

The mechanism by which FGF23/FGFR-1 activates NOX2-ROS in vascular endothelial cells in the context of severe heat stroke-induced acute lung injury

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Abstract

Background: The high mortality rate of severe heat stroke is mainly related to multiple organ dysfunction syndrome (MODS), and respiratory failure caused by acute lung injury (ALI) is a significant factor in the development of MODS during the course of severe heat stroke. Previous research has demonstrated that severe heat stroke-induced acute lung injury (sHS-ALI) is associated with an increase in reactive oxygen species (ROS) in vascular endothelial cells (VECs), but the specific initiating factors and intermediate mechanisms involved are unclear.

Methods: In this study, the mRNA profiles of mouse lung tissues were analysed using high-throughput sequencing. Genome-wide knockout was performed using CRISPR-Cas9 technology to identify a cohort of differentially expressed genes that promote human umbilical vein endothelial cells survival after heat stress. The expression of key proteins [fibroblast growth factor 23 (FGF23), phosphorylated fibroblast growth factor receptor-1 (p-FGFR-1), FGFR-1, phosphorylated phospholipase C- γ 2 (p-PLC- γ 2), PLC- γ 2, p-p47^{phox}, p67^{phox}, p22^{phox}, p40^{phox}, and nicotinamide adenine dinucleotide phosphate oxidase isoform 2 (NOX2)] involved in the FGF23/FGFR-1 mechanism was examined using western blotting and immunohistochemistry.

Results: In this study, we first screened sHS-ALI target genes by cross-comparison *in vivo* and *in vitro* and found that FGF23 is the upstream promoter of sHS-ALI. Subsequent investigations involving the interference or inhibition of FGF23 expression revealed that FGF23 induced FGFR-1 Y766 phosphorylation during heat stress-induced VECs damage. In addition, FGF23 participated in NOX2 activation and ROS accumulation and was involved in the process of sHS-ALI. These findings indicated that the FGFR-1 Y766 site mutation strongly suppressed the production of p-PLC- γ 2 and heat stress-induced NOX2-ROS activation in VECs. More importantly, mutation of the FGFR-1 Y766 phosphorylation site had no effect on FGF23 expression, and it was impossible to significantly induce the expression of p-PLC- γ 2. Moreover, NOX2-ROS activation was inhibited, even in the presence of heat stress, the recombinant FGF23 protein, or combined stimulation.

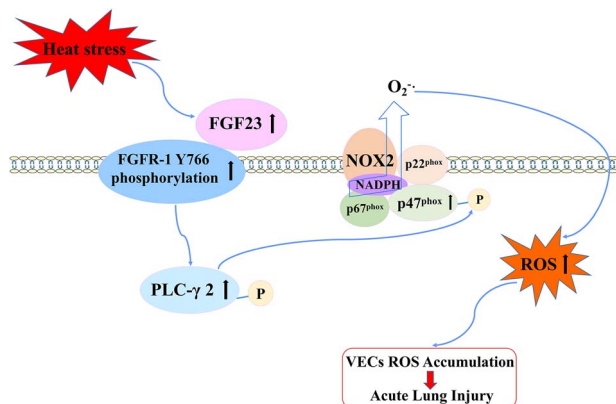
Conclusions: This study confirmed that FGF23/FGFR1 signalling, as an upstream priming factor, mediated NOX2-ROS activation in VECs after heat stress, thus participating in the sHS-ALI process. FGFR-1 Y766 phosphorylation is essential for FGF23/FGFR-1 signalling activation in VECs, which is involved in sHS-ALI. These findings further clarify the mechanism underlying sHS-ALI and contribute to reducing the mortality and morbidity of severe heat stroke.

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Graphical Abstract



Keywords: Severe heat stroke; Heat stress; Fibroblast growth factor 23; Fibroblast growth factor receptor-1; NOX2; Reactive oxygen species

Highlights

- FGF23 is a key upstream initiating factor involved in severe heat stroke-induced acute lung injury (sHS-ALI) and is a potential therapeutic target for intervention in the treatment of severe heat stroke.
- FGF23/FGFR1 signalling, an upstream priming factor that mediates NOX2 activation, enhances NADPH oxidase activity and ROS accumulation in vascular endothelial cells (VECs) after heat stress, thus participating in the process of sHS-ALI.
- FGFR-1 Y766 phosphorylation is critical for FGF23/FGFR-1 activation through PLC- γ 2 phosphorylation and further promotes NOX2-ROS activation in VECs after heat stress, and this process is involved in sHS-ALI.

Background

Heat stroke refers to a condition in which the human body is exposed to high temperature and high humidity for a long time and the central body temperature exceeds 40°C. These conditions can result in abnormalities in electrolyte acid–base metabolism and central nervous system dysfunction, which are symptoms of acute heat-induced illnesses that, in extreme cases, can progress to severe heat stroke [1,2]. Multiple organ dysfunction syndrome (MODS) frequently occurs in patients with severe heat stroke, and mortality rates can range from 40 to 70% [3,4]. Heat waves are summertime weather events that directly affect human health and have become one of the most noticeable aspects of global climate change in recent years due to global warming [5,6]. Severe heat stroke is becoming more common each year due to the high intensity and prolonged duration of heat waves, which has had a negative impact on people's lives, productivity, and even health in various areas [5,6]. However, little is known currently about the pathophysiology of severe heat stroke and how MODS is caused by it. As a result, it is critical to investigate the pathophysiology of severe heat stroke and develop appropriate preventative and therapeutic measures.

The respiratory system is one of the target organs most commonly damaged by severe heat stroke, and it has been established that the high fatality rate of this condition is mostly associated with MODS [7,8]. According to reports, respiratory failure caused by acute lung injury (ALI) is a significant precipitating factor of MODS during severe heat stroke, with respiratory failure occurring in 45–98.8% of patients with severe heat stroke prior to MODS [7,8]. Acute pulmonary congestion and pulmonary oedema are the primary clinical signs of ALI, which can develop in the early stages of severe heat stroke and cannot be stopped by active cooling therapy. Previous research conducted by our team also revealed that animals with ALI may experience pathological changes as a result of low-intensity heat stress. As heat-stress intensity and

duration increase, animals with severe heat stroke are more likely to experience lung tissue damage, which can manifest as bleeding, exudation, and damage to the alveolar epithelial cells in the alveolar cavity, among other symptoms [9,10]. However, the exact mechanism causing severe heat stroke-induced ALI (sHS-ALI) has not been determined.

The function of vascular endothelial cells (VECs) in the pathophysiology of MODS in severe heat stroke has garnered increasing interest in recent years due to advancements in research on this condition [11,12]. According to current theory, coagulopathies, systemic inflammatory responses resulting from vascular endothelial platforms, and direct heat exposure combine intricately to cause severe heat stroke and MODS progression [11,13]. The typical features of severe heat stroke have been validated using animal experimental investigations and clinical studies. These features include widespread injury to organ VECs, an inflammatory response, the creation of microthrombi, and significant bleeding and necrosis [11,14,15]. The data unequivocally show that VECs damage is a significant aspect of severe heat stroke and a key target cell in MODS onset and progression in severe heat stroke. As the first barrier in lung tissue to withstand heat stress, pulmonary microvascular endothelial cells (PMVECs) are crucial for the development of pulmonary oedema and the pulmonary inflammatory response that results from severe heat stroke. According to our team's earlier experimental findings, heat stress may cause PMVECs to undergo apoptosis and oxidative damage, which exacerbates the sHS-ALI process [16]. However, the exact mechanism by which PMVECs contribute to sHS-ALI at the molecular level, including the precise initiation event and interactions between different signalling pathways, remains unclear.

The involvement of reactive oxygen species (ROS) in heat stress in VECs has increasingly gained attention in recent years as research on severe heat stroke has progressed [12,16]. Nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase is one of the primary enzymes that mediates ROS formation in VECs, and it is one of the many sources of ROS. The seven major subunits of NADPH oxidase include dual oxidase1-2 (DUOX1-2) and nicotinamide adenine dinucleotide phosphate oxidase isoforms 1-5 (NOX1-5). The primary producers of ROS in VECs are NOX2 and NOX4. Research has indicated that upon stimulation by Angiotensin II (Ang II) and inflammatory substances (e.g. tumor necrosis factor- α , TNF- α), NOX2 activity is upregulated in VECs without influencing NOX4 activity. This leads to excess ROS and ultimately endothelial dysfunction [17,18]. It has been demonstrated that NOX2 (gp91^{phox}), p22^{phox}, p47^{phox}, p67^{phox}, p40^{phox}, and the small G protein Rac form a protein complex known as activated NOX2. When NADPH oxidase is inactive, p47^{phox}, p67^{phox}, and p40^{phox} separate from NOX2 and p22^{phox} and form complexes in the cytoplasm. When p47^{phox} is activated, it migrates to the membrane and is phosphorylated to form biologically active NADPH oxidase complexes with NOX2 and p22^{phox}, initiating subsequent reactions that produce ROS and contribute to the regulation of cellular functions [18,19]. Therefore, more research is required to determine whether heat stress activates NADPH oxidase, which is crucial for ROS accumulation in VECs and the subsequent development of sHS-ALI. The upstream signalling molecules induced by heat stress resulting from NOX2-ROS activation must also be identified.

Here, mRNA sequencing of mouse lung tissue was performed to identify the transcriptionally differentially expressed genes (DEGs) associated with sHS-ALI to identify the upstream initiating factors of sHS-ALI. To identify prospective DEG cohorts that support human umbilical vein endothelial cells (HUVECs) survival under heat stress, CRISPR-Cas9 technology was utilized to screen for full-genome knockouts (19 050 genes) in HUVECs. In light of the aforementioned findings and additional verification data, it is hypothesized that fibroblast growth factor 23 (FGF23) may be an upstream initiating factor in sHS-ALI. Future research is needed to confirm this theory and further elucidate the mechanism by which FGF23 mediates NOX2-ROS activation in VECs following heat stress and participates in the sHS-ALI process.

Methods

Isolation of primary PMVEVs

Primary PMVECs were isolated from lung tissues of healthy newborn C57BL/6 J mice. Fresh lung tissues were isolated from the mice and washed with PBS supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 100 μ g/ml) (Invitrogen). The pleura was carefully removed to obtain ~1 mm of the outer edge of the remaining lung tissue without large blood vessels, and the lung tissue was again washed with phosphate buffer saline (PBS) mixed with antibiotics. The tissues were cut into pieces, placed in 60-mm cell dishes (Corning), and cultured in dulbecco's modified eagle medium (DMEM); (Gibco) supplemented with 10% foetal bovine serum (FBS, Pricella), 1% penicillin-streptomycin and 30 μ g/l bovine endothelial-mitogen (GlpBio). The tissue culture mixture was incubated in a humidified incubator at 37°C with a 5% CO₂ atmosphere. The tissue was removed after 60 h and the medium was changed every 2 days thereafter. After reaching 85% confluence, the cells were treated with 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco). Cell isolates

were characterized by their cobblestone morphology and immunofluorescence staining for CD31 and von Willebrand factor.

Cell culture and treatments

HUVECs and PMVECs maintained in culture media culture dishes were sealed with parafilm and immersed for 2 h in a circulating water bath at 37 $\pm$ 0.5°C (control) or at 43 $\pm$ 0.5°C (heat stress) [12,20]. Culture medium was replaced with fresh medium, and the cells were further incubated at 37°C for the indicated times.

Lentivirus production and GeCKO library purification

Lentivirus was produced as previously described by Hsu *et al.* [21]. Briefly, 4 μ g of a lentiCRISPR plasmid library (#1000000048, Addgene) was cotransfected with 2 μ g of pVSVg (#8454, Addgene) and 6 μ g of psPAX2 (#12260, Addgene) packing plasmids into human 293 T cells in a 10 cm² dish using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h, the medium was collected and centrifuged at 3000 rpm at 4°C for 10 min to pellet the cell debris. The supernatant was filtered through a 0.45- μ m filter (Pall, KA2DBLP6G).

