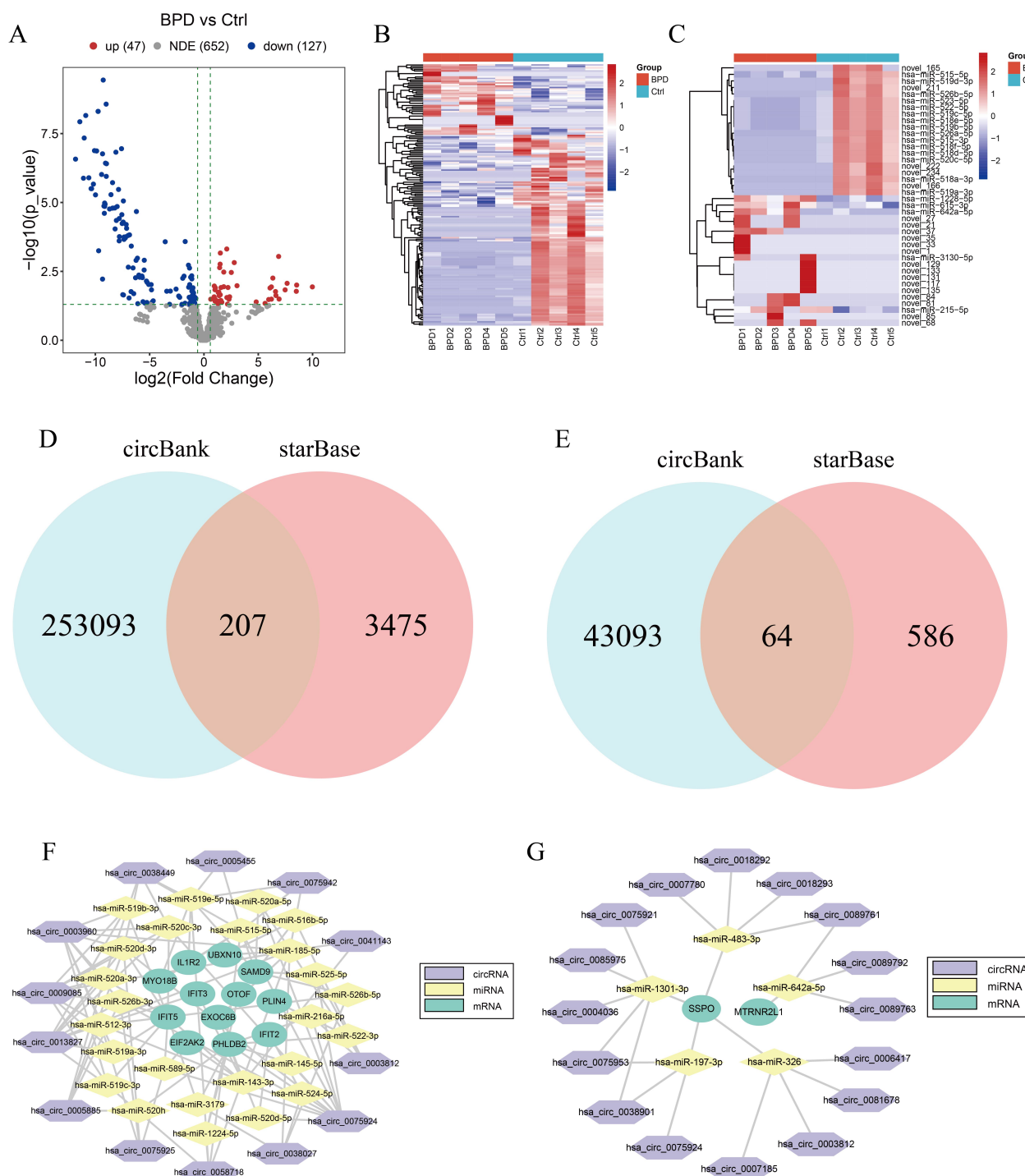


Fig. 2. Selection of aberrantly expressed microRNAs (miRNAs) in peripheral blood of BPD and construction of Circular RNA (circRNA)-miRNA-mRNA networks. (A) Volcano plot depicting aberrantly expressed miRNAs in BPD versus controls. (B) Expression patterns of miRNAs with aberrant expression across BPD and control peripheral blood. (C) The first twenty miRNAs with down- or up-regulation in BPD. (D) Venn plot of the shared interactions of down-regulated miRNAs with circRNAs between the circBank and starBase databases. (E) Venn plot illustrating the interactions between up-regulated miRNAs and circRNAs shared by the circBank and starBase databases. (F) The network of circRNAs, down-regulated miRNAs, and up-regulated mRNAs. (G) The network of circRNAs, up-regulated miRNAs, and down-regulated mRNAs.

