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RESEARCH ARTICLE

PDK4 Enhanced Glycolysis to Drive Pulmonary Vascular Remodeling in Broiler Ascitic Syndrome

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ABSTRACT

Broiler ascites syndrome (AS), also known as pulmonary hypertension syndrome, is a metabolic disease in fast-growing broilers characterized by hypoxia-induced pulmonary vascular remodeling (PVR), including excessive proliferation of pulmonary arterial smooth muscle cells (PASMCs), vascular wall thickening, and fibrosis. However, the role of pyruvate dehydrogenase kinase 4 (PDK4), a key regulator of glucose metabolism, in this process remains unclear. In this study, blood gas and biochemical analyses revealed hypoxemia and acidosis in AS broilers. Histological and molecular assays demonstrated increased pulmonary arterial wall thickness, enhanced PASMCs proliferation, and reduced apoptosis. Transcriptomic and protein analyses showed that PDK4 was significantly upregulated, along with activation of glycolysis and pyruvate metabolism pathways. In vitro, hypoxia (3% O_2, 48 h) promoted PASMCs proliferation, inhibited apoptosis, enhanced glycolysis, and increased lactate production, accompanied by elevated PDK4 expression. PDK4 overexpression reproduced these effects, whereas inhibition by dichloroacetate (DCA) reversed them. Furthermore, lactate treatment promoted PASMCs proliferation and G1/S cell cycle transition. These results indicate that PDK4 drives glycolysis and promotes PASMCs proliferation via lactate-induced cell cycle progression, thereby participating in PVR in AS broilers.

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INTRODUCTION

Broiler ascites syndrome (AS) is a nutritional and metabolic hypoxia induction and genetic selection toward rapid growth, mainly manifested as right ventricular hypertrophy and effusion (Kang *et al.*, 2025). Its underlying pathophysiological mechanism is closely associated with hypoxia-induced pulmonary vascular remodeling (PVR) (Chen *et al.*, 2025). That is induced by low atmospheric oxygen partial pressure in high altitude areas or low ventilation density in crowded enclosed farms. Atmospheric low oxygen partial pressure leads to failure of pulmonary gas exchange and low systemic blood oxygen saturation, which lead to progressive development of broilers AS (Khajali *et al.*, 2016). Broiler ascites syndrome is a serious challenge for intensive broiler farming. With the continuous selection for rapid growth traits in broiler breeding, its incidence is gradually increasing, especially in high altitude hypoxic areas (Wideman *et al.*, 2013; Khajali, 2022**)**. Buzdugan *et al.* reported that broiler ascites syndrome was the primary cause of culling in the UK from 2015 to 2017, leading to significant economic losses (Buzdugan *et al.*, 2021). Although nutrition intervention can alleviate the ascites symptoms to some extent (Xiang *et al.*, 2002; Pan *et al.*, 2005; Mohammadalipour *et al.*, 2017), the irreversible PVR limits the effect of nutrition intervention on preventing and controlling AS. Therefore, elucidating the molecular mechanism of PVR is very important for treating AS and reducing losses in poultry industry.

PVR is characterized by structural and functional changes of vasculature, induced by hypertrophy, proliferation, migration, and anti-apoptotic phenotypes of pulmonary arterial smooth muscle cells (PASMCs), which

is the main cellular component of pulmonary vascular wall (Tajsic *et al.*, 2011; Stenmark *et al.*, 2018**)**. Hypoxia induces abnormal proliferation and apoptosis resistance of PASMCs via multiple signaling pathways and is regarded as the driver of PVR (Liu *et al.*, 2022; Li *et al.*, 2024). Recently, it is reported that hypoxia disturbs the cellular energy metabolism of PASMCs. Enhanced glycolysis was observed in cardiomyocytes of AS broilers (Li *et al.*, 2022). However, the regulatory mechanism of metabolic change is still unknown.

Cellular glucose and lipid metabolism are controlled by pyruvate dehydrogenase (PDH) which is suppressed and phosphorylated by pyruvate dehydrogenase kinase (PDK) to limit entry of pyruvate to the tricarboxylic acid cycle. The family of PDKs consists of four isoforms, the dysregulated expression and activity of which are associated with various physiological and pathological processes, and metabolic disorders (ex: atherosclerosis, diabetes) and cancer (Li *et al.*, 2020; Ma *et al.*, 2023; Wu *et al.*, 2024). The most common isoform is Pdk4 that is found in high-energy-needing tissues, including the heart, adipose tissue, and skeletal muscle (Jeong *et al.*, 2012). Recent findings have noted a higher PDK4 level in the pulmonary blood in AS broilers (Huang *et al.*, 2024), but the specific effect of PDK4 on the PVR process of AS broilers is still not characterized. So far, the role of functional interaction between PDK4 and PASMCs behavior, and how effective this interaction is to the AS-associated PVR in broilers, is unfamiliar.