Heat stress resistance screen using the GeCKO library

Cells were transduced with the GeCKO library at a multiplicity of infection (MOI) of 0.4 to ensure that most cells received only one viral construct. Briefly, 2x10⁷ HUVECs per 10-cm dish were plated in DMEM containing 10% foetal calf serum. The lentivirus was added to each dish with 8 μ g/ml polybrene (Sigma). At 48 h after infection, the culture medium was replaced with fresh medium containing 1 μ g/ml puromycin. At 96 h after puromycin selection, the cells were subjected to heat stress for 48 h, and the surviving cells were collected for genomic DNA isolation.

Plasmid construction and stable transfection

The pcDNA3.1-FGF23 Y766F and pcDNA3.1-Empty vectors were constructed by Cyagen Biosciences (Guangzhou, China). Cells were transfected with empty vector (pcDNA3.1) or the indicated constructs (pcDNA3.1-FGF23 Y766F) using Lipofectamine 3000. Culture medium containing G418 was used to select stable transfectants. After 48 h of incubation at 37°C in a humidified atmosphere of 5% CO₂, the cells were exposed to heat stress for further experiments.

Small interfering RNA transfection

Small interfering RNA (siRNA) targeting FGF23 was designed and synthesized by Cyagen Biosciences (Guangzhou, China). The sequence of the FGF23 gene and its scrambled sequence are shown as follows: sense and antisense siRNAs, 5'-GAUCCACAAGAAUGGCCAATT-3' and 5'-AUGGCCAUUCUUGUGGAUUCTT-3', respectively. For the scrambled control, the sense and antisense siRNAs used were 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3', respectively. At 24 h prior to transfection, cells were plated onto a 6-well plate (Nest, Biotech, China) at 30-50% confluence and were then transfected into cells using TurboFect™ siRNA Transfection Reagent (Fermentas, Vilnius, Lithuania) according to the

manufacturer's protocol. After 48 h of incubation at 37°C in a humidified atmosphere of 5% CO₂, the cells were exposed to heat stress for further experiments.

FGF23 assay

Extracellular FGF23 levels in culture medium were evaluated using a mouse FGF23 enzyme linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Cells were seeded in 12-well plates and subjected to heat stress as described above. Then, the cell supernatant was collected, and FGF23 assays were performed. The absorbance was measured at 450 nm with an iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). The data were normalized to the FGF23 levels and are expressed as a percentage of the respective levels measured in the control set as 100%.

Measurements of ROS

After exposure to 43°C heat stress for 2 h, the cells were further incubated at 37°C for the indicated times. Intracellular and mitochondrial ROS (O₂^{•-}) was detected using the fluorescent probe dihydroethidium (DHE; Molecular Probes). The cells were incubated with 1 μM DHE (red fluorescence) for 30 min at 37°C in the dark. The fluorescence intensity of intracellular ROS (O₂^{•-}) images were captured using laser scanning confocal microscopy.

Animals

Pathogen-free 6–8-week-old male C57BL/6 mice were purchased from the Experimental Animal Center of Southern Medical University in Guangzhou, P. R. China (Certification: SCXK (YUE) 2021–0041). The animals were housed individually under controlled environmental conditions with a 12-hour light/dark cycle and unrestricted access to food and water throughout the study. All efforts were made to reduce the number of animals used and to minimize animal discomfort. The experimental protocols were approved by the Animal Care and Use Committee of Southern Medical University, Guangzhou, China. None of the authors are members of this committee. Care of the animals was in accordance with the National Institutes of Health Guidelines as well as with the Chinese National Guidelines.

Induction of severe heat stroke

The animals in the heat stress group were placed in a prewarmed incubator maintained at 35.5 ± 0.5°C with a relative humidity of 60 ± 5% in the absence of food and water. The rectal core temperature (*T_c*) was continuously monitored with a rectal thermometer until it reached 42°C. The animals in the control group were sham-heated at a temperature of 25 ± 0.5°C and a humidity of 35 ± 5% for a time comparable to that of the severe heat stress (HS) group [12,20]. The mice were sacrificed at the indicated times after severe heat stress, and serum and lung tissue were isolated.

Experimental groups and drug administration

The first experiment revealed that FGF23 is involved in regulating the progression of sHS-ALI. Mice were randomized into the control group, the FGF23 recombinant protein group (FGF23 recombinant protein, 100 mg/kg body weight, intraperitoneal), the severe heat stroke (HS)

group, or the FGF23 Ab group (FGF23 neutralizing antibody, 10 mg/kg body weight, intraperitoneal). Further experiments revealed that FGF23 causes phospholipase C-γ2 (PLC-γ2) phosphorylation, thereby facilitating NOX2 activation and ROS accumulation by inducing fibroblast growth factor receptor 1 (FGFR-1) Y766 phosphorylation during HS-ALI. Mice were randomized into the control group, the HS group, the FGF23 Ab group, the FGFR-1 inhibitor group (SU5402, 10 mg/kg body weight, intraperitoneal), and the PLC-γ2 inhibitor U73122 group (U73122, 30 mg/kg body weight, intraperitoneal), with *n* = 6 per group.

Whole transcriptome library preparation and RNA-sequencing

Total RNA from the lung tissue of control mice and HS mice was extracted using a TRIzol reagent kit according to the manufacturer's protocol, and eukaryotic mRNA was enriched using oligo(dT) beads. The enriched mRNA was cut into short fragments using fragmentation buffer, diluted to 100 ng/μL, and reverse transcribed to cDNA using random primers. Second-strand cDNA was synthesized using deoxyribonucleoside triphosphates (dNTPs), Deoxyribonuclease I (DNase I), Ribonuclease H (RNase H), and buffer solution, and purified with a QiaQuick PCR extraction kit. After end repair and the addition of a poly(A) tail, the fragments were connected to an Illumina sequencing adapter and selected by agarose gel electrophoresis. The sequences were amplified using PCR and sequenced on the Illumina HiSeq2500 platform by Gene Denovo Biotechnology Co. The clean reads were filtered and aligned with ribosomal RNA. DEseq2 was used to determine differences between groups, and sequences with a *l*fold changel ≥ 2 and *Q* < 0.005 were considered to be differentially expressed.

Western blot analysis

Protein was extracted from cells or aortic endothelium from all treatment groups using a total protein extraction kit or a mitochondria fractionation kit (Beyotime Institute of Biotechnology, China) following the manufacturer's protocols. The protein concentrations of the extracts were determined using an enhanced BCA protein assay kit (Beyotime Institute of Biotechnology, China). Western blot analysis was performed as described previously using the following antibodies: FGF23, phosphorylated (p)-FGFR-1, FGFR-1, p-PLC-γ2, PLC-γ2, p-p47^{phox}, p67^{phox}, p22^{phox}, p40^{phox}, and NOX2 (all used at 1 : 1000; Abcam or Cell Signaling Technology). An horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibody was used as the secondary antibody (Zhongshan Inc., China), and the signal was visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Histopathological evaluation of lung tissue

To visualize changes in morphology, lung tissue was harvested, fixed in 4% paraformaldehyde, embedded in paraffin and sliced into 5 μm-thick sections before being stained with hematoxylin-eosin (H&E) staining. H&E staining was evaluated by a pathologist who was blinded to the experimental groups. The severity of the pathological injury to the lung tissue was evaluated based on the presence of pulmonary interstitial oedema and alveolar haemorrhage. The injury

scores ranged from 0 (normal) to 4 (extremely severe), and the total score was calculated.

Ultrastructural observation with transmission electron microscope

Lung tissue was cryosectioned in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h and then washed in PBS for 2 days. The samples were then fixed in 1% osmic acid, dehydrated with ethanol and acetone gradients, embedded in epoxy resin, and cut into ultrathin sections. The tissue sections were stained with uranyl acetate and lead citrate and then observed under an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry for lung tissue was performed on paraffin-embedded sections as described. Primary antibodies against FGF23 (1 : 500, Abcam), p-FGFR-1 (1 : 200, Abcam), p-PLC- γ 2 (1 : 200, Abcam), phosphorylated p47phox (p-p47^{phox}; 1 : 250, Abcam) and NOX2 (1 : 500, Abcam) were diluted in PBS containing 1% BSA and incubated on slides overnight at 4°C. The next day, the slides were washed thrice in PBS and incubated with the appropriate HRP-conjugated secondary antibodies (1 : 2, Pervision) for 60 min at room temperature; staining was developed using DAB+ Chromogen (Dako). Then, five random fields of each section at 200 $\times$ were photographed under an Olympus microscope, and the numbers of FGF23-, p-FGFR-1-, p-PLC- γ 2-, p-p47^{phox}- and NOX2-positive cells are presented as the average number of positive cells per field.

Terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling staining

A cell apoptosis assay of lung tissue was performed using a terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling (TUNEL) kit (Roche, USA). The lung sections from each sample (4 μ m) were deparaffinized and rehydrated with ethanol at concentrations of 100, 95, and 75% (v/v) for 5 min each. After rinsing with distilled water, proteinase K (10 μ g/ml, Roche) was added to the lung sections for 15 min at 37°C, and the sections were then washed again three times in Tris-buffered saline (TBS; each time for 5 min). Equilibrium solution was added to each lung section for 10 min. A TUNEL assay kit (Roche, USA) was used to determine the percentage of apoptotic cells in the lung tissue. The signals were detected using a fluorescence microscope (Olympus, Tokyo, Japan). DAPI (Roche, USA) was used to stain the nucleus. TUNEL-positive cells were counted in three fields of a lung section.

Statistical analysis

All the data were analysed for statistical significance using SPSS 13.0 software (SPSS, Chicago, IL, USA). The data are expressed as the means $\pm$ SDs from at least three independent experiments performed in duplicate. Differences between two groups were assessed using Student's *t* test. Statistical comparisons of the results among multiple groups were performed using one-way analysis of variance. A *P* value < 0.05 was considered to indicate statistical significance.

Results

Gene ontology term enrichment analysis and Kyoto encyclopedia of genes and genomes pathway analysis of the upregulated DEGs in the lung tissue of sHS-ALI mice

Both H&E staining and transmission electron microscopy (TEM) revealed significant lung injury after severe heat stroke, and the histological grade of the HS group was significantly greater than that of the control group ([supplementary Fig. 1](#), see online supplementary material). mRNA profiles in mouse lung tissues were analysed using high-throughput sequencing. A total of 30 731 mRNAs were identified from lung specimens, and based on the criteria of fold change ≥ 2 and *P* value ≤ 0.05 , a total of 2006 genes were identified as differentially expressed between the control group and HS group. Approximately 1169 genes were markedly upregulated ([supplementary Table 1](#), see online supplementary material), suggesting that these 1169 DEGs were involved in sHS-ALI mice. In addition, 837 genes were significantly downregulated >2-fold in the HS group compared with the control group. The volcano and scatter plots of all genes are shown in [Fig. 1a](#). As shown in [Fig. 1b](#) and [c](#), the upregulated DEGs in sHS-ALI were studied using gene ontology (GO) enrichment analysis and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis.

Genome-wide knockout using CRISPR-Cas9 technology to screen for DEG knockout cohorts that promote HUVECs survival after heat stress

Previous studies have shown that VECs injury is involved in the formation of sHS-ALI. Here, we performed genome-wide knockout using CRISPR-Cas9 technology to identify DEG knockout cohorts that promote HUVECs survival after heat stress. We introduced a GeCKO library containing 123 411 sgRNAs targeting 19 050 human genes into lentiviral vectors and generated a pool of cells in which every targeted gene theoretically carried a loss-of-function mutation. After the pooled cells were treated at 43°C for 2 h and further incubated at 37°C for 48 h, viable cells were collected for analysis of the expressed sgRNAs. The sgRNAs amplified from the surviving cells were determined using next-generation sequencing, and the candidate genes were ranked depending on the number of unique sgRNAs vs. the number of next-generation sequencing reads. As shown in [Fig. 1d](#) and [Supplementary Table 2](#) (see online supplementary material), two separate experiments were conducted, 10 011 genes were copied, and 597 genes in which three sgRNAs appeared in a single gene in at least one experiment were selected for GO and KEGG analysis. These results also indicated that knocking out these 597 DEGs could promote HUVECs survival during heat stress. As shown in [Fig. 2e](#) and [f](#), GO enrichment analysis and KEGG pathway analysis were performed for 597 knockout DEGs.