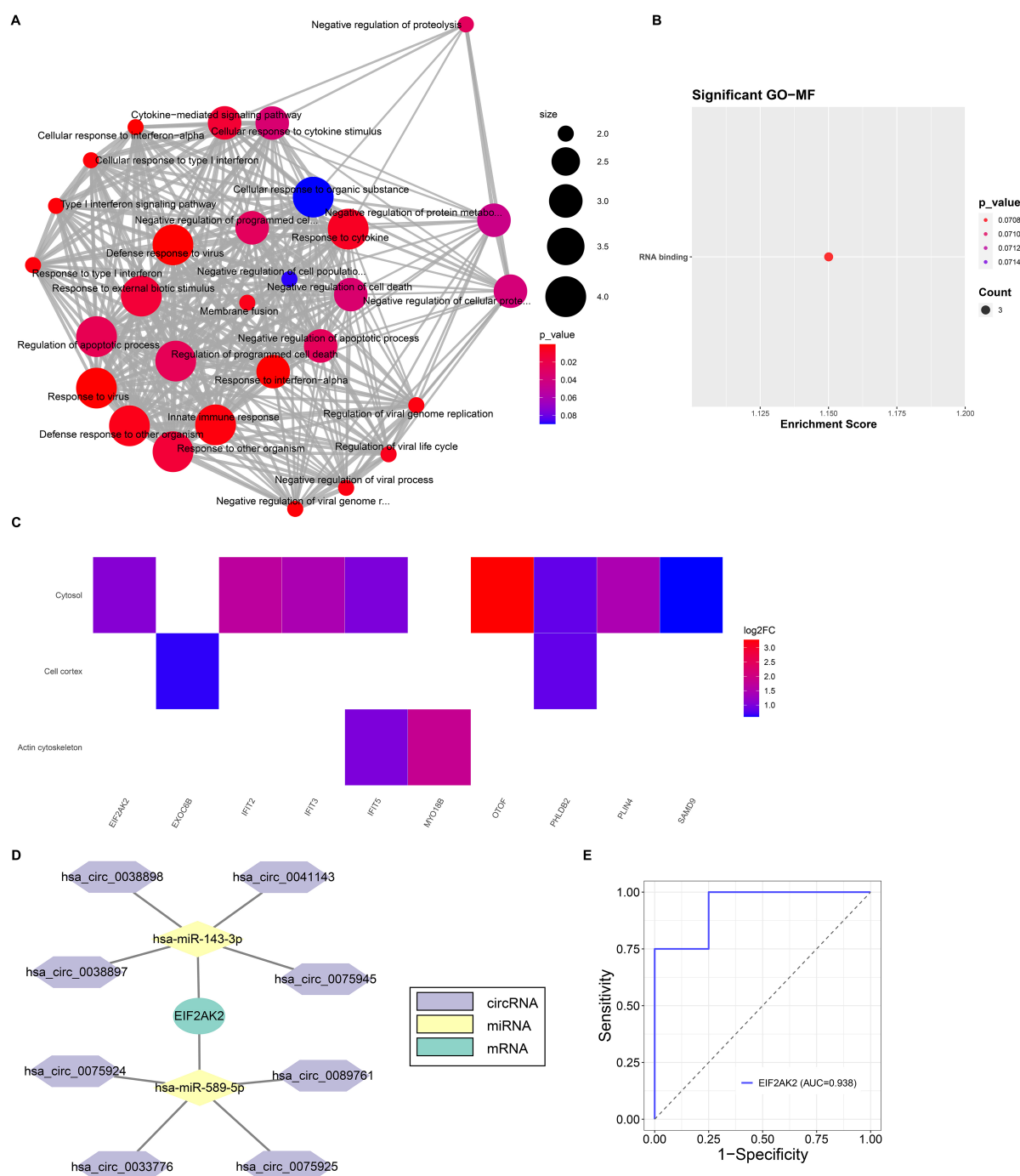


Fig. 3. Biological implications of mRNAs in the circRNA-miRNA-mRNA networks, and generation of a necroptosis-based sub-network. (A) The biological process network of mRNAs in the networks. (B) The significant molecular function. (C) The cellular components associated with mRNAs in the networks. (D) Generation of a necroptosis-based subnetwork. (E) Receiver operator characteristic curve (ROC) of eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*) in BPD diagnosis. GO, Gene Ontology; MF, molecular function; FC, fold change.

Biological Implications of mRNAs in the CircRNA-MiRNA-mRNA Networks

Various immunity-associated biological processes such as response to interferon-alpha, type I interferon, innate immune response, and response to cytokine, were no-

tably enriched by mRNAs in the circRNA-miRNA-mRNA networks (Fig. 3A). They exhibited the molecular function of RNA binding (Fig. 3B). Cellular components such as cytosol, cell cortex, and actin cytoskeleton were remarkably linked with such mRNAs (Fig. 3C).