We hypothesized in the study that PDK4 increases broiler AS glycolysis in PASMCs to promote the high output of PVR. We prove that PDK4 stimulates glycolysis and excess lactate produced in response to active glycolysis supports the further development and cell growth of PASMCs. The outcomes confirm causal relationships existing between glycolysis and PVR in AS broilers, with PASMC-derived PDK4 as a clinical agent of potentially correcting PVR in AS broilers.

MATERIALS AND METHODS

Experimental Birds: All broilers were obtained from Sunner Group, a commercial chicken farm located in Gansu Province, China. AS broilers and healthy broilers (n=6) were housed in the same cage within the same breeding shed. All AS broilers were found to have abdominal cavities filled with clear ascitic fluid and a right ventricle free wall to total ventricle weight ratio (RV/TV) ≥0.25 were classified as having ascites syndrome(Qiao *et al.*, 2019). The calculation methods for RV/TV and organ indices were performed as described by Liu *et al.* (2025).

Biochemical Assays: Blood gas and biochemical analyses were performed using a Wondfo animal-specific blood gas biochemical analyzer (BGA-101). Blood gas parameters, Lactate, and pH (W46616405H, Guangzhou Wondfo Biotech Co. China) values were measured in accordance with the manufacturer's instructions.

Histological Techniques: After routine paraffin embedding and sectioning of tissue samples, perform the following steps: **(1) H&E Staining:** After deparaffinization and rehydration, sections were stained with hematoxylin, differentiated, blued, and then counterstained with eosin, followed by dehydration, clearing, and mounting with neutral resin (Liu *et al.,* 2025; Hussain *et al.*, 2021; Karakurt *et al.*, 2026). **(2) IHC Staining:** The experiment was performed as described by Huang et al. (Huang *et al.*, 2024; Song *et al.*, 2024). Sections were first baked, then deparaffinized, rehydrated, and subjected to antigen retrieval and blocking. Subsequently, sections were incubated with primary and secondary antibodies, and signals were visualized using DAB. After dehydration through a graded ethanol series and clearing in xylene, sections were mounted with neutral resin.

Cell culture and experimental treatments: Pulmonary arteries were isolated from 18-day-old broiler embryos (South China Biological Medicine). Cells were inoculated into culture dishes with DMEM (G4511, Servicebio, China) supplemented with 10% fetal serum (LY-BFS-003, YiLang, China) and antibiotics (SW-CJ-2D, Thermo Fisher, USA). The cells were cultured in a 5% CO_2 incubator at 37°C. When the cells are passaged to the 3rd to 5th generations, they can be used in experiments involving hypoxic treatment, transient transfection for PDK4 plasmid overexpression, and treatment with sodium dichloroacetate (B7174, DCA, APEXBIO, United States) as well as L-Lactate (876-56-1, Aladdin, China). The hypoxic condition is maintained with 3% O_2, 5% CO_2, and 92% N_2 (Qiao *et al.*, 2022).

CCK-8: PASMCs were seeded into a 96-well cell culture plate. After the specified treatment, 10μL of CCK-8 reagent was added to each well containing 100μL of culture medium, following the kit instructions (C6005, NCEBiotech, Jiangsu, China) (Wang *et al.*, 2025). The absorbance at 450nm was then measured, and cell viability (%) was calculated based on the measured values.

Western blot (WB): Total proteins were extracted from tissues/cells as described previously. Proteins were separated by 10% SDS-PAGE (0227, Biosharp, Canada) and transferred onto PVDF membranes (ISEQ15150, Sigma, Germany). Subsequent steps included blocking with milk, followed by incubation with primary and secondary antibodies (Liu *et al.*, 2024; Shakeel *et al.*, 2024). Band images were captured, and relative protein levels were calculated using β-actin as the internal reference. The primary antibody usage is provided in Table 1.

Table 1: List of antibodies.

Names	Dilution rate	Company	Country	Application
PDK4	1:200	ABclonal	China	IHC
α-SMA	1:100	WanLeiBio	China	IHC
Ki-67	1:200	Zen-Bio	China	IHC
PCNA	1:200	ABclonal	China	IHC
VEGF	1:2000	Bioss	China	WB
Caspase3	1:1000	Proteintech	China	WB
APAF1	1:1000	ABclonal	China	WB
PCNA	1:2000	ABclonal	China	WB
CyclinD1	1:10000	Proteintech	China	WB
Bcl2	1:1000	ABclonal	China	WB
PDK4	1:1000	ABclonal	China	WB
β-actin	1:10000	Proteintech	China	WB

RT-qPCR: Total RNA extraction was performed using the Trizol (AG21102, AG, China) reagent (Liu *et al.*, 2024). Reverse transcription was performed using an ABClonal Reverse Transcription Kit. Quantitative real-time PCR (qPCR) was conducted using SYBR Green RT-qPCR reagents (RK20433, ABClonal, China). Relative mRNA expression levels were computed by means of the 2^−ΔΔCt method, and β-actin served as the internal control for normalization. Primer sequences are listed in Table 2.

Table 2: Primer sequences of the genes.