Cross-comparison of *in vivo* and *in vitro* experiments to screen common target DEGs that promote HUVECs survival after knockout during heat stress and participate in sHS-ALI

To search for commonly expressed DEGs involved in promoting HUVEC survival after heat stress (597 DEGs) and participating in sHS-ALI (1169 DEGs), further experiments were conducted to compare and screen the experimental data

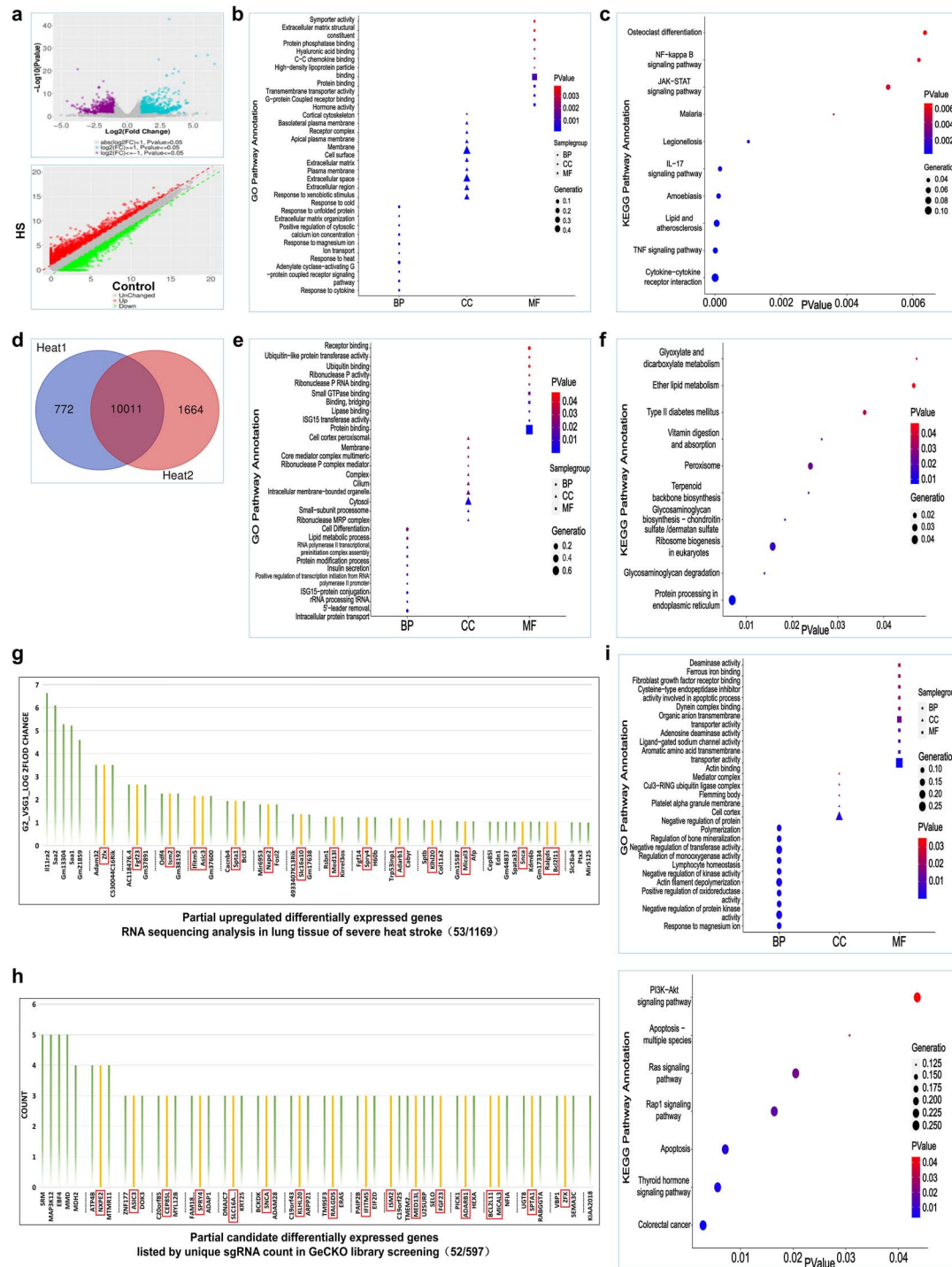


Figure 1. Cross-comparison of *in vivo* and *in vitro* experiments to screen common target DEGs that promote HUVECs survival after knockout during heat stress and participate in sHS-ALI. Animals in the control group were sham heated at $25 \pm 0.5^\circ\text{C}$ and a humidity of $35 \pm 5\%$ for a time comparable to that of the HS group. The animals in the HS group were placed in a temperature-controlled chamber (ambient temperature $35.5 \pm 0.5^\circ\text{C}$ and $60 \pm 5\%$ relative humidity) and their rectal core temperature (T_c) was continuously monitored with a rectal thermometer until it reached 42°C . The mice were sacrificed 6 h after HS and the serum and lung tissue were isolated. **(a)** Volcano and scatter plots show up-regulated and down-regulated DEGs in lung tissue from severe heat stroke. **(b)** GO annotation of up-regulated DEGs in lung tissue from severe heat stroke. **(c)** KEGG pathway annotation of up-regulated DEGs in lung tissue from severe heat stroke. Genome-wide knockout using CRISPR-Cas9 technology was used to screen for knockout gene cohorts that promote HUVECs survival after heat stress. **(d)** Venn diagram of two GeCKO sorting results; there are 10 011 overlapping genes. **(e)** GO annotation of 597 genes in which three sgRNAs appeared in a single gene in at least one experiment in HUVECs from heat stress. **(f)** KEGG pathway annotation of 597 genes in which three sgRNAs appeared in a single gene in at least one experiment in HUVECs from heat stress. **(g)** Partial upregulated DEGs in RNA sequencing analysis (53/1169; DEGs in the red rectangle represent commonly expressed DEGs). **(h)** Partial candidate genes listed by unique sgRNA count in GeCKO library screening (52/597; DEGs in the red rectangle represent commonly expressed DEGs). **(i)** GO annotation of 17 common DEGs both in sHS-ALI and that promote HUVECs survival after knockout during heat stress. KEGG pathway annotation of common DEGs both in sHS-ALI and that promote HUVECs survival after knockout during heat stress

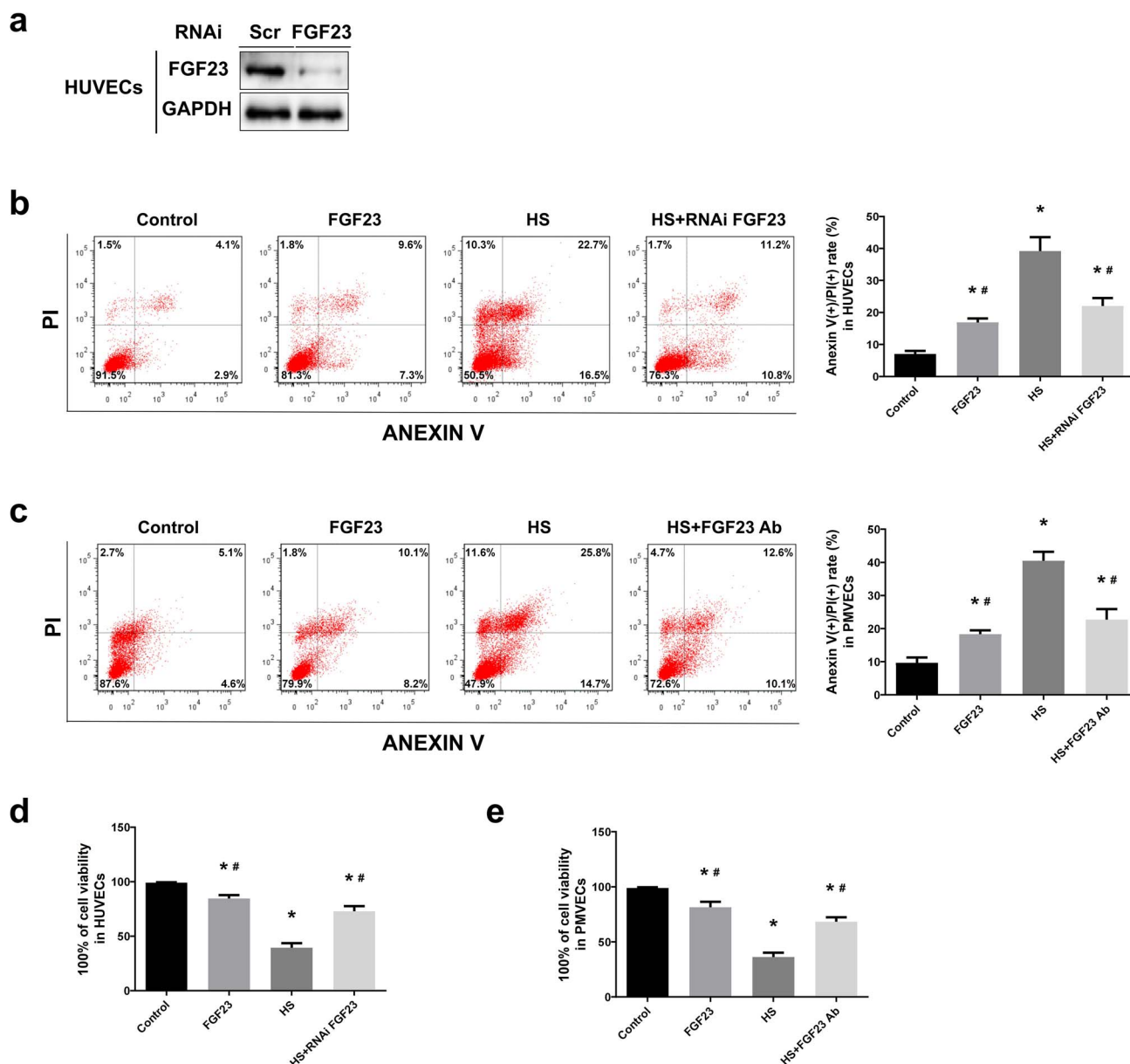


Figure 2. FGF23 is involved in VECs damage after heat stress. HUVECs were transfected with FGF23 siRNA 48 h in advance, exposed to a temperature of 43°C for 2 h, and then further incubated at 37°C for 6 h. The FGF23 recombinant protein-treated group was used to stimulate HUVECs or PMVECs for 2 h with FGF23 recombinant protein (10 ng/ml). In the HS + FGF23 Ab-treated group, PMVECs were incubated with FGF23 neutralizing antibody (5 μ g/ml) for 2 h in advance, and then exposed to 43°C for 2 h and incubated at 37°C for the indicated duration of 6 h. (a) Interference efficiency of FGF23 in HUVECs was detected by western blot. (b) Overall apoptosis rate of HUVECs in each group was detected by Annexin-V/PI, and the overall apoptosis rate of HUVECs in each group was analyzed in the histogram. (c) Overall apoptosis rate of PMVECs in each group was detected by Annexin-V/PI, and the overall apoptosis rate of PMVECs in each group was analyzed in the histogram. (d) Cell viability of HUVECs in each group was measured by CCK-8. (e) Cell viability of PMVECs in each group was measured by CCK-8. * $P < 0.05$, compared to the control group; # $P < 0.05$, compared to the HS group. CCK-8 Cell counting kit-8

in vivo and *in vitro*. As shown in Fig. 1g and h, a total of 17 common DEGs were found to be involved in sHS-ALI and promoted HUVECs survival after knockout during heat stress. As shown in Fig. 1i, GO enrichment analysis was performed for these 17 commonly expressed DEGs. KEGG pathway analysis revealed that 7 DEGs were involved in seven pathways, namely, colorectal cancer, thyroid hormone signalling pathway, apoptosis, Rap1 signalling pathway, Ras signalling pathway, apoptosis-multiple species, and PI3K-Akt signalling pathway (Fig. 1i), and these 7 DEGs included RALGDS, FGF23, SLC16A10, MED13L, SPTA1, RALGDS, and

BCL2L11. Further review of previous studies revealed that FGF23 can cause oxidative stress injury or inflammatory responses through the above pathways, such as the Rap1, Ras, PI3K-Akt, and other signalling pathways, and aggravate tissue and organ damage in chronic kidney disease, cardiovascular disease, asthma, chronic obstructive pulmonary disease, acute burn, and other diseases [22–26]. Our team's previous studies revealed that sHS-ALI is related to oxidative stress and the inflammatory response of VECs, and it is hypothesized that FGF23 potentially represents an upstream target gene during sHS-ALI.