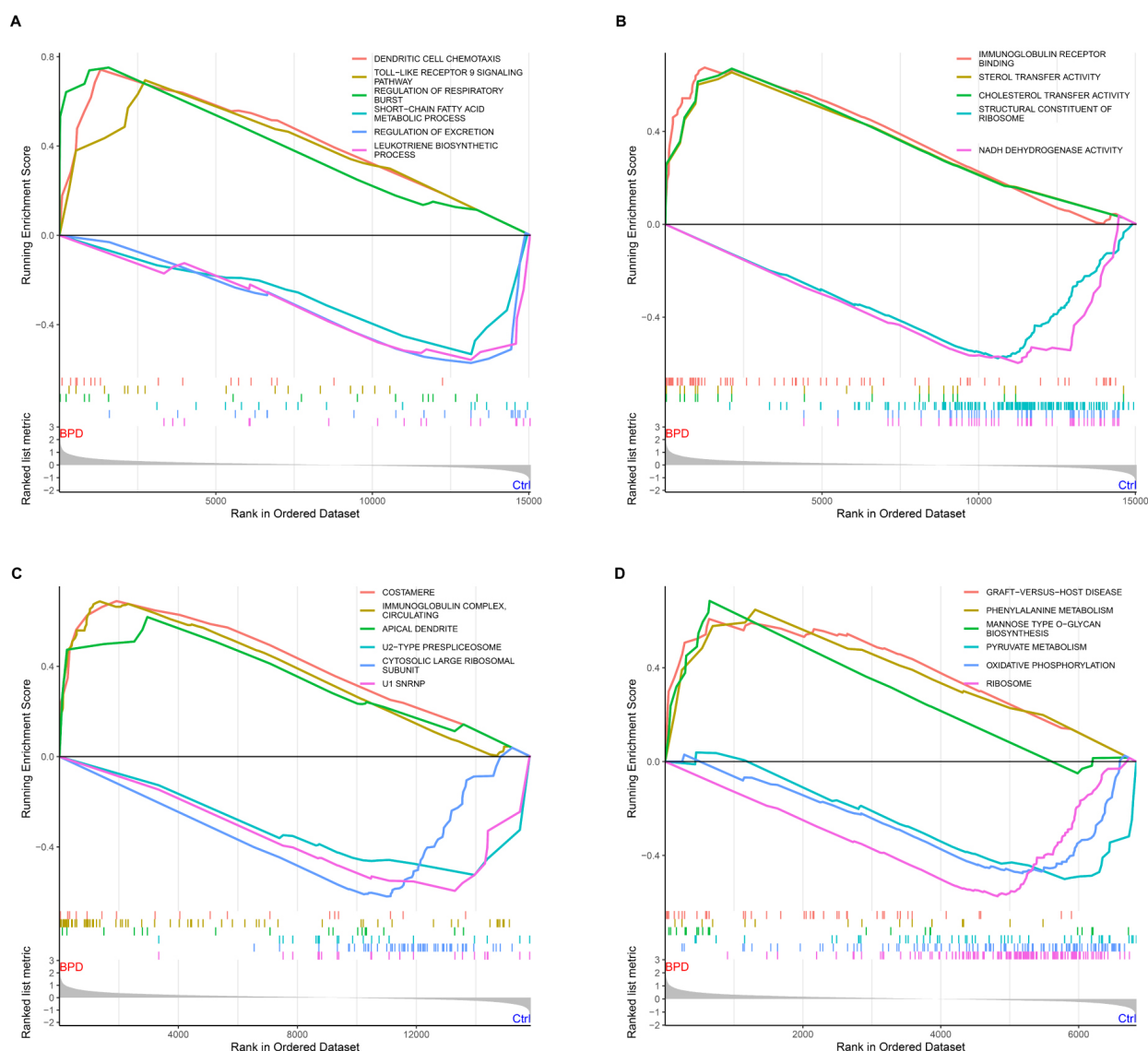


Fig. 4. Exploration of biological role of *EIF2AK2*. (A–D) Gene set enrichment analysis (GSEA) of the differences in (A) biological processes, (B) molecular functions, (C) cellular components, and (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in low versus high *EIF2AK2* expression samples.

Construction of a Necroptosis-Based Subnetwork and *EIF2AK2* in BPD Diagnosis

Among the mRNAs in the circRNA-miRNA-mRNA networks, *EIF2AK2* was a necroptosis gene. Subsequently, a necroptosis-based subnetwork was constructed. As illustrated in Fig. 3D, hsa_circ_0075945/hsa_circ_0038897/hsa_circ_0038898/hsa_circ_0041143-mediated hsa-miR-143-3p, while hsa_circ_0075924/hsa_circ_0075925/hsa_circ_0033776/hsa_circ_0089761-mediated hsa-miR-589-5p, were identified as potential regulator of *EIF2AK2* expression. Moreover, it was also demonstrated that *EIF2AK2* effectively diagnosed BPD in our RNA-seq dataset (area under the curve (AUC) = 0.938) (Fig. 3E).

Exploration of Biological Role of *EIF2AK2*

It was found that high *EIF2AK2* expression was associated with regulation of respiratory burst, dendritic cell chemotaxis, and toll-like receptor 9 signaling pathway (Fig. 4A). The negative correlations of *EIF2AK2* with regulation of excretion, leukotriene biosynthetic process, and short-chain fatty acid metabolic process were also observed. In Fig. 4B, up-regulated *EIF2AK2* samples exhibited the enrichment in immunoglobulin receptor binding, cholesterol transfer activity, and sterol transfer activity, while *EIF2AK2* down-regulation samples showed the enrichment in nicotinamide adenine diphosphate (NADH) dehydrogenase activity, NADH dehydrogenase (ubiquinone) activity, and structural constituent of ribosome. More-