Gene name	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
β-actin	CCGTGCTGTGTTCCCATCTA	TCTGGGCTTCATCACCAACG
PDK4	TGCAATCACCAAAGGTCACCA	TGCAGTAGCTGAAGCTGTGTT
β-actin	CCGTGCTGTGTTCCCATCTA	TCTGGGCTTCATCACCAACG
PDK4	TGCAATCACCAAAGGTCACCA	TGCAGTAGCTGAAGCTGTGTT
CyclinD1	GACTTTTGTGGCTCTGTGCG	TGTTCTTGGCAGGCTCGTA
Bcl2	GATCGTCGCCTTCTTCGAGT	GGCCTCATACTGTTGCCGTA
APAF1	ATGGCAGGTTGTGGAAAGAC	TATGAGGAGCCCTGCTTTGT
Bax	TCCTCATCGCCATGCTCAT	CCTTGGTCTGGAAGCAGAAGA
Caspase3	CGGACTGTCATCTCGTTCA	TGGCTTAGCAACACACAAAC
Caspase8	GAGCTGGCGTCTCTGAAGTT	CTTCCCGCTTCCTTACAGTG
Caspase9	GCACTGCCTGATCTTCAACA	CTGAAACGCTTCTCCAGCTT
MCT1	GAGCTCTACTTCTGCGTGGG	TCGCCAGCCAAATACACCAA
MCT4	TCTGTAGCGTGTGCGTCAAT	CATGGAGGCAAAAAGGCCAC
E2F1	CCATCGACGTGTTCCTCTGT	CTGTGCCTGGGAGCAGTG
PKM2	CCACAGTGTACACCACAGGG	CTGTTTGGTCATTGCGGGTG
GLUT1	GATGGCTTTGTCCTTTGAGATGC	CAAAGATGCTGGTGGAGTAGTAG
HK2	CTGCTGAAGGAGGCAATCCA	GCTTCCCAGGGTTAAGGGAC
LDHA	GCACTTTCCAAGTAGGTCAAATCC	AGTCTTTGGTTTCACGTTGTGT
CyclinA	AAGCACGGATGAACTCCAGG	CTTGTTGTTTCCCAGCGAGC
CyclinB3	ATCACCAACGCTCACAAGAAC	AGGCTCCACAGGAACATCTG
CyclinC	TTGCCTTGCATCCTTCTGCT	TGCTGCTACCTCTTGCTTGG
CyclinE1	CCCAGCCCAGAATTGGTAGC	GCAAGAGGAAAGCCGATGTG
PCNA	CCCTGAAGGACCTCATCACTG	CATGCTGTTTAGGTTGACGCC
TGF-β1	CTCTCTCTGTGCTGCTGGAT	ATGAAGGAGAACTCGGGTGG
TGF-β3	TGGCTGTCCTTCGATGTCAC	CAAGATGTCCCCATTGGGCT
HIF-1α	TGAGAGAAATGCTTACACACAG	TGATGGGTGAGGAATTGGTTCAC
EPAS1	GCTGACAAGGAGAAGAAGAGGA	TGGGAACTGACATTGTGGGG
ULK1	GAGCAAGAGCACACCGACCC	ATTTTCAGGGCAGCAATCTCCATCAC
CITED2	CTGCCGCCCAATGTCATAGA	TCGTTCTGTCCCAACCACAG
IL1RL1	AGCGATGAATGAAATGGG	ACCGACAGTCACGAGCAG

Measurement of cellular metabolites: For the determination of cellular lactate, glucose, and lactate dehydrogenase (LDH), specific kits were employed: Lactate Assay Kit (KTB1100, Abbkine, China), Glucose Assay Kit with O-toluidine (S0201S, Beyotime, China), and LDH Assay Kit (C0016, Beyotime, China). Detection of the target substances was conducted in accordance with the respective kit protocols.

Cellular oxygen consumption rate: Cells from 6-well plates were collected and loaded into the instrument's sample compartment. After allowing cellular respiration to stabilize, basal respiratory parameters (Basal) were measured. Subsequently, Oligomycin (Oligo, 5nM, 495455, Sigma, Germany), CCCP (1μM, C2759, Sigma, Germany), rotenone (Rot, 0.5μM, R8875, Sigma, Germany), and antimycin A (AMA, 2.5μM, A8674, Sigma, Germany) were added sequentially (Smolina *et al.*, 2023).

Flow cytometry: This included cell apoptosis detection (k2003, APEXBIO, United States), reactive oxygen species (ROS) detection (S0033S, Beyotime, China), and cell cycle detection (c1052, Beyotime, China) (Li *et al.*, 2025). Collected cells were treated and stained accordingly following the kit instructions.

High-throughput RNA sequencing (RNA-Seq): Total pulmonary artery RNA was extracted with Trizol. After library preparation and Illumina Novaseq sequencing, raw data were quality-controlled and mapped. DEGs were subjected to GO enrichment analysis via clusterProfiler and KEGG pathway analysis using KOBAS 3.0 (Huan *et al.*, 2022).