Heat stress results in increased FGF23 levels both *in vivo* and *in vitro*

To assess FGF23 expression both *in vivo* and *in vitro* after heat stress, first, FGF23 expression was detected in the serum of mice with severe heat stroke. FGF23 serum levels were significantly increased in mice with severe heat stroke (supplementary Fig. 2a and c, see online supplementary material), and FGF23 mRNA and protein expression in the lung tissue of mice with severe heat stroke were significantly increased (supplementary Fig. 2b and c). Heat stress increased the levels of FGF23 mRNA and protein in HUVECs and PMVECs and increased FGF23 levels in the media used to grow HUVECs and PMVECs (supplementary Fig. 2d–j).

FGF23 is involved in sHS-ALI by mediating VECs damage after heat stress

To clarify the role of FGF23 in VECs after heat stress, RNA interference (RNAi) was used to knock out FGF23 expression in HUVECs, and western blot analysis revealed that FGF23 expression could not be detected after interference (Fig. 2a). As shown in Fig. 2b–e, the overall apoptosis rate of VECs significantly increased after heat stress, whereas cell viability significantly decreased. However, after FGF23 expression was inhibited with RNAi or an FGF23 neutralizing antibody, the overall apoptosis rate of HUVECs or PMVECs decreased significantly, and the viability of HUVECs or PMVECs significantly improved after heat stress. In addition, the overall apoptosis rate of HUVECs and PMVECs increased with FGF23 recombinant protein stimulation alone, whereas cell viability decreased (Fig. 2b–e).

Subsequent *in vivo* experiments showed that the FGF23 neutralizing antibody significantly inhibited FGF23 expression in the lung tissue of mice after severe heatstroke (Fig. 3a). TUNEL staining revealed that apoptosis in the lung tissue of mice with severe heat stroke was significantly attenuated after treatment with the anti-FGF23 neutralizing antibody (Fig. 3b). The H&E staining results showed that the anti-FGF23 neutralizing antibody significantly reduced pathological injury to the lung tissue of mice with severe heat stroke, and the effect primarily manifested as reduced exudate in the alveolar cavity, relief of capillary dilation, congestion and bleeding, and reduced neutrophil exudation after severe heat stroke (Fig. 3c). Electron microscopy revealed that the anti-FGF23 neutralizing antibody reduced lung tissue damage in mice with severe heat stroke, and the main manifestations were alleviation of the dissolution and destruction of alveolar epithelial cells, reduced swelling of capillary endothelial cells, and reduced exudation and bleeding in the alveolar cavity (Fig. 3d). In addition, stimulation of mice with recombinant FGF23 protein resulted in increased FGF23 expression in mouse lung tissue (Fig. 3a), increased apoptosis (Fig. 3b), and promoted lung injury (Fig. 3c and d). These results suggest that FGF23 is an important and critical upstream regulator of VECs injury after heat stress and sHS-ALI.

FGF23 is involved in NOX2 activation and ROS accumulation in VECs after heat stress

To observe the effect of heat stress on oxidative stress, NOX2 activation and ROS accumulation in HUVECs, PMVECs, and lung tissues of mice with severe heat stroke were detected. Heat stress induced p-p47^{phox}, p67^{phox}, p22^{phox}, and NOX2 protein expression (Fig. 4a–c), and enhanced NADPH enzyme activity in HUVECs, PMVECs and lung tissues of

mice with severe heat stroke (Fig. 4h–j). Moreover, heat stress also induced the cell membrane translocation of p47^{phox} and p67^{phox} and the accumulation of ROS in both HUVECs and PMVECs (Fig. 4d–g). In addition, the immunohistochemical results showed that p-p47^{phox} and NOX2 expression was significantly increased in the lung tissue of mice with severe heat stroke (Fig. 7d and e).

Further experiments revealed that after FGF23 expression was inhibited by RNAi or an FGF23 neutralizing antibody, the expression of p-p47^{phox}, p67^{phox}, p22^{phox}, and NOX2; cell membrane translocation of p47^{phox} and p67^{phox}; ROS accumulation; and NADPH oxidase activity were inhibited in HUVECs and PMVECs subject to heat stress (Fig. 5a and b and d–g). However, the addition of the recombinant FGF23 protein significantly stimulated the phosphorylation of p47^{phox}, p67^{phox}, p22^{phox}, and NOX2; the cell membrane translocation of p47^{phox} and p67^{phox}; ROS accumulation; and the enhancement of NADPH oxidase activity in PMVECs (Fig. 5c and f–g). These results suggest that FGF23, an upstream priming factor, mediates NOX2 activation and enhances NADPH oxidase activity and ROS accumulation in VECs after heat stress, thereby participating in the formation of sHS-ALI.

FGF23 causes PLC- γ 2 phosphorylation to participate in NOX2-ROS activation by inducing FGFR-1 Y766 phosphorylation during sHS-ALI

Heat stress induced FGFR-1 Y766 and PLC- γ 2 phosphorylation in HUVECs, PMVECs, and lung tissues of mice with severe heat stroke (Fig. 6a–c and Fig. 7b). Upon the use of RNAi or neutralizing antibody to interfere with or inhibit FGF23 expression, FGFR-1 Y766 and PLC- γ 2 phosphorylation was inhibited in VECs subjected to heat stress or in the lung tissue of mice with severe heat stroke (Fig. 6d and e and Fig. 7b and c). In addition, FGFR-1 Y766 expression and PLC- γ 2 phosphorylation were induced by the recombinant protein FGF23 in PMVECs (Fig. 6f).

The FGFR-1 inhibitor SU5402 was used to significantly inhibit the expression of p-PLC- γ 2, p-p47^{phox}, p67^{phox}, p22^{phox}, and NOX2 in HUVECs and PMVECs after heat stress (Fig. 6g and h). Moreover, p47^{phox} and p67^{phox} membrane translocation, NADPH oxidase activity, and ROS accumulation were inhibited by the FGFR-1 inhibitor in HUVECs and PMVECs after heat stress (Fig. 6i–m). The expression of p-FGFR-1 Y766, p-PLC- γ 2, p-p47^{phox}, and NOX2 in the lung tissue of mice with severe heat stroke was significantly inhibited by the FGFR-1 inhibitor SU5402 (Fig. 7b–e). SU5402 also significantly reduced the number of apoptotic cells in the lung tissue of mice (Fig. 7f) and alleviated lung injury due to severe heat stroke (Fig. 7g and h). However, SU5402 did not inhibit FGF23 expression in the lung tissue of mice with severe heatstroke (Fig. 7a). These results suggest that FGF23 induces FGFR-1 Y766 phosphorylation and further activates PLC- γ 2 phosphorylation during heat stress, which promotes NOX2 activation and ROS accumulation in VECs and thus participates in the process of sHS-ALI.

Interference with PLC- γ 2 significantly inhibited NOX2-ROS activation during sHS-ALI

To determine the influence of PLC- γ 2 on NOX2 activation and ROS accumulation after heat stress, this study used RNAi to interfere with PLC- γ 2 in PMVECs and detected the effect of RNAi interference. PLC- γ 2 expression was

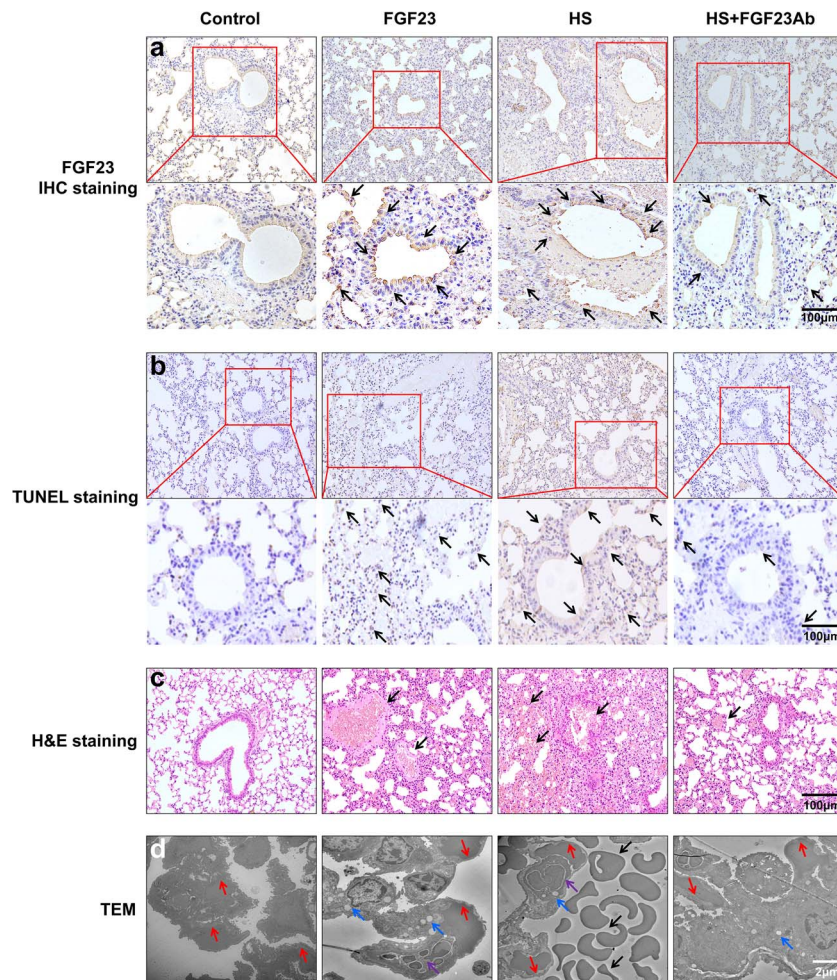


Figure 3. FGF23 is involved in regulating the progression of sHS-ALI. Mice were put through the same HS assay as previously described. In the FGF23 recombinant protein-treated group, FGF23 recombinant protein (100 mg/kg body weight) was administered by intraperitoneal injection. In the FGF23 Ab-treated group, FGF23 neutralizing antibody (10 mg/kg body weight) was administered by intraperitoneal injection. **(a)** Changes of FGF23 in lung tissues of mice in each group were detected by immunohistochemistry (IHC); the black arrows represent positive cells. **(b)** TUNEL staining was used to detect apoptosis in lung tissues of mice in each group; black arrows represent positive cells. **(c)** Pathological changes in lung tissue of mice in each group were stained by H&E; black arrows represent intracavicular haemorrhage. **(d)** Ultrastructural changes in lung tissue in each group were observed by transmission electron microscope. Red, blue, purple, and black arrows represent PMVECs, lamellar bodies of type II alveolar epithelial cells, neutrophils, and intracavicular haemorrhage, respectively.

significantly reduced in PMVECs after RNAi interference (supplementary Fig. 3a, see online supplementary material). Moreover, interference with PLC- γ 2 significantly inhibited heat stress-induced p-p47^{phox}, p67^{phox}, p22^{phox}, and NOX2 expression and inhibited the membrane translocation of p47^{phox} and p67^{phox}, NADPH oxidase activity, and ROS accumulation in PMVECs (supplementary Fig. 3b–f). The expression of p-p47^{phox}, p-p47^{phox}, and NOX2 in the lung tissue of mice with severe heat stroke was significantly inhibited after the application of the PLC- γ 2 inhibitor U73122 (Fig. 7c–e). U73122 also significantly inhibited apoptosis in the lung tissue of mice with severe heat stroke (Fig. 7f) and alleviated lung injury (Fig. 7g and h). However, U73122 had no significant effect on FGF23 or p-FGFR-1 expression in the lung tissue of mice with severe heat stroke (Fig. 7a and b), and interference with the expression of PLC- γ 2 also had no significant effect on FGF23 or p-FGFR-1 expression in PMVECs (supplementary Fig. 3b). These results further confirmed that FGF23/FGFR-1 is involved in NOX2 activation and ROS accumulation in VECs after heat

stress by inducing PLC- γ 2 phosphorylation, thereby causing sHS-ALI.