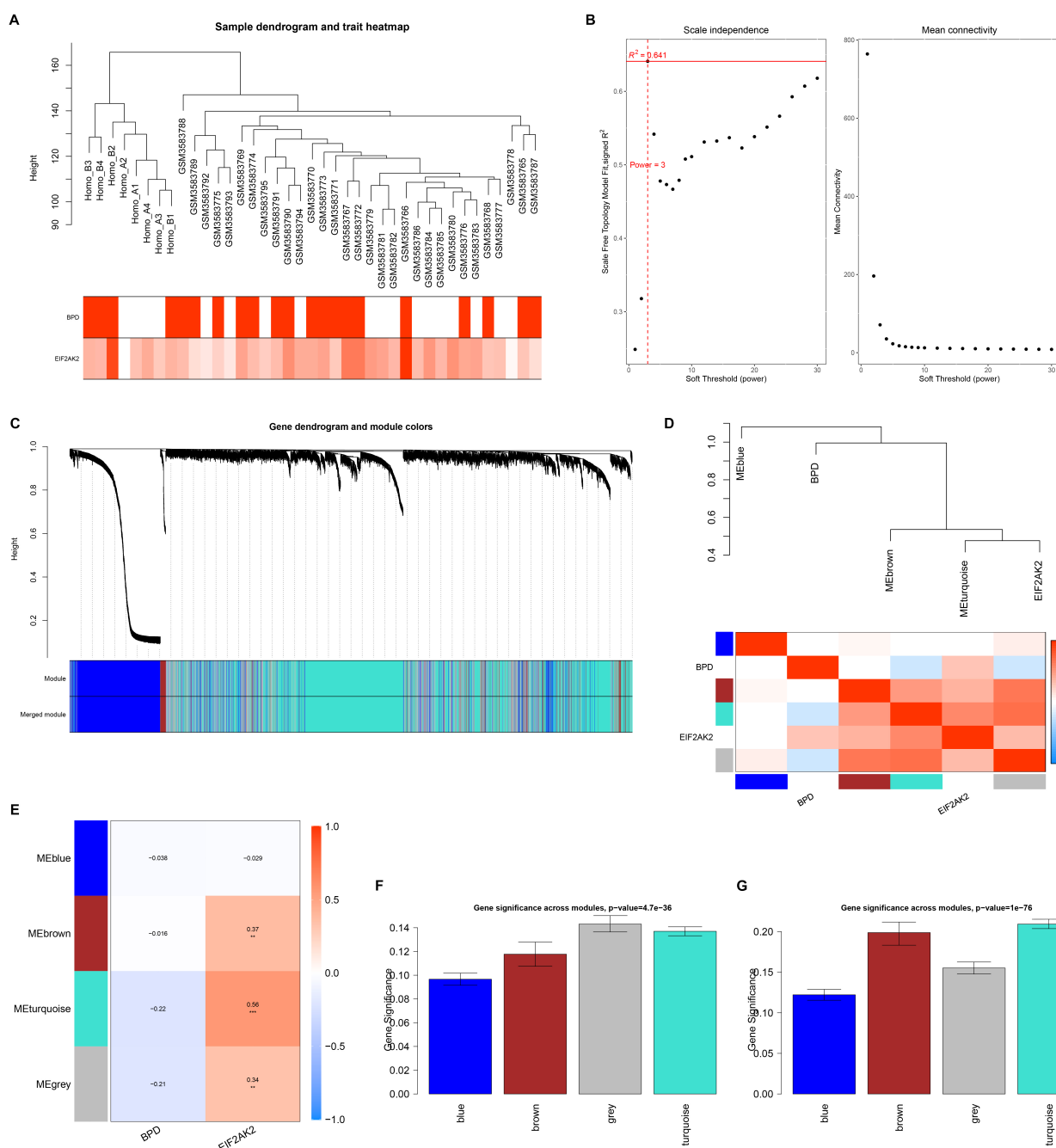


Fig. 5. Establishment of *EIF2AK2*-relevant co-expression modules. (A) Cluster dendrogram and heatmap of BPD and *EIF2AK2*. (B) Scale independence and mean connectivity under soft thresholding values. (C) Gene dendrogram and merged modules. (D) Relationships of modules with BPD and *EIF2AK2*. (E) Correlation analysis on modules with BPD and *EIF2AK2*. (F) Barplot illustrating the gene significance for BPD with modules. (G) Barplot demonstrating the gene significance for *EIF2AK2* with modules. $**p < 0.01$, $***p < 0.001$.

over, costamere, immunoglobulin complex, circulating, and apical dendrite presented the positive interactions with *EIF2AK2* up-regulation (Fig. 4C). Meanwhile, U1 SNRNP, cytosolic large ribosomal subunit, and U2-type prespliceosome were negatively associated with *EIF2AK2* expression. In addition, mannose type O-glycan biosynthesis, phenylalanine metabolism, and graft-versus-host disease

were enriched by up-regulated *EIF2AK2* specimens. While *EIF2AK2* down-regulation specimens showed enrichment in ribosome, pyruvate metabolism, and oxidative phosphorylation (Fig. 4D).

Establishment of EIF2AK2-Relevant Co-Expression Modules

Samples with mRNA expression profiles were utilized for WGCNA (Fig. 5A). To ensure that the constructed network adhered to the scale-free network features, soft thresholding value of 3 was chosen (Fig. 5B). Next, gene dendrogram was established, and four modules were merged (Fig. 5C). Turquoise module exhibited the strongest connection with *EIF2AK2* (Fig. 5D,E; $r = 0.56$). In addition, this module was strongly linked with BPD and *EIF2AK2* (Fig. 5F,G). Thus, genes in turquoise module might correlate to *EIF2AK2*. We selected genes with $r > 0.6$ as *EIF2AK2*-relevant genes (*IFI44*, *IFI44L*, *IGKV2-29*, *SIGLEC1*, *CXCL10*, *BATF2*, *SERPING1*, *MCOLN2*, *IFNG*, *OASL*, *IFIT3*, *HERC5*, *CARMIL3*, *IGKV3-20*, *IFIT2*, *KLHDC7B*, *IGHV3-15*, *IFI6*, *OAS3*, *IFITM3*, *MX1*, *TGFBR3*, *CD274*, *GBP1*, *IGHV3-7*, *AIM2*, *SPATS2L*, *GBP5*, *TNFAIP6*, *ISG15*, and *CLEC4E*).