Construction of overexpression plasmid: The chicken PDK4 sequence was amplified using homologous recombination, and the pEGFP-N1 vector was used to construct a PDK4-overexpressing plasmid. Primer sequences were designed as follows:

Forward sequence: TACCGGACTCAGATCTCGAG ATGAAGGCCGCCCGTATCG;

Reverse sequence: TGGTGGCGACCGGTGGATCCTC AGAGAGCTGCAGTCTGCTTT.

Statistical analysis: Results are expressed as the Means±SEM of experiments. Group comparisons were performed using unpaired two-tailed Student's t-test for two groups, and one-way or two-way ANOVA for single- and multi-factor multiple groups, respectively. $P<0.05$ was defined as statistically significant.

RESULTS

Gross autopsy: A comparison was made between six naturally occurring ascites syndrome (AS) broilers and six healthy broilers raised at high altitude. AS broilers exhibited a trend toward lower body weight relative to healthy broilers (Fig. 1D). Necropsy findings revealed abdominal distension in AS broilers, with the entire abdominal cavity filled with clear, transparent ascites (Fig. 1A).

Cardiac damage: AS broilers exhibited right ventricular dilation and hypertrophy, accompanied by thinning of the ventricular wall (Fig. 1B). The heart index and RV/TV ratio were significantly increased (Figs. 1E, F; $P<0.01$, $P<0.0001$). Histological analysis revealed disrupted myocardial architecture, including widened inter-fiber spaces, fiber disorganization, fragmentation, and erythrocyte infiltration (Fig. 1I).

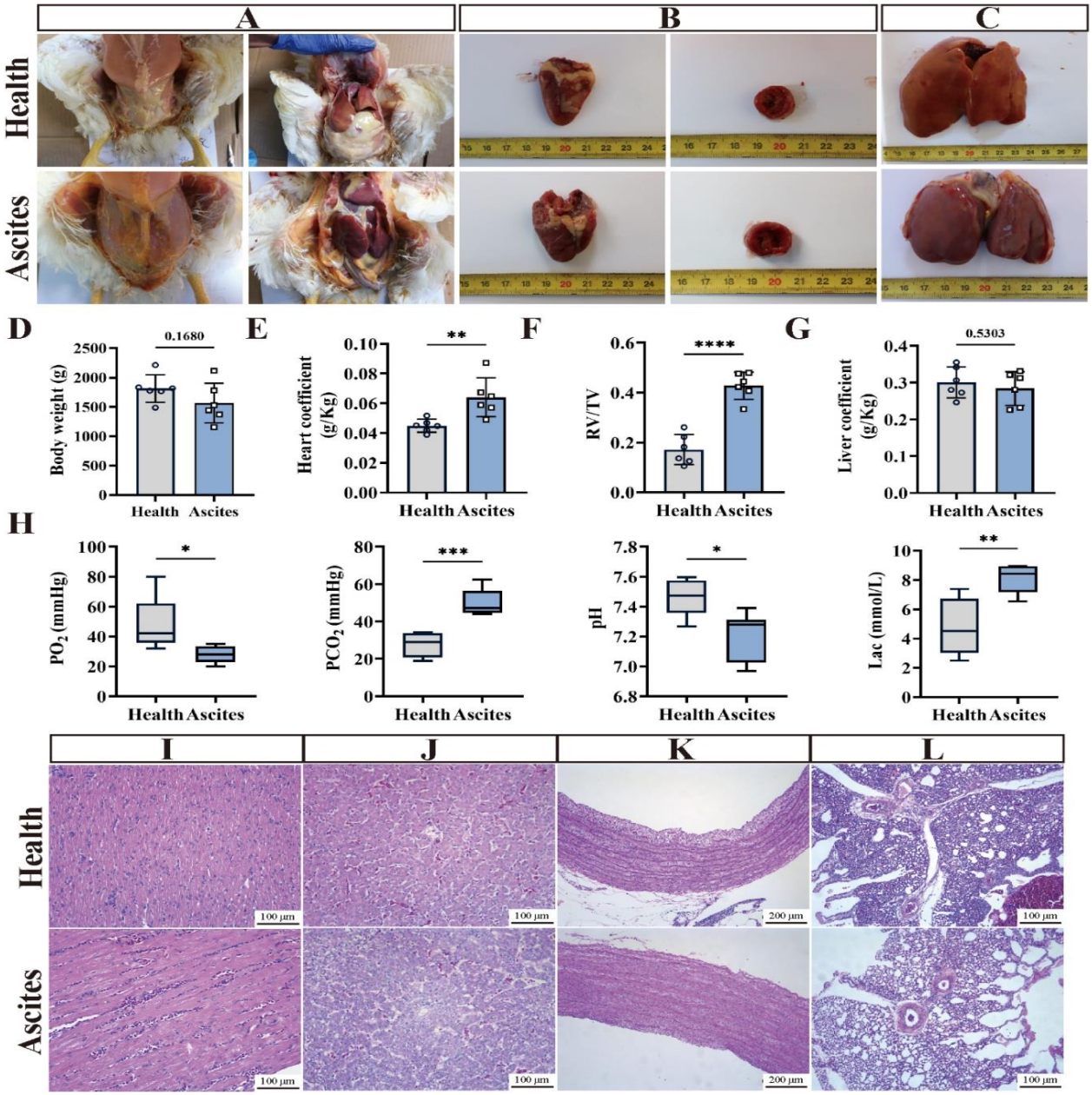

Fig. 1: Physiological and pathological changes in AS broilers. (A) Abdominal cavity anatomy; (B) Hearts and cross-sections; (C) Livers; (D) Body weight; (E) Heart index; (F) RV/TV; (G) Liver index; (H) Blood gas parameters (PO_2, PCO_2, pH, Lac); (I–L) H&E staining of myocardium, liver, pulmonary artery, and lung.