The FGFR-1 Y766 phosphorylation site is the key to FGF23/FGFR-1-induced NOX2-ROS activation in VECs induced by heat stress

Previous experimental results have demonstrated that FGF23 induces phosphorylation of its receptor FGFR-1 Y766, which further leads to PLC- γ 2 phosphorylation, thus promoting NOX2-ROS activation in VECs after heat stress. It is suggested that the phosphorylation of FGFR-1 Y766 in the FGF23 receptor is critical to activating the downstream signal of VEC injury during heat stress. Therefore, mutations at the FGFR-1 Y766 phosphorylation site were created in subsequent experiments. Specifically, the FGFR-1 Y766 site was mutated to alanine (Y766F), and a schematic diagram of the specific mutation sites is shown in Fig. 8a. Mutation of FGFR-1 Y766 significantly inhibited the expression of p-FGFR-1 Y766, p-PLC- γ 2, and NOX2 in PMVECs after heat stress

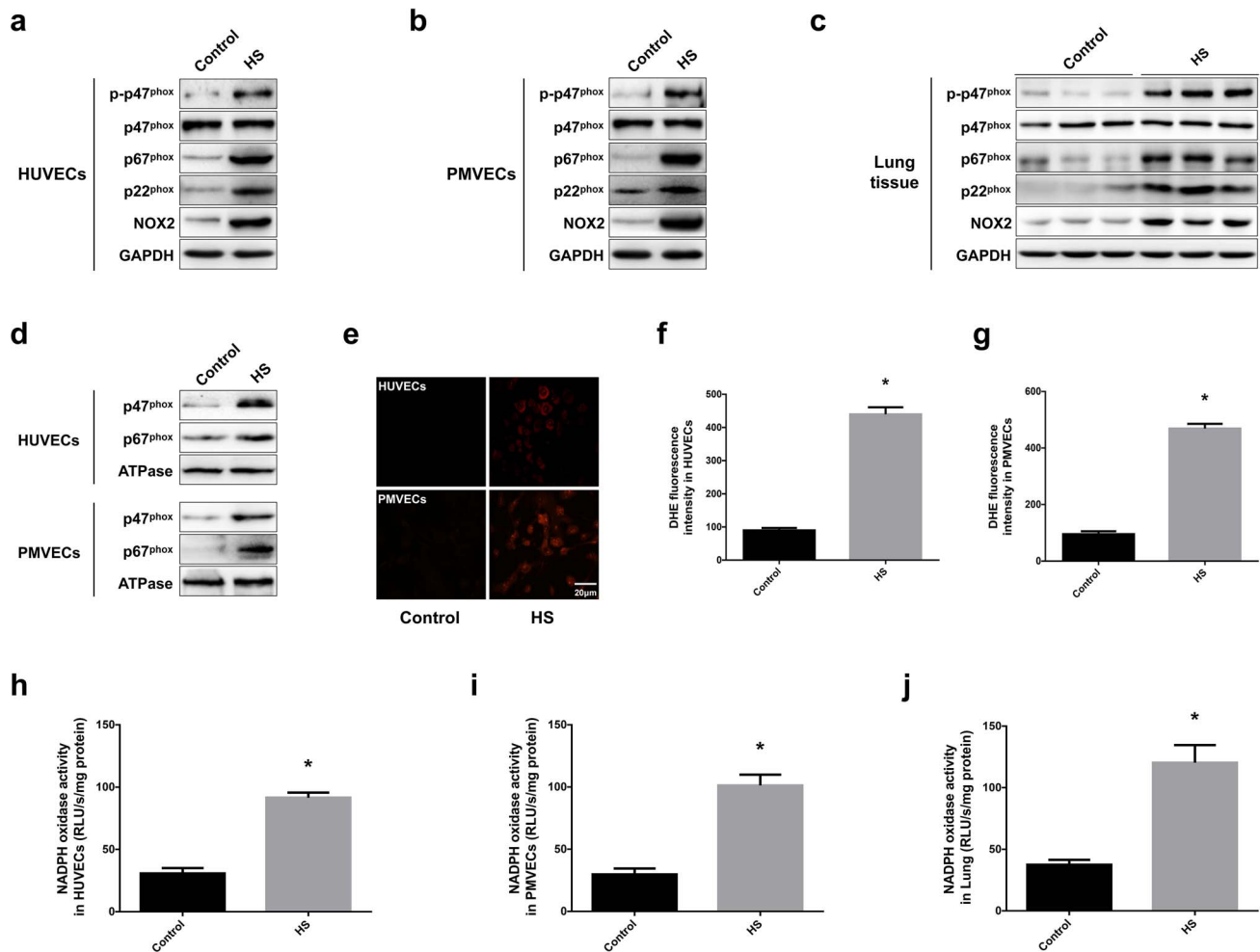


Figure 4. Heat stress-induced NOX2-ROS activation in VECs. The HUVECs or PMVECs were exposed to HS (43°C) for 2 h and then incubated at 37°C for the indicated duration of 6 h. Animals in the HS group were placed in a temperature-controlled chamber (ambient temperature $35.5 \pm 0.5^\circ\text{C}$ and $60 \pm 5\%$ relative humidity) and their rectal core temperature (T_{c}) was continuously monitored with a rectal thermometer until it reached 42°C . The mice were sacrificed 6 h after HS and lung tissue was isolated. Expression of p-p47^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, and NOX2 in (a) HUVECs, (b) PMVECs, and (c) lung tissue was detected western blot (d) Cell membrane translocation of p47^{phox} and p67^{phox} in HUVECs and PMVECs was detected by western blot. (e) Accumulation of ROS in HUVECs and PMVECs was detected by immunofluorescence and is analysed in histograms for (f) HUVECs and (g) PMVECs. Histogram analysis of NADPH oxidase activity in HUVECs (h) and PMVECs (i), and NADPH oxidase activity in lung tissue (j). * $P < 0.05$, compared to the control group, $n = 6$

but had no significant effect on FGF23 expression (Fig. 8b). Co-immunoprecipitation results also showed that mutations at the Y766 phosphorylation site of FGFR-1 inhibited the binding of p-FGFR-1 to p-PLC- γ 2 and NOX2 but had no effect on FGF23 (Fig. 8c). Moreover, after heat stress, the cell membrane translocation of p47^{phox} and p67^{phox} (Fig. 8b) NADPH oxidase activity, ROS accumulation, and overall cell apoptosis rate were inhibited, whereas PMVEC viability improved (Fig. 8d–g). More importantly, in the case of the FGFR-1 Y766 mutation, p-PLC- γ 2 and NOX2 expression could not be significantly induced in PMVECs even with the use of the recombinant FGF23 protein, heat stress, or combined stimulation (Fig. 9a). Moreover, cell membrane translocation of p47^{phox} and p67^{phox}, NADPH oxidase activity, ROS accumulation, and overall apoptosis rate were significantly inhibited (Fig. 9b–e), whereas cell viability was significantly restored (Fig. 9b–f). In addition, the use of recombinant FGF23 protein, heat stress, or combined stimulation in PMVECs with the FGFR-1 Y766 mutation did not affect FGF23 expression (Fig. 9a). These results suggest that

FGFR-1 Y766 phosphorylation is the key to the FGF23/FGFR-1-mediated induction of PLC- γ 2 phosphorylation and further mediates NOX2-ROS activation in VECs after heat stress, which is involved in sHS-ALI.

Discussion

Long-term exposure to high temperatures and humidity can cause a number of harmful stress reactions, including severe heat stroke and MODS, which pose a direct threat to human life [2,3]. The increasing global mortality rate from severe heat stroke in recent years is a stark reflection of the detrimental effects of high temperatures on public health [5,6]. Severe heat stroke is becoming more common and fatal as a result of global warming and increasing heat-wave frequency and intensity. Therefore, it is essential to investigate the underlying causes of severe heat stroke and develop strategies for its treatment and prevention.

Research has demonstrated that one of the first and most commonly affected target organs in cases of severe heat stroke is the respiratory system and that respiratory failure caused