Biological Roles and Interactions of EIF2AK2-Relevant Genes

Further analysis unveiled the close connections of *EIF2AK2*-relevant genes with immunity-associated biological processes, including the innate immune response, type I interferon, etc. (Fig. 6A). These genes were found to possess molecular functions of 2'-5'-oligoadenylate synthetase activity, adenylyltransferase activity, GTP binding, etc. (Fig. 6B). In addition, cellular components such as immunoglobulin complex, circulating, plasma membrane were notably enriched by *EIF2AK2*-relevant genes (Fig. 6C). Immunity-associated pathways were potentially modulated by these genes, especially Interleukin (IL)-17 signaling pathway and cell adhesion molecules (Fig. 6D–F). These *EIF2AK2*-relevant genes exhibited the tight connections at the protein level (Fig. 6G).

Molecular Classification of BPD Based upon EIF2AK2-Relevant Genes

Utilizing consensus clustering method, we classified BPD infants into two molecular subtypes based upon *EIF2AK2*-relevant genes (Fig. 7A). These *EIF2AK2*-relevant genes exhibited the higher transcript level in cluster2 compared with cluster1 (Fig. 7B). Cluster1 demonstrated the higher enrichment of regulation of animal organ formation, negative regulation of platelet activation, and an endocardial cushion formation, while showing the lower enrichment of negative regulation of viral genome replication, response to interferon-beta, and cellular response to type I interferon (Fig. 7C). As illustrated in Fig. 7D, ephrin receptor activity, cytoskeletal adaptor activity, and intermediate filament binding were notably enriched in cluster1, with immunoglobulin receptor binding, immunoglobulin binding, and antigen binding being enriched in cluster2. Photoreceptor disc membrane, nurd complex, and myosin II complex were enriched in cluster1, while immunoglob-

ulin complex, immunoglobulin complex, circulating, and condensed nuclear chromosome kinetochore presented the enrichment in cluster2 (Fig. 7E). Additionally, cluster1 was notably enriched by maturity onset diabetes of the young, phototransduction, and basal cell carcinoma, while cluster2 was enriched by graft-versus-host disease, other glycan degradation, and antigen processing and presentation (Fig. 7F).

EIF2AK2 Knockdown Alleviates Apoptosis of Hyperoxia-Induced BEAS-2B Cells

To stimulate BPD, lung epithelial cells BEAS-2B were exposed to hyperoxia. It was proven that *EIF2AK2* expression was remarkably up-regulated in hyperoxia-induced BEAS-2B cells ($p < 0.001$, Fig. 8A–C). Specific siRNAs against *EIF2AK2* were transfected into BEAS-2B cells for silencing *EIF2AK2* expression ($p < 0.0001$, Fig. 8D). Apoptosis was notably increased in hyperoxia-induced BEAS-2B cells, which was significantly alleviated by *EIF2AK2* silencing ($p < 0.0001$, Fig. 8E,F).

Discussion

BPD is a multifactorial chronic pulmonary pathology and a dominating factor in premature illness and death [32]. Its initiation and progression involve numerous factors, among which premature birth represents the most critical reason of BPD. Necroptosis affects the molecular and biological mechanisms of BPD and further development [8]. The work elucidated the molecular alterations in peripheral blood of BPD, with the specific focus on the post-transcriptional mechanisms of necroptosis gene *EIF2AK2*.

This study revealed the up-regulation of *IFIT3*, *OTOF*, *IFIT2*, *CPSF1P1*, *IL1R2*, *UBXN10*, *PHLDB2*, *MYO18B*, *ITPRIPL1*, *SAMD9*, *EIF2AK2*, *PLIN4*, *IFIT5*, and *EXOC6B* in peripheral blood of BPD, while *MTRNR2L1*, *RN7SL396P*, and *SSPO* exhibited the down-regulation. These aberrantly expressed mRNAs potentially resulted in BPD pathogenesis. Nonetheless, no study has proven their implication in BPD. Based upon aberrantly expressed mRNAs and miRNAs, we established the circRNA-miRNA-mRNA networks that unveiled the post-transcriptional regulatory mechanisms of mRNAs with aberrant expression. The mRNAs in the network were notably linked with diverse immunity-associated biological processes, such as response to interferon-alpha, type I interferon, innate immune response, and response to cytokine. The previous research characterized the evolution of the innate immune response in BPD murine models [33]. Macrophage-derived IL-6 trans-signaling has been proven as a target in the pathogenesis of BPD. IL-1 receptor antagonist may prevent murine BPD driven by perinatal inflammation and hyperoxia [34].

Among the circRNA-miRNA-mRNA networks, *EIF2AK2* necroptosis gene emerged as a key player,