Liver damage: The liver showed congestion, edema, and blunt edges (Fig. 1C). Histological examination demonstrated hepatocyte vacuolation, disorganized arrangement, and inflammatory cell infiltration (Fig. 1J), although no significant difference in liver index was observed.

Pulmonary damage: H&E staining revealed marked pulmonary interstitial expansion and thickening of small pulmonary arterial walls (Figs. 1K, L). Pulmonary arterial smooth muscle exhibited hypertrophy and hyperplasia, with increased medial thickness and structural disorganization.

Abnormalities in blood gas indicators: Blood gas analysis showed significantly decreased venous PO_2 ($P<0.05$) and increased PCO_2 ($P<0.001$), along with reduced pH ($P<0.05$) and elevated lactate levels ($P<0.01$), indicating hypoxemia and metabolic acidosis in AS broilers.

Excessive proliferation of PASMCs is involved in PVR of AS broilers: PVR is a core pathological feature of AS broiler, and the excessive proliferation of PASMCs plays a pivotal regulatory role in this process. Morphological and molecular biological evaluations have established the abnormal proliferation of PASMCs in the AS broilers in a natural state. Quantitative indicators of PVR, including mean medial thickness of pulmonary arterioles (mMTPA%) and the ratio of medial area to total vascular area (WA/TA%), were significantly increased (Figs. 2C, 2E; $P<0.0001$), indicating thickening of the pulmonary arterial media. In addition, the expression of proliferation markers PCNA ($P<0.01$), Ki67, and α-SMA ($P<0.05$) was significantly elevated in pulmonary vessels (Figs. 2A, 2B).