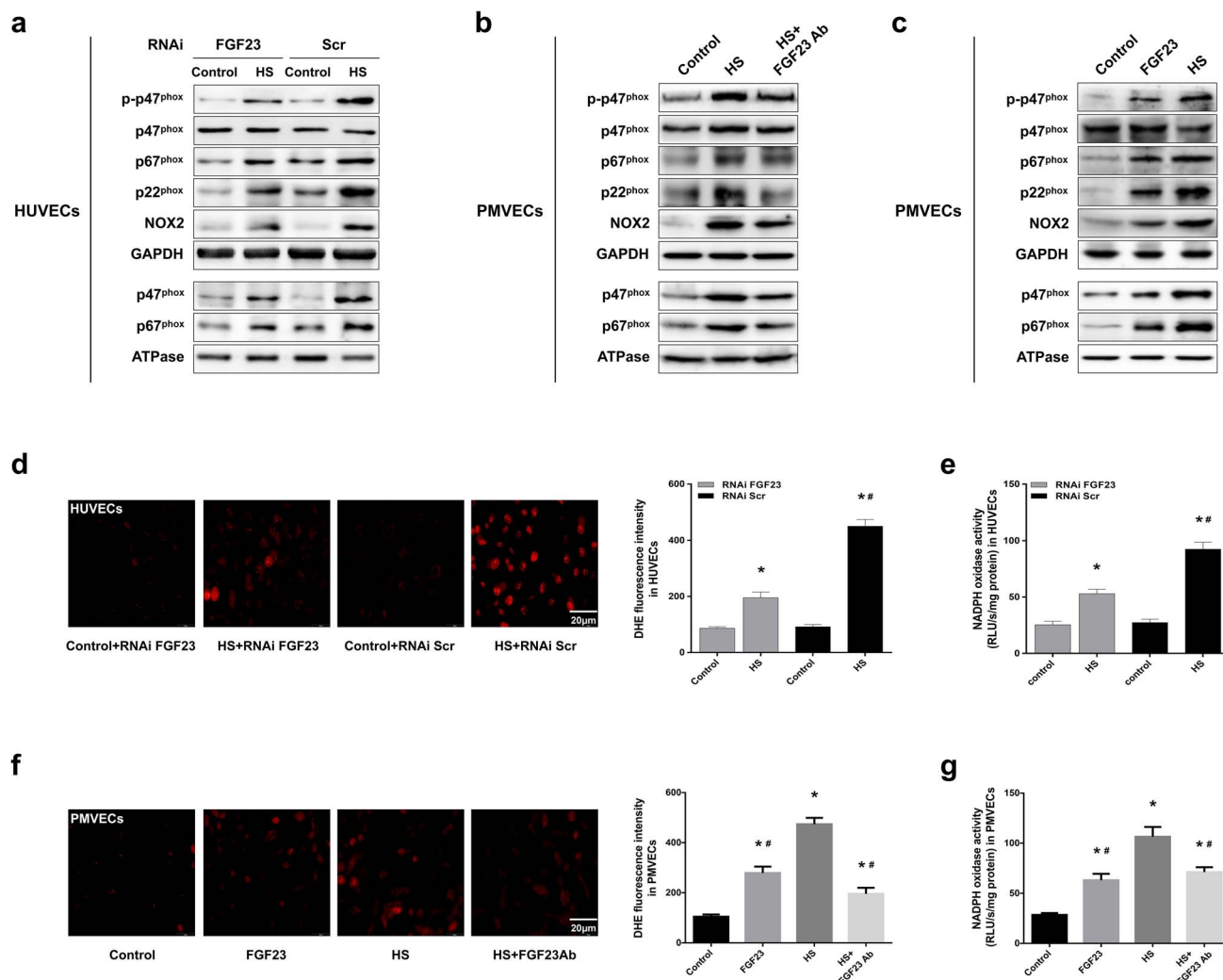


Figure 5. FGF23 is involved in NOX2-ROS activation in VECs after heat stress. HUVECs were transfected with FGF23 siRNA 48 h in advance, exposed to a temperature of 43°C for 2 h, and then further incubated at 37°C for 6 h. The FGF23 recombinant protein-treated group was used to stimulate HUVECs or PMVECs for 2 h with FGF23 recombinant protein (10 ng/ml). In the HS + FGF23 Ab-treated group, PMVECs were incubated with FGF23 neutralizing antibody (5 μg/ml) for 2 h in advance, then exposed to 43°C for 2 h and incubated at 37°C for the indicated duration of 6 h. **(a)** Western blot analysis of p-p47^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, NOX2 expression, and cell membrane translocation of p47^{phox} and p67^{phox} in HUVECs after interference with FGF23. **(b)** Western blot analysis of p-p47^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, NOX2 expression, and cell membrane translocation of p47^{phox} and p67^{phox} in PMVECs after using FGF23 neutralizing antibody. **(c)** Western blot analysis of p-p47^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, NOX2 expression, and cell membrane translocation of p47^{phox} and p67^{phox} in PMVECs after using FGF23 recombinant protein. **(d)** Immunofluorescence was used to detect ROS accumulation, and histogram analysis of ROS accumulation in HUVECs after interference with FGF23 is shown. **(e)** Histogram analysis of NADPH oxidase activity in HUVECs after interference with FGF23. **(f)** Immunofluorescence was used to detect ROS accumulation, and histogram analysis of ROS accumulation in PMVECs after using FGF23 neutralizing antibody or FGF23 recombinant protein is shown. **(g)** Histogram analysis of NADPH oxidase activity in PMVECs after using FGF23 neutralizing antibody or FGF23 recombinant protein. **P* < 0.05 compared to the control group or the RNAi-FGF23 control group; #*P* < 0.05 compared to the HS group or the RNAi-FGF23 HS group

by ALI plays a significant role in the development of MODS in cases of severe heat stroke [7,8]. The degree of severe lung injury cannot be averted by active cooling treatment, according to both clinical observations and experimental observations of animals with severe heat stroke [7,9,27]. ALI can also occur in the early stages of severe heat stroke. Furthermore, PMVECs injury plays a role in the sHS-ALI process [16,28]. The exact molecular injury mechanism to PMVECs involved in ALI needs to be further clarified, even though ALI has been confirmed to occur in the early stages of severe heat stroke, and PMVECs are involved in the occurrence and development of sHS-ALI. Moreover, the upstream initiation factors mediating sHS-ALI remain unknown.

Total RNA was isolated from the lung tissue of mice suffering from severe heat stroke to identify the upstream starting factors of sHS-ALI. Additionally, mRNA sequencing was performed to identify the transcriptional DEGs of sHS-ALI. In addition, 123 411 sgRNAs that target 19 050 human genes were assembled into a GeCKO library. Furthermore, HUVECs with whole-genome knockout (19 050 genes) were screened using CRISPR-Cas9 technology to identify prospective knockout DEG cohorts for HUVECs that would enhance cell survival under heat stress. Through the aforementioned experiments, it was possible to identify a cohort of 592 knockout DEGs that promoted HUVECs survival after heat stress, indicating that knocking out these genes might promote

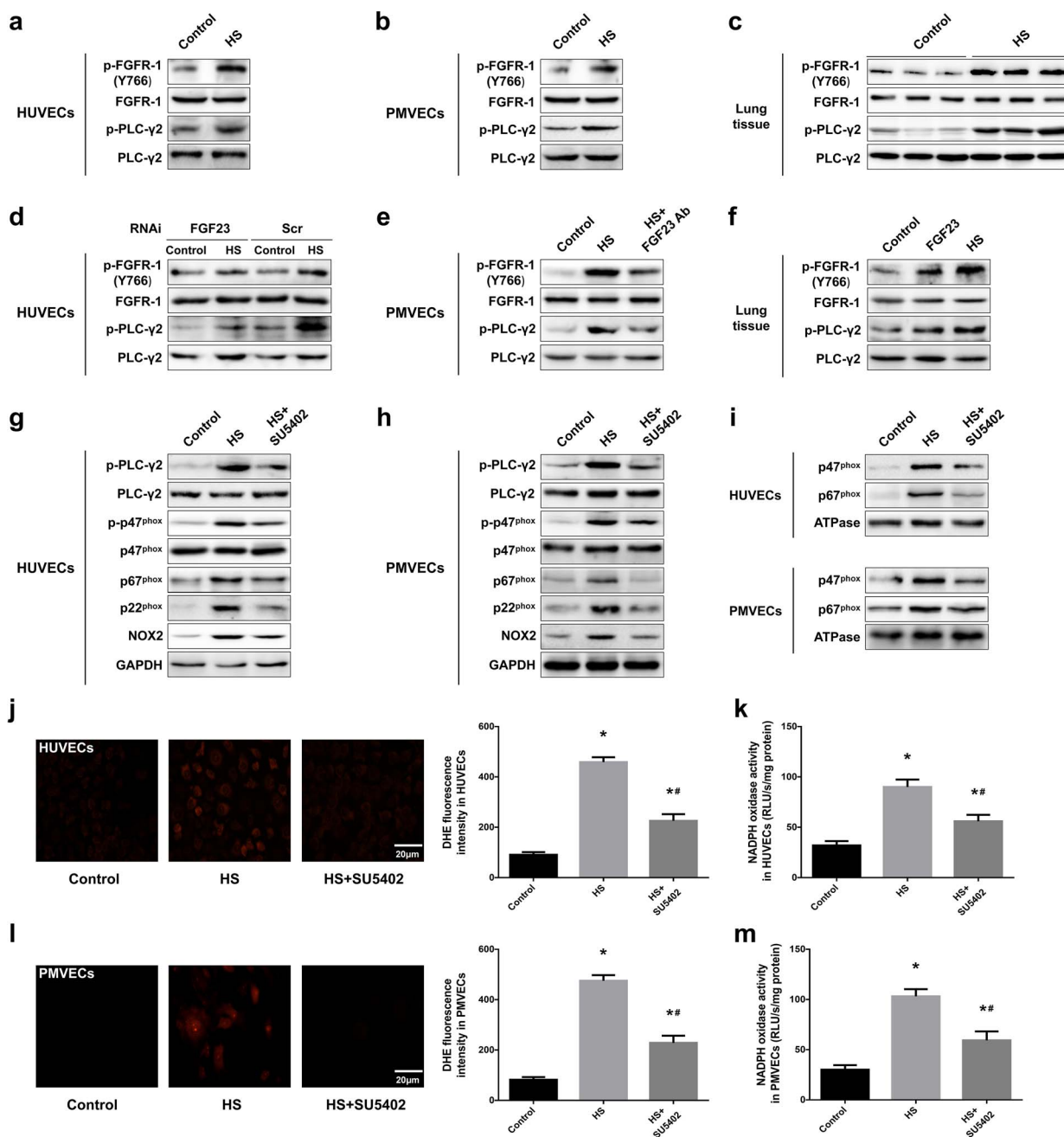


Figure 6. FGF23 causes PLC-γ2 phosphorylation to participate in NOX2-ROS activation by inducing FGFR-1 Y766 phosphorylation during heat stress-induced VECs damage. Treatment of the HUVECs transfection group, FGF23 recombinant protein group, and HS + FGF23 Ab-treated group was the same as previously described; the SU5402 group was incubated with HUVECs or PMVECs with SU5402 (1 μM) in advance for 30 min, and then exposed to a temperature of 43°C for 2 h and incubated at 37°C for the indicated duration of 6 h. Expression of p-FGFR-1, FGFR-1, p-PLC-γ2, and PLC-γ2 in (a) HUVECs, (b) PMVECs, and (c) lung tissue was detected by western blot. (d) Western blot analysis of p-FGFR-1, FGFR-1, p-PLC-γ2, and PLC-γ2 expression in HUVECs after interference with FGF23. (e) Western blot analysis of p-FGFR-1, FGFR-1, p-PLC-γ2, and PLC-γ2 expression in PMVECs after using neutralizing antibody with FGF23. (f) Western blot analysis of p-FGFR-1, FGFR-1, p-PLC-γ2 and PLC-γ2 expression in PMVECs after using FGF23 recombinant protein. Western blot analysis was performed to detect p-PLC-γ2, PLC-γ2, p47^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, and NOX2 expression in (g) HUVECs and (h) PMVECs after using FGFR-1 inhibition. (i) Cell membrane translocation of p47^{phox} and p67^{phox} was detected by western blot after using FGFR-1 inhibition. (j) Immunofluorescence was used to detect ROS accumulation, and histogram analysis of ROS accumulation in HUVECs after using FGFR-1 inhibition is shown. (k) Histogram analysis of NADPH oxidase activity in HUVECs after using FGFR-1 inhibition. (l) Immunofluorescence was used to detect ROS accumulation, and histogram analysis of ROS accumulation in PMVECs after using FGFR-1 inhibition is shown. (m) Histogram analysis of NADPH oxidase activity in PMVECs after using FGFR-1 inhibition. **P* < 0.05, compared to the control group; #*P* < 0.05, compared to the HS group

HUVECs survival after heat stress. In total, 1169 DEGs were upregulated in the lung tissues of mice with severe heat stroke, suggesting that these genes may participate in

sHS-ALI. The 17 DEGs identified above were found to be involved in promoting HUVECs survival after heat stress induction and to participate in sHS-ALI. Additional