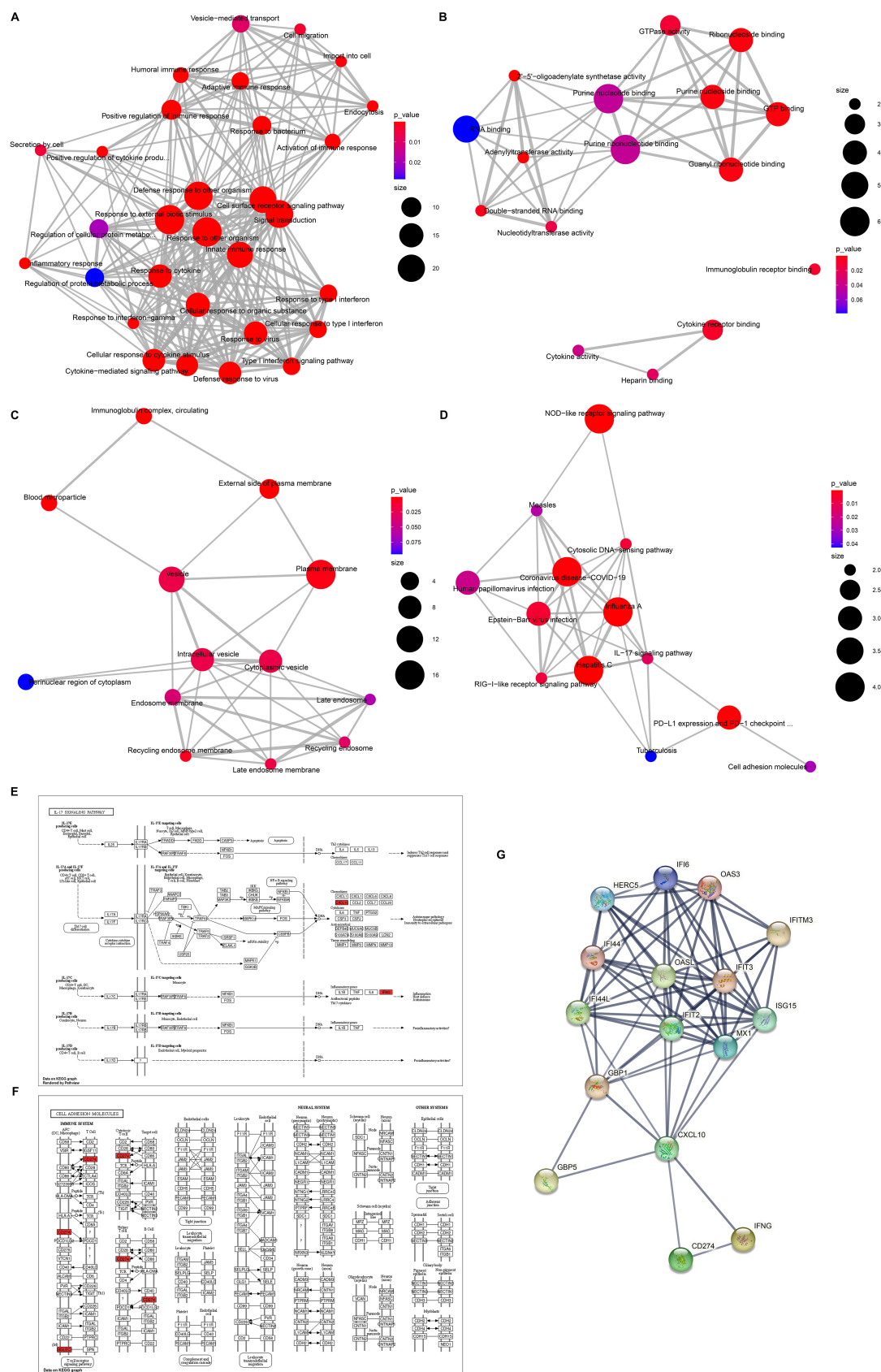


Fig. 6. Biological roles and interactions of *EIF2AK2*-relevant genes. (A–D) The networks of (A) biological processes, (B) molecular functions, (C) cellular components, and (D) KEGG pathways based upon *EIF2AK2*-relevant genes. (E,F) The graphs of Interleukin (IL)-17 signaling pathway and cell adhesion molecules. (G) The protein-protein interaction (PPI) network of *EIF2AK2*-relevant genes.