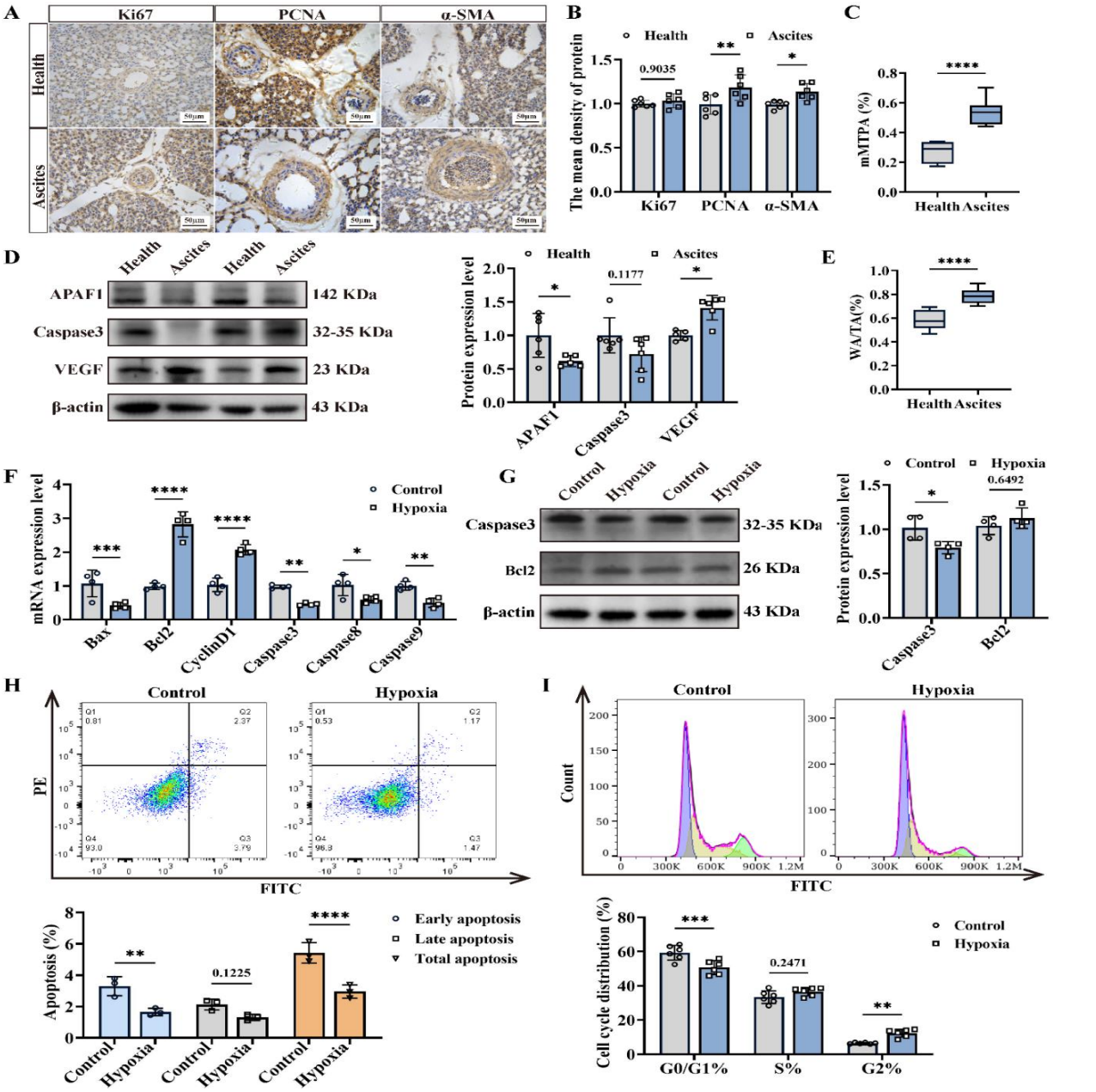

Fig. 2: Hypoxia-induced PASMCs proliferation and its association with PVR in AS broilers. (A–B) Immunohistochemistry of Ki67, PCNA, and α-SMA with quantitative analysis in lung tissues; (C) mMTPA%; (D) Western blot of APAF1, Caspase3, and VEGF in lung tissues; (E) WA/TA%; (F) mRNA expression of Bcl2, CyclinD1, Bax, and Caspase3/8/9 in PASMCs; (G) Western blot of Bcl2 and Caspase3 in PASMCs; (H) Apoptosis rate of PASMCs; (I) Cell cycle analysis of PASMCs.

To further investigate the relationship between hypoxia and PASMC proliferation, primary PASMCs were cultured under hypoxic conditions (3% O_2). Hypoxia promoted cell proliferation, with upregulation of proliferation-related genes/proteins (Bcl2, CyclinD1) and downregulation of apoptosis-related genes/proteins (Bax, Caspase3/8/9) (Figs. 2F, 2G). Flow cytometry analysis showed a reduced apoptotic rate (Fig. 2H; $P<0.01$) and accelerated cell cycle progression from G1 to S and G2 phases (Fig. 2I; $P<0.01$). Additionally, VEGF expression was increased, while apoptosis-related proteins (APAF1, Caspase3) were decreased in AS broilers (Fig. 2D).

PDK4 is highly expressed in the pulmonary vasculature and PASMCs of AS broilers: To identify key regulatory genes involved in PVR, differentially expressed genes (DEGs) between pulmonary artery tissues of AS broilers and healthy controls were identified using transcriptome sequencing, applying the following criteria: Padj<0.05 and |Log2FoldChange|>1 (Fig. 3A). The reliability of transcriptome data was further validated by RT-qPCR. Consistent with sequencing results, PDK4 expression was significantly increased in the AS group (Fig. 3B). Further validation demonstrated that PDK4 expression was elevated in broiler lungs, pulmonary arteries, and hypoxia-treated PASMCs. Western blot analysis showed increased PDK4 protein levels in pulmonary artery tissues (Fig. 3G) and hypoxic PASMCs (Fig. 3H), while immunohistochemistry revealed enhanced PDK4 staining in pulmonary vasculature (Fig. 3E). In addition, hypoxia-induced upregulation of PDK4 at the mRNA level was confirmed by qPCR (Fig. 3F). Together, these findings indicate that PDK4 could be a significant regulatory gene in PVR of AS. KEGG and GO pathway enrichments (Fig.

3C and D) showed that DEGs were engaged in various signaling pathways related to AS pathogenesis, such as, but are not confined to, the extracellular matrix, vasculature development, cell cycle, glucose metabolic process, lactate metabolic process, and apoptosis pathways. Besides, glucose and pyruvate metabolism pathways, in which PDK4 is involved, were significantly activated during AS progression. These findings indicate that PDK4 is associated with metabolic reprogramming in PVR of AS broilers.

Hypoxia induces enhanced glycolysis in PASMCs to promote lactate production: Glycolysis is essential for cell survival. Under hypoxic conditions (3% O_2), glucose consumption in PASMCs was significantly increased compared with normoxia (Fig. 4F; P<0.01). RT-qPCR analysis showed elevated mRNA expression of key glycolytic enzymes, including GLUT1 and HK2 (Fig. 4C; P<0.01). Enhanced glycolytic activity was accompanied by increased lactate production (Fig. 4E; P<0.05), elevated intracellular LDH levels (Fig. 4D; P<0.01), and upregulated expression of LDHA, as well as lactate transporters MCT1 and MCT4 (Fig. 4C; P<0.001). Mitochondrial respiratory function was further assessed (Fig. 4A). Results showed that oxygen consumption rates in PASMCs were significantly reduced under hypoxic conditions, with varying degrees of suppression observed in basal respiration (Basal), mitochondrial ATP production capacity, maximal respiration, and non-mitochondrial respiration. This was accompanied by increased ROS production (Fig. 4B). These findings indicate that hypoxia enhances PASMC glycolytic capacity while suppressing mitochondrial function, leading to increased lactate production.