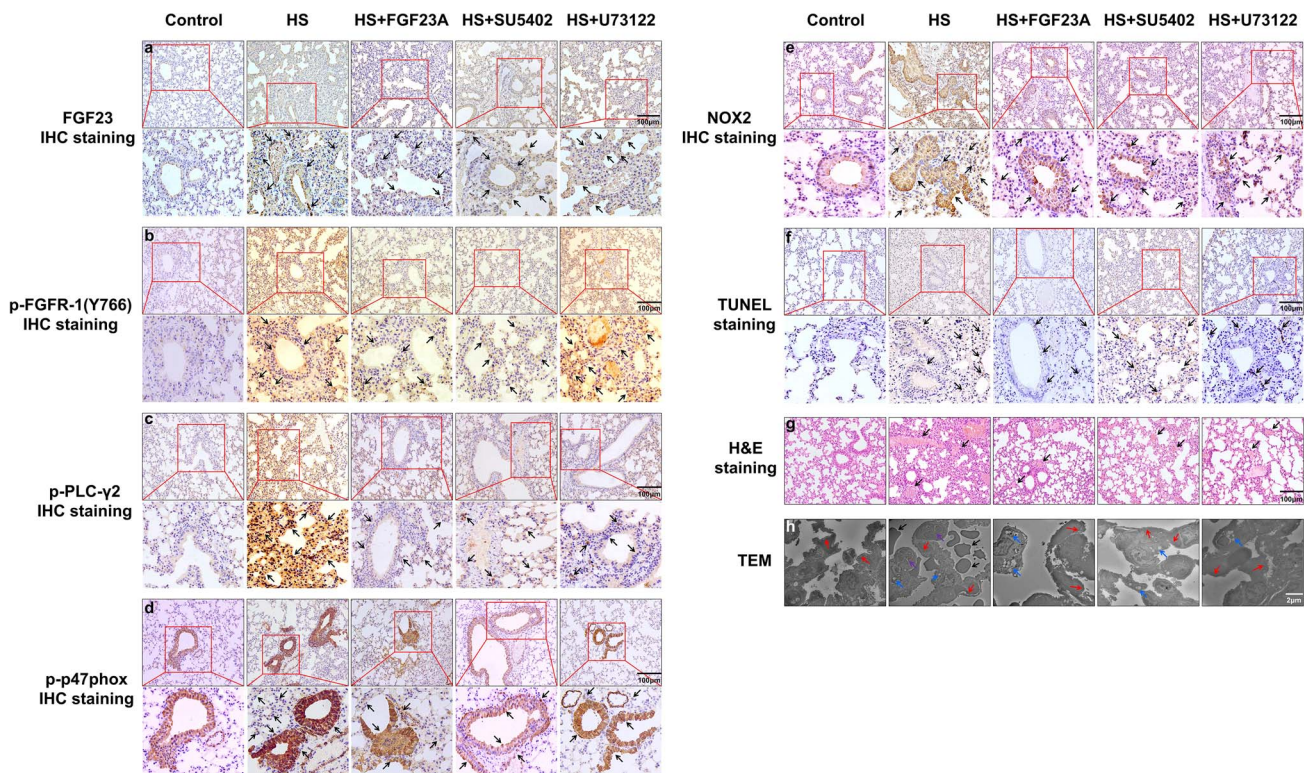


Figure 7. FGF23 causes PLC-γ2 phosphorylation to participate in NOX2-ROS activation by inducing FGFR-1 Y766 phosphorylation during sHS-ALI. Mice were put through the same HS assay as previously described. In the FGF23 Ab-treated group, FGF23 neutralizing antibody (10 mg/kg body weight) was administered by intraperitoneal injection. In the FGFR-1 inhibitor SU5402-treated group, 10 mg/kg body weight was administered by intraperitoneal injection. In the PLC-γ2 inhibitor U73122-treated group, 30 mg/kg body weight was administered by intraperitoneal injection. Changes in (a) FGF23, (b) p-FGFR-1, (c) p-PLC-γ2, (d) p-p47phox, and (e) NOX2 in lung tissues of mice in each group were detected by immunohistochemistry; the black arrows represent positive cells. (f) TUNEL staining was used to detect apoptosis in lung tissues of mice; black arrows represent positive cells. (g) H&E staining shows the pathological changes of lung tissue of mice in each group; and the black arrows represent intracavicular haemorrhage. (h) Ultrastructural changes of lung tissue in each group were observed by transmission electron microscope. The red, blue, purple, and black arrows represent PMVECs, lamellar bodies of type II alveolar epithelial cells, neutrophils, and intracavicular hemorrhage, respectively

KEGG pathway analysis identified 7 genes involved in seven signal transduction pathways, including RALGDS, FGF23, SLC16A10, MED13L, SPTA1, RALGDS, and BCL2L11.

Previous research has demonstrated that the primary cause of sHS-ALI is the accumulation of ROS in heat-stressed VECs [16]; preliminary investigations have also demonstrated the involvement of different cell death patterns caused by ROS accumulation (apoptosis, necrotic apoptosis, pyrodeath, etc.) [12,16,28], although the precise initiating factors and intermediate mechanisms involved remain unclear. Further analysis of the literature showed that FGF23, one of the aforementioned potential genes that may mediate sHS-ALI, mainly enhances oxidative stress and, consequently, endothelial dysfunction in the context of chronic kidney disease and cardiovascular disease [22,23]. Furthermore, FGF23 has become a significant predictor of acute burn, chronic obstructive pulmonary disease, renal disease, and the rapid progression of these conditions in people with chronic kidney disease [22–26]. Accordingly, FGF23 is proposed in this study as a putative upstream target gene during sHS-ALI. Here, changes in FGF23 levels in the lung tissue and serum of severe heat stroke mice and VECs subject to heat stress were detected to exclude false-positives from previous *in vivo* and *in vitro* screening results and to ascertain whether FGF23 is involved in heat stress-mediated VECs damage and sHS-ALI. The findings demonstrated a significant increase in FGF23 levels

in serum, lung tissue from mice suffering from severe heat stroke, and HUVECs and PMVECs following heat stress. More importantly, the results demonstrated that a FGF23 neutralizing antibody significantly reduced lung tissue injury and apoptosis in mice with severe heatstroke. RNAi interferes with FGF23 levels, and the use of a FGF23 neutralizing antibody significantly decreases the overall apoptosis rate and improves viability in VECs after heat stress. Furthermore, the use of recombinant FGF23 alone may exacerbate VECs damage and promote the development of ALI. These findings unequivocally show that FGF23 is a crucial and significant upstream regulator of VECs damage under heat stress. It is also possible that FGF23 contributes to the process of sHS-ALI by mediating VECs damage, although more research is needed to completely understand the mechanism involved.

Numerous studies have connected heat stress to oxidative stress, proposing a synergistic increase in cell death given that heat-stressed cells were found to produce more ROS [12,16,28]. Our earlier research demonstrated that heat stress causes intracellular ROS to accumulate in VECs and that manganese superoxide dismutase (MnSOD) overexpression successfully decreases oxidative stress-induced damage to VECs; however, more research is still needed to determine the exact mechanism involved [12]. It has been demonstrated that the multicomponent NADPH oxidase is crucial for

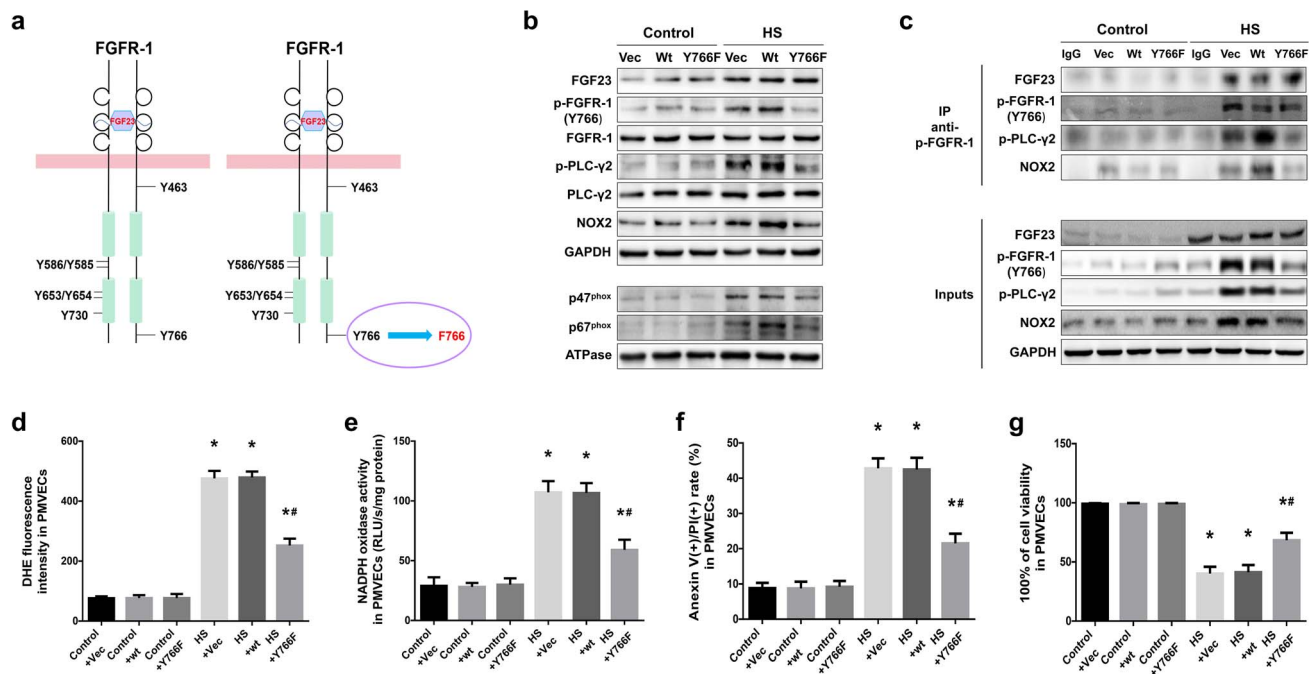


Figure 8. FGFR-1 Y766 phosphorylation site is the key for FGF23/FGFR-1 signaling to induce heat stress-mediated NOX2-ROS activation. PMVECs were transfected with FGFR-1 Y766 wild-type (wt) or FGFR-1 Y766F plasmid for 48 h, followed by heat stress at 43°C for 2 h, and then incubated at 37°C for the indicated duration of 6 h. **(a)** Schematic diagram of FGFR-1 Y766 phosphorylation site mutation. **(b)** Expression of FGF23, p-FGFR-1, FGFR-1, p-PLC-γ2, PLC-γ2, and NOX2, as well as cell membrane translocation of p47^{phox} and p67^{phox} were detected by western blot. **(c)** Binding of p-FGFR-1 to FGF23, p-PLC-γ2, and NOX2 were measured by co-immunoprecipitation (IP). Histogram analysis of **(d)** ROS accumulation, **(e)** NADPH oxidase activity, **(f)** overall apoptosis rate, and **(g)** cell viability was measured by CCK-8. **P* < 0.05, compared to the control + Vec group; #*P* < 0.05, compared to the HS + Vec group

redox-dependent signalling. The cytoplasmic p40–p47–p67^{phox} complex must be directed toward the membrane-bound heterodimeric p22–NOX2 flavocytochrome for NADPH oxidase to become activated. This process also triggers oxidative stress reactions that increase ROS generation [29,30]. Oxidative stress inhibition can be achieved by removing and renewing damaged proteins and organelles before they cause damage to tissues and cells. ROS produced by oxidative stress can cause damage and ageing of cell membrane lipoproteins [29,31]. However, when the stimulation is intense and prolonged, the cell's capacity to self-clear diminishes, and equilibrium is disrupted, which activates NADPH oxidase and catalyses the rapid synthesis of additional ROS [29,31]. Research indicates that NOX2 is the primary NADPH oxidase that causes ROS generation and drives VECs dysfunction when Ang II and inflammatory substances (such as TNF-α) activate VECs [17,18]. In addition, the possible mechanism linking FGF23 to cardiovascular disease is its effect on the markers of oxidative stress [32]. Studies have also shown that excess FGF23 may primarily promote NOX2-induced ROS production-associated oxidative stress and thus VECs dysfunction in chronic kidney disease [33]. In this study, heat stress increased NADPH enzyme activity in the lung tissues of mice suffering from severe heat stroke and VECs, as did the expression of p-p47^{phox}, p67^{phox}, p22^{phox}, and NOX2 proteins. In addition, heat stress caused ROS accumulation in VECs as well as the translocation of p47^{phox} and p67^{phox} to cell membranes. Subsequent investigations revealed that FGF23 neutralizing antibodies or RNAi targeting FGF23 levels in HUVECs significantly reduced the expression of p-p47^{phox}, p67^{phox}, p22^{phox}, and NOX2 induced by heat stress. Additionally, ROS accumulation, p47^{phox} and

p67^{phox} cell membrane translocation, and NADPH oxidase activity were also inhibited. On the other hand, stimulation of PMVECs with recombinant FGF23 protein had the opposite effect. Mice suffering from severe heat stroke also showed inhibited p-p47^{phox} and NOX2 expression in their lung tissue upon exposure to an FGF23 neutralizing antibody. These findings imply that FGF23, an upstream priming factor, contributes to the production of sHS-ALI by mediating NOX2 activation, increasing NADPH oxidase activity, and causing ROS accumulation in VECs during heat stress.

Although its molecular mechanism and signalling pathway are still unknown, previous experimental results have shown that FGF23 mediates NOX2-ROS accumulation in VECs during heat stress and participates in the process of sHS-ALI. FGF23 is dependent on the FGFR in both healthy and diseased states, according to earlier research [33,34]. Excess FGF23 or decreased FGFR activity may be harmful in pathological circumstances. Through the transmembrane protein Klotho, FGF23 can increase the affinity between FGF23 and FGFR. This allows FGF23 to bind closely to four different FGFR subtypes, with FGFR-1 exhibiting the highest affinity [34]. Subsequent investigations revealed that FGF23, independent of Klotho, can directly activate the downstream pathway by binding to its receptors (FGFR-1, FGFR-4 etc.) [35,36]. The extracellular part of FGFR-1, a transmembrane receptor tyrosine kinase, is where its ligand fibroblast growth factor (FGF) binds [37]. The intracellular active area of tyrosine kinases is phosphorylated when FGFs attach to the FGFR-1 extracellular section. This process also controls key signalling proteins, such as PLC-γ, by initiating a cascade of cellular biological signals [38].