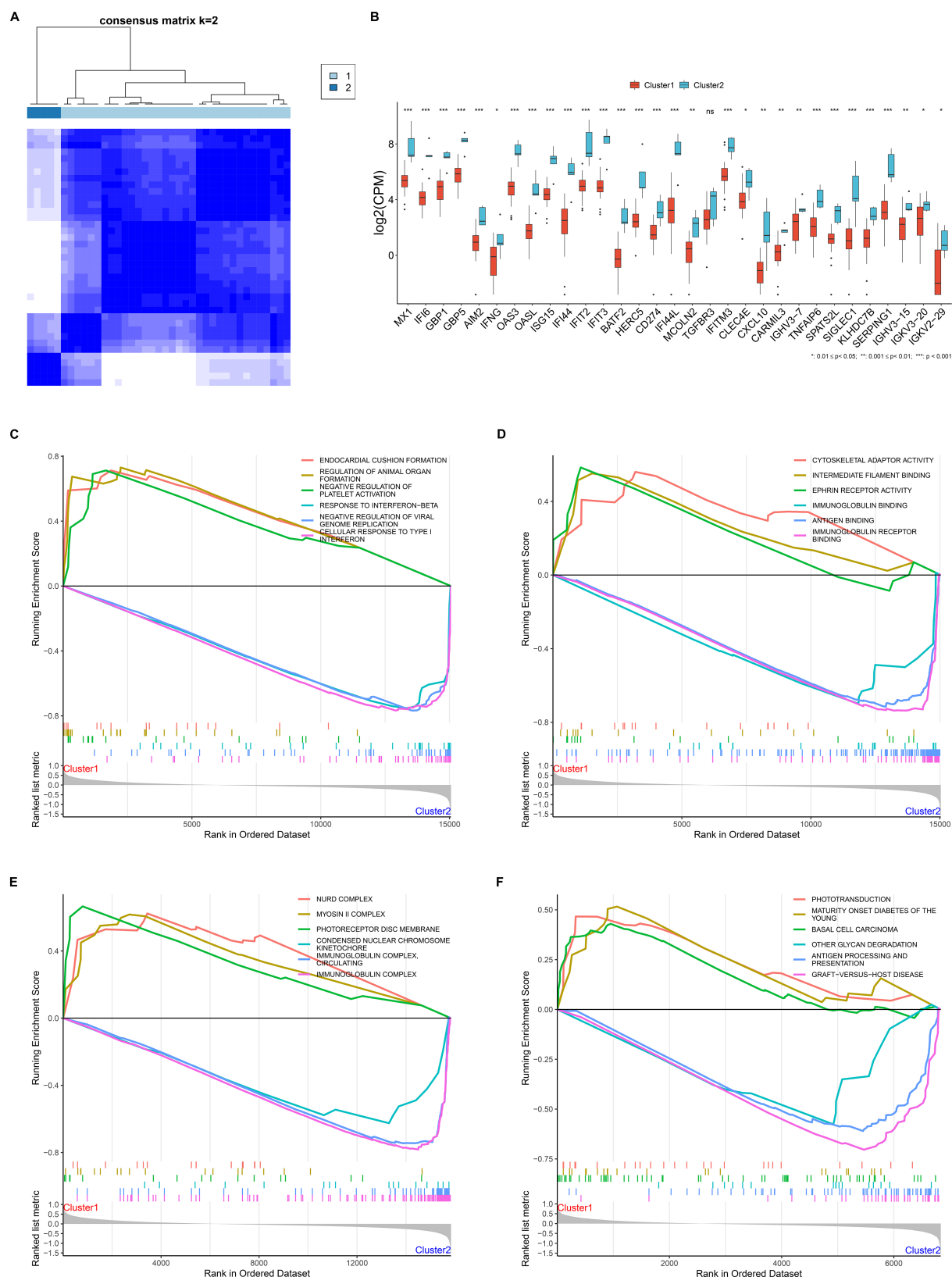


Fig. 7. Molecular classification of BPD based upon *EIF2AK2*-relevant genes. (A) Consensus matrix at $k = 2$. (B) Transcript level of *EIF2AK2*-relevant genes in two molecular subtypes. (C–F) Different enrichment of (C) biological processes, (D) molecular functions, (E) cellular components, and (F) KEGG pathways in two subtypes. ns: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