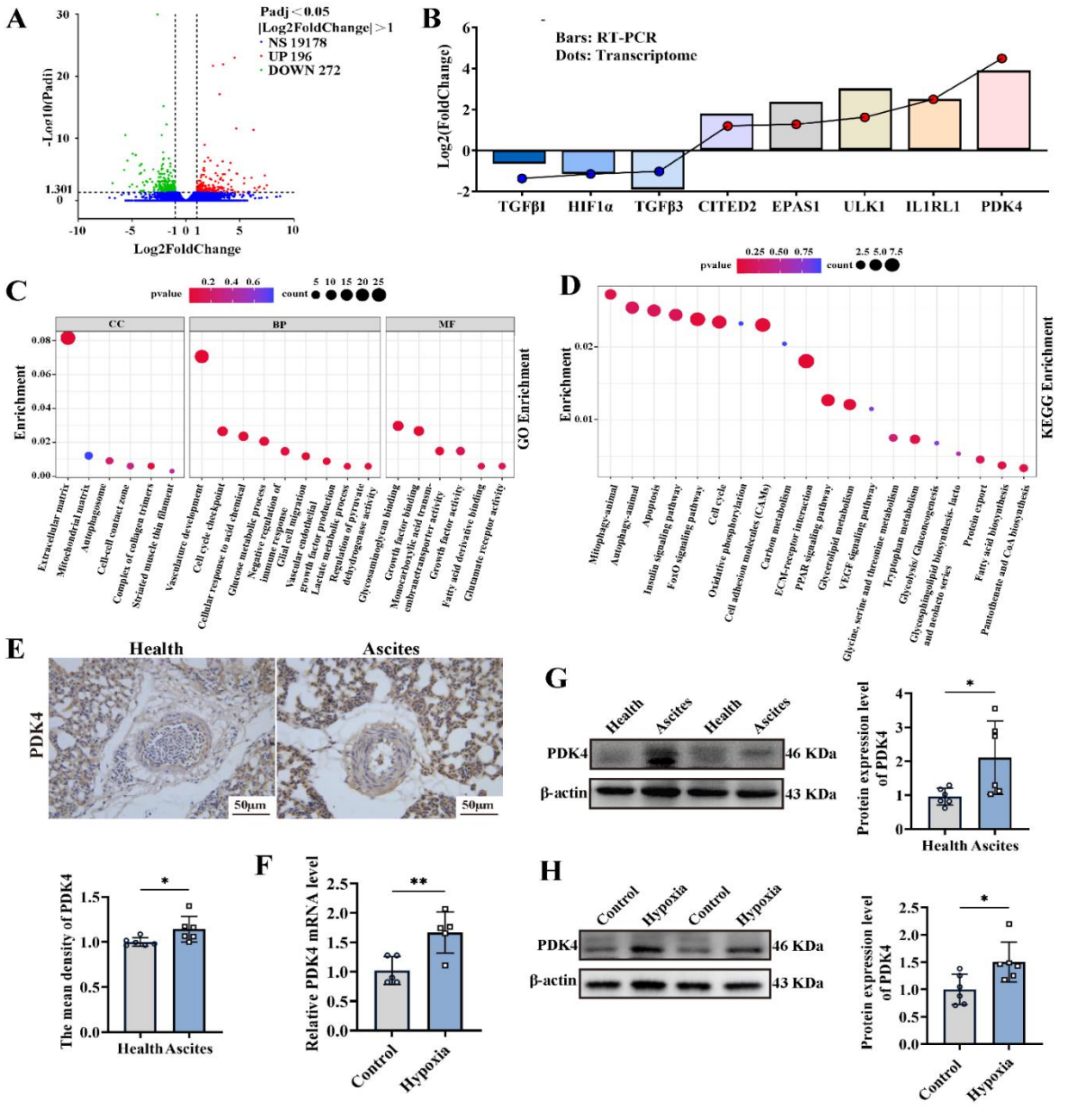

Fig. 3: PDK4 expression is upregulated in pulmonary vasculature of ascitic broilers and hypoxic PASMCs. (A) Volcano plot of DEGs; (B) RT-qPCR validation of selected genes; (C) GO enrichment; (D) KEGG enrichment; (E) Immunohistochemistry of PDK4 in ling tissues; (F) PDK4 mRNA expression; (G–H) Western blot of PDK4 in tissues and PASMCs.