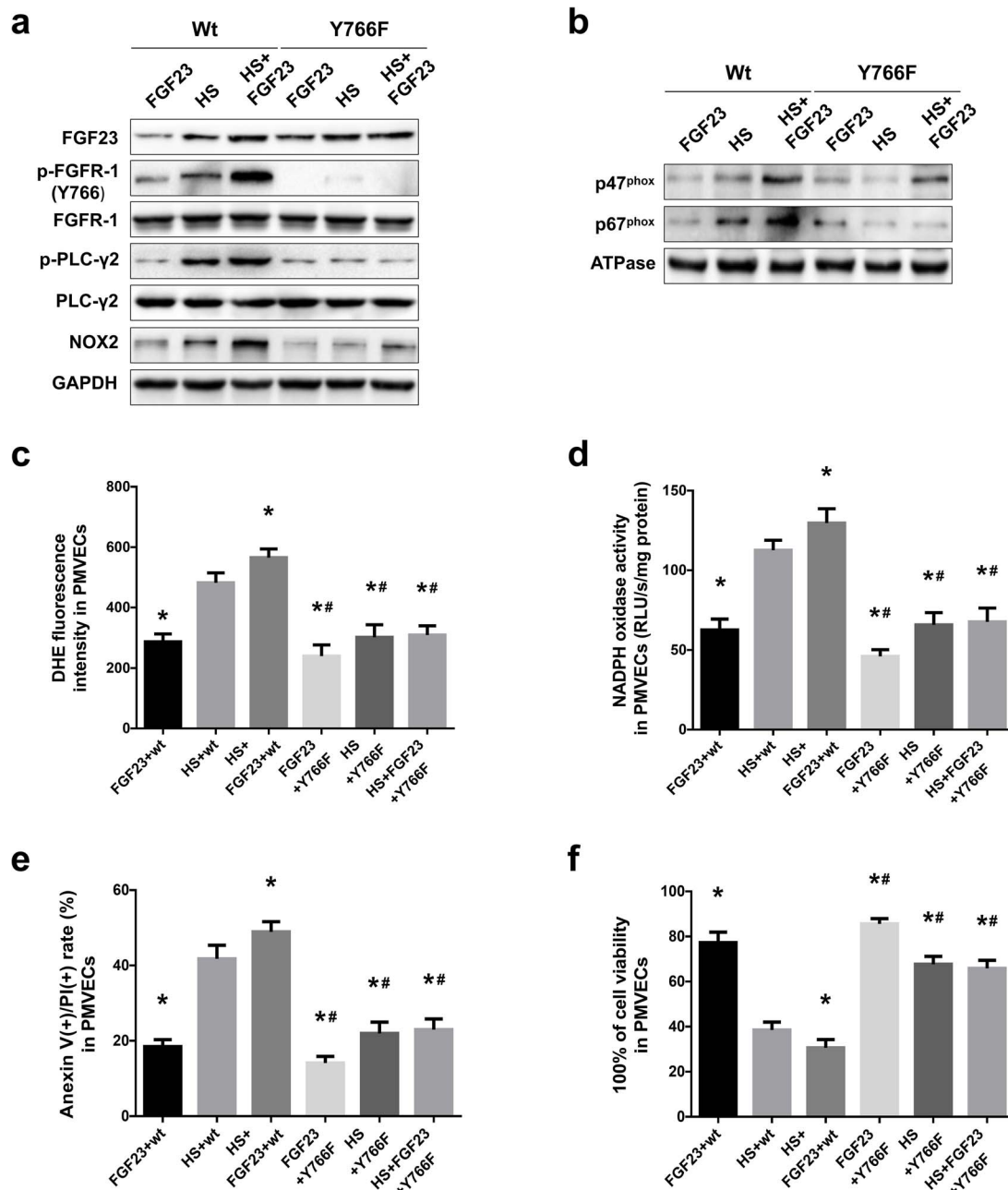


Figure 9. The FGFR-1 Y766 phosphorylation site is the key to FGF23/FGFR-1-induced NOX2-ROS activation after heat stress. PMVECs were transfected with FGFR-1 Y766 wild-type (wt) or FGFR-1 Y766F plasmid for 48 h, then treated with/without FGF23 recombinant protein for 2 h, stimulated with/without heat stress at 43°C for 2 h, and then incubated at 37°C for the indicated duration of 6 h. (a) Expression of FGF23, p-FGFR-1, FGFR-1, p-PLC-γ2, PLC-γ2, and NOX2 was detected by western blot. (b) Cell membrane translocation of p47^{phox} and p67^{phox} was detected by western blot. Histogram analysis of (c) ROS accumulation, (d) NADPH oxidase activity, and (e) overall apoptosis rate. (f) Cell viability was measured by CCK-8.

* $P < 0.05$, compared to the HS wt group; # $P < 0.05$, compared to the HS + FGF23 Y766F group

FGF23 has an impact on various facets of the growth state, including metabolic control, cell survival and proliferation, and individual growth and development [38]. Furthermore, earlier research demonstrated that FGF23, independent of Klotho, can directly interact with FGFR to cause atherosclerotic cardiovascular disease [39]. Here, in VECs and lung tissues of mice suffering from acute heat stroke, heat stress enhanced the expression of p-FGFR-1 Y766. An FGF23 neutralizing antibody or FGF23 deletion dramatically reduced p-FGFR-1 Y766 expression in VECs, whereas recombinant FGF23 protein increased p-FGFR-1

Y766 expression in PMVECs. FGF23 contributes to the process of sHS-ALI after it primarily mediates VECs damage through the phosphorylation of its receptor FGFR-1 at Y766.

Additionally, it has been established that certain phosphorylation sites on FGFR-1 are associated with particular roles in FGFR-1 signal transduction. For instance, Y463 in the near-membrane region binds the small articulator Crk, Y653 and Y654 are critical for kinase activity in the second kinase domain, and Y766 is the PLC-γ binding site [38]. Furthermore, when FGFs attach to the receptor, FGFR dimerization causes the tyrosine residues of the intracellular

domain to become phosphorylated. This can be directly triggered by interactions with the FGF receptor substrate 2 α , PLC- γ , or transcriptional activator 1/3/5 (STAT1/3/5), which activate downstream signalling pathways. The FGFR-1 Y766 site can stimulate the phosphorylation and activation of PLC- γ after binding, which subsequently triggers the activation of downstream signalling pathways [40]. On the other hand, mutation of the Y766 site of FGFR-1 can prevent FGFR-1 from binding to PLC- γ and activating downstream signalling pathways. Furthermore, research has demonstrated that PLC- γ comprises two subtypes, PLC- γ 1 and PLC- γ 2, and that phosphorylation of PLC- γ 2 is a critical step in oxidative stress activation [41]. PLC- γ 2 plays a central role in upregulating calcium influx-related oxidative stress downstream signalling [42]. Additionally, suppression of PLC- γ 2 phosphorylation is involved in emodin-mediated inhibition of oxidative stress by lipopolysaccharide stimulation in rat peritoneal macrophages [43]. The results showed that p-PLC- γ 2 expression increased in VECs induced by heat stress and in the lung tissues of mice with severe heat stroke. The expression of p-PLC- γ 2 was significantly inhibited after FGF23 interference or blockade in VECs and lung tissues of mice with severe heat stroke. Recombinant FGF23 protein stimulation also enhanced p-PLC- γ 2 expression in PMVECs. Moreover, NOX2-ROS activation, p-PLC- γ 2 expression, and NADPH oxidase activity in VECs were markedly suppressed by the FGFR-1 inhibitor SU5402. In mice suffering from severe heat stroke, SU5402 also markedly reduced lung tissue damage and suppressed the production of p-PLC- γ 2, p-p47^{phox}, and NOX2. Furthermore, interference with PLC- γ 2 may considerably prevent heat stress-induced NOX2 activation and ROS accumulation. On the other hand, FGF23 and p-FGFR-1 expression remains unaffected. Further experiments were conducted to mutate the FGFR-1 Y766 phosphorylation site. The findings demonstrated that mutation of the FGFR-1 Y766 phosphorylation site strongly reduced p-FGFR-1 Y766 and p-PLC- γ 2 expression, NOX2-ROS activation, and the total rate of heat-induced overall cell apoptosis. More importantly, after mutation of the FGFR-1 Y766 phosphorylation site, p-PLC- γ 2 expression could not be significantly induced, and NOX2-ROS activation and the overall apoptosis rate were inhibited, even when the recombinant FGF23 protein, heat stress, or combined stimulation were used. However, there was no effect on FGF23 expression. According to these findings, FGFR-1 Y766 phosphorylation plays a crucial role in FGF23/FGFR-1-mediated activation of PLC- γ 2 phosphorylation. Additionally, NOX2-ROS activation in VECs during heat stress is further promoted, which is linked to sHS-ALI.

Nevertheless, some unanswered scientific questions remain. (1) Although earlier research has suggested that FGF23 can directly interact with FGFR to cause disease development without the help of its coreceptor Klotho, this aspect has not been investigated, and these findings need to be confirmed in further research. (2) We only examined FGF23 in detail among all the typical target DEGs that support HUVECs survival after deletion during heat stress as identified by sHS-ALI screening *in vivo* and *in vitro*. Extensive investigations on whether additional prospective DEGs contribute to the development of sHS-ALI and the potential underlying mechanism should be performed in subsequent research. (3) In addition, this study was limited to HUVECs and PMVECs and did not discuss the mechanism of endothelial tissue and cell damage in other tissues and organs after severe heat stroke, given that

the endothelial tissue and cells in each organ have their own characteristics. Whether the possible mechanism of HUVECs and PMVECs damage after severe heat stroke also occurs in endothelial tissues and cells of other organs must be verified by subsequent studies.

Conclusions

In conclusion, FGF23 is a pivotal upstream initiating factor of sHS-ALI and a potential therapeutic target for the treatment of severe heat stroke. Additionally, FGF23/FGFR1 signalling, acting as an upstream priming factor, mediates NOX2 activation and enhances NADPH oxidase activity and ROS accumulation in VECs following heat stress, thereby contributing to the process of sHS-ALI. FGFR-1 Y766 phosphorylation is essential for FGF23/FGFR-1 activation of PLC- γ 2 phosphorylation and additional NOX2-ROS activation in VECs following heat stress, and these processes are implicated in sHS-ALI. These results have significant ramifications for mechanism-based treatment plans in the future for individuals suffering from severe heat stroke.

Abbreviations

Ab: Antibody; ALI: Acute lung injury; DEGs: Differentially expressed genes; DHE: Dihydroethidium dihydroethidium; FGF: Fibroblast growth factor; FGFR: Fibroblast growth factor receptor; HS: Heat stroke; HUVECs: Human umbilical vein endothelial cell; MODS: Multiple organ dysfunction syndrome; NADPH: Nicotinamide adenine dinucleotide phosphate; NOX2: nicotinamide adenine dinucleotide phosphate oxidase isoform 2; PLC- γ 2: Phospholipase C- γ 2; PMVECs: Pulmonary microvascular endothelial cells; RNAi: RNA interference; ROS: Reactive oxygen species; sHS-ALI: Severe heat stroke-induced acute lung injury; siRNA: Small interfering RNA; VECs: Vascular endothelial cells; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; enzyme linked immunosorbent assay (ELISA); manganese superoxide dismutase (MnSOD); hematoxylin-eosin (H&E) staining; horseradish peroxidase (HRP); deoxyribonucleoside triphosphates (dNTPs); Deoxyribonuclease I (DNase I); Ribonuclease H (RNase H); multiplicity of infection (MOI); dulbecco's modified eagle medium (DMEM); phosphate buffer saline (PBS), tumor necrosis factor- α , TNF- α , Angiotensin II (Ang II).

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Author contributions

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Li Li (Supervision [lead], Validation [lead], Writing—review & editing [lead])

Supplementary data

Supplementary data is available at *Burns & Trauma* online.

Ethics approval

Pathogen-free 6- to 8-week old male C57BL/6 mice were purchased from the Experimental Animal Center of the Southern Medical University in Guangzhou, P.R. China (Certification: SCXK (YUE) 2021-0041). The experimental protocols were approved by the Animal Care and Use Committee of the Southern Medical University, Guangzhou, China. None of the authors are members of this committee. The care of the animals was in accordance with the National Institutes of Health Guidelines, as well as with those of the Chinese National.

Conflict of interest

None declared.

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