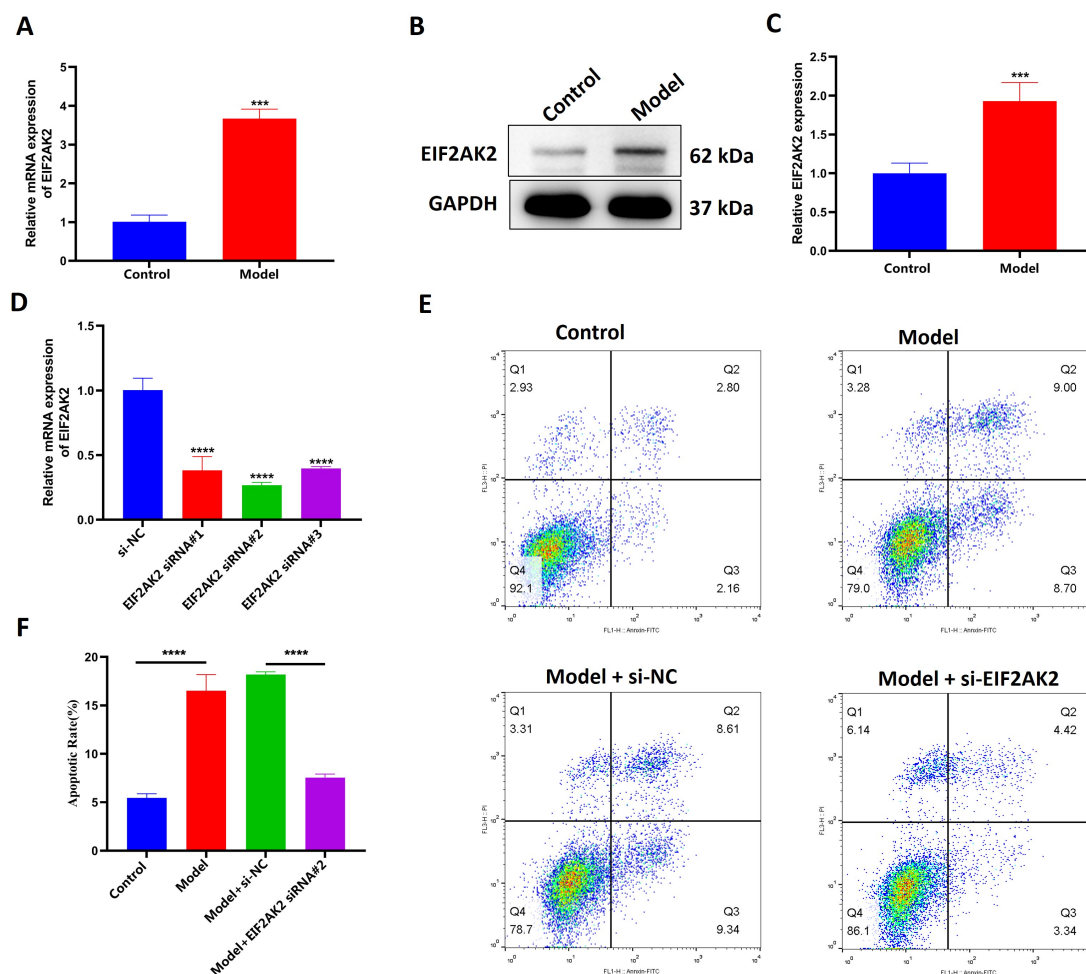


Fig. 8. *EIF2AK2* knockdown alleviates apoptosis of hyperoxia-induced BEAS-2B cells. (A) Real time-polymerase chain reaction (PCR) of *EIF2AK2* expression in normoxia- or hyperoxia-treated BEAS-2B cells. (B,C) Western blot of *EIF2AK2* expression in normoxia- or hyperoxia-induced BEAS-2B cells. (D) Real time-PCR of *EIF2AK2* expression in BEAS-2B cells transfected with specific siRNAs targeting *EIF2AK2*. (E,F) Apoptosis of BEAS-2B cells with hyperoxia or/and *EIF2AK2* siRNA transfection. *** $p < 0.001$; **** $p < 0.0001$. $n = 3$. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; si-NC, transfected with knockdown control siRNA; si-*EIF2AK2* (*EIF2AK2* siRNA), transfected with siRNA specifically knockdown *EIF2AK2* expression.

exhibiting promising potential for diagnosing BPD individuals. *In vitro* experiments demonstrated that silencing *EIF2AK2* attenuated apoptosis of hyperoxia-induced BEAS-2B cells. *EIF2AK2* exerts an essential role in both pathological and physiological processes. For instance, *EIF2AK2* mediates stress response as well as cell cycle progression [35]. *EIF2AK2* variants are associated with neurodevelopmental disorder [36]. In BPD, aberrantly expressed *EIF2AK2* were post-transcriptionally modulated by hsa_circ_0075945/hsa_circ_0038897/hsa_circ_0038898/hsa_circ_0041143-mediated hsa-miR-143-3p as well as hsa_circ_0075924/hsa_circ_0075925/hsa_circ_0033776/hsa_circ_0089761-mediated hsa-miR-589-5p. miR-143-3p represses leukemia cell proliferation by inhibiting *KAT6A* expression [37]. Hsa-miR-143-3p influences the pulmonary inflammatory factors and cell apoptosis in mycoplasma pneumonia through mediating

TLR4/MyD88/NF- κ B signaling [38]. Hsa-miR-143-3p controls *TGF- β 1*-mediated cellular proliferation as well as extracellular matrix generation in airway smooth muscle [39]. Hypermethylation in hsa-miR-589-5p promoter leads to *HDAC5* overexpression, thus promoting malignancy in non-small cell lung carcinoma [40]. Despite these associations, experimental verification is required to prove the post-transcriptional regulatory mechanisms of *EIF2AK2*.

Thirty-one *EIF2AK2*-relevant genes (*IFI44*, *IFI44L*, *IGKV2-29*, *SIGLEC1*, *CXCL10*, *BATF2*, *SERPING1*, *MCOLN2*, *IFNG*, *OASL*, *IFIT3*, *HERC5*, *CARMIL3*, *IGKV3-20*, *IFIT2*, *KLHDC7B*, *IGHV3-15*, *IFI6*, *OAS3*, *IFITM3*, *MX1*, *TGFB3*, *CD274*, *GBP1*, *IGHV3-7*, *AIM2*, *SPATS2L*, *GBP5*, *TNFAIP6*, *ISG15*, and *CLEC4E*) were determined, which may classify BPD into two unique molecular subtypes. Nonetheless, this molecular classification will be further verified in larger datasets.

Conclusions

Altogether, we proposed that necroptosis gene *EIF2AK2* acts as a key molecular determinant of BPD, and its aberrant expression was post-transcriptionally regulated by hsa_circ_0075945/hsa_circ_0038897/hsa_circ_0038898/hsa_circ_0041143-mediated hsa-miR-143-3p as well as hsa_circ_0075924/hsa_circ_0075925/hsa_circ_0033776/hsa_circ_0089761-mediated hsa-miR-589-5p. Additionally, a novel molecular classification of BPD was put forward based upon *EIF2AK2*-relevant molecules. It was experimentally proven that *EIF2AK2* knockdown alleviated apoptosis of hyperoxia-induced BEAS-2B cells. These findings enhance our understanding of the role of necroptosis in pathogenesis of BPD. Moreover, targeting the necroptosis gene *EIF2AK2* as a novel molecular therapy could potentially alleviate the progression of BPD.

Abbreviations

BPD, Bronchopulmonary dysplasia; mRNA, messenger RNA; miRNA, microRNA; ROC, receiver operator characteristic curve; WGCNA, weighted correlation network analysis; AUC, area under the curve; RIPK1, receptor interacting protein kinase 1; MLKL, mixed lineage kinase domain-like protein; circRNAs, circular RNAs; FC, fold change; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene set enrichment analysis; PPI, protein-protein interaction.

Availability of Data and Materials

All experimental data included in this study can be obtained by contacting corresponding author if needed.

Author Contributions

WJL, YHQ and LRL designed the research study. WJL and XKW performed the research. ZML and YYY provided help and advice on the experiments. QNH analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Eighth Affiliated Hospital of Guangxi Medical University (GYYXLL-20210601-18) and written informed consent was obtained from each patient in accordance with institutional guidelines. Human subjects, human materials or human data were conducted in accordance with the principles outlined in the Declaration of Helsinki.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.23812/j.biol.regul.homeost.agents.20243806.389>.

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