Lactate accelerates the cell cycle progression of PASMCs to promote their proliferation: To explore the specific role of lactate in PVR of AS broilers, broiler PASMCs were incubated with lactate (0–50mM) for 24 or 48h (Fig. 5A). CCK-8 assays showed that lactate promoted PASMCs proliferation at concentrations below 15 mM after 48 h of incubation. Treatment with 15 mM lactate for 48h significantly reduced the apoptosis rate (Fig. 5D; P<0.05). RT-qPCR and Western blot analyses further demonstrated upregulation of proliferation-related genes and proteins, accompanied by downregulation of apoptosis-related markers (Fig. 5C and E). Cell cycle analysis revealed an increased proportion of PASMCs in the S phase following lactate treatment (Fig. 5F; P<0.05). Consistently, the expression of key regulators of the G1/S transition, including CyclinA, CyclinD1 (P<0.001), CyclinE1, and E2F1 (P<0.05), was elevated, along with increased CyclinD1 protein levels (Fig. 5E). These results indicate that lactate promotes PASMCs proliferation by driving cell cycle progression, thereby exacerbating PVR. that lactate promotes PASMCs proliferation by facilitating cell cycle progression, thereby contributing to PVR.

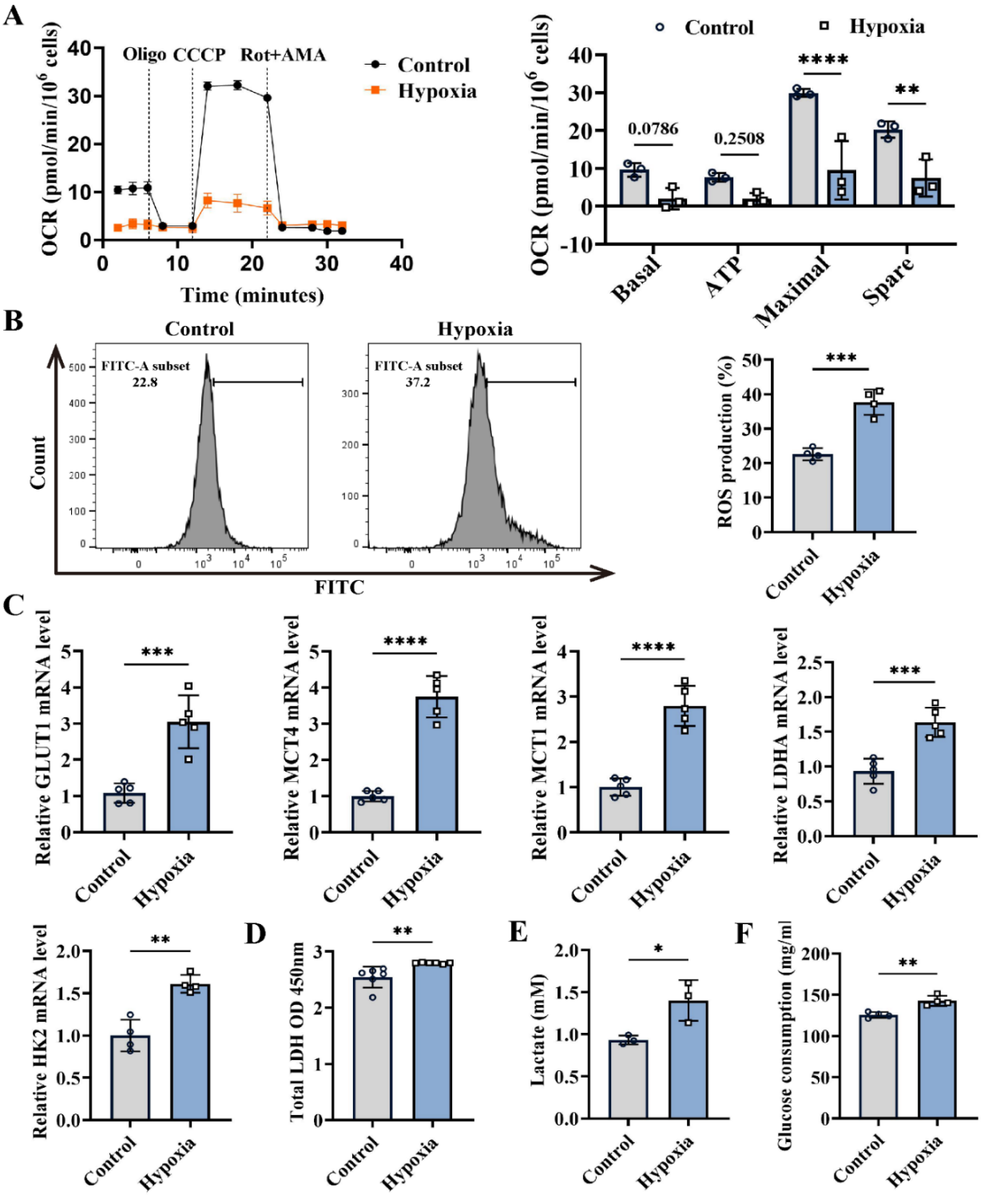

Fig. 4: Effects of hypoxia on glycolysis and lactate production. (A) OCR and mitochondrial respiration; (B) ROS levels; (C) mRNA expression of glycolytic genes; (D) Intracellular LDH; (E) Lactate production; (F) Glucose consumption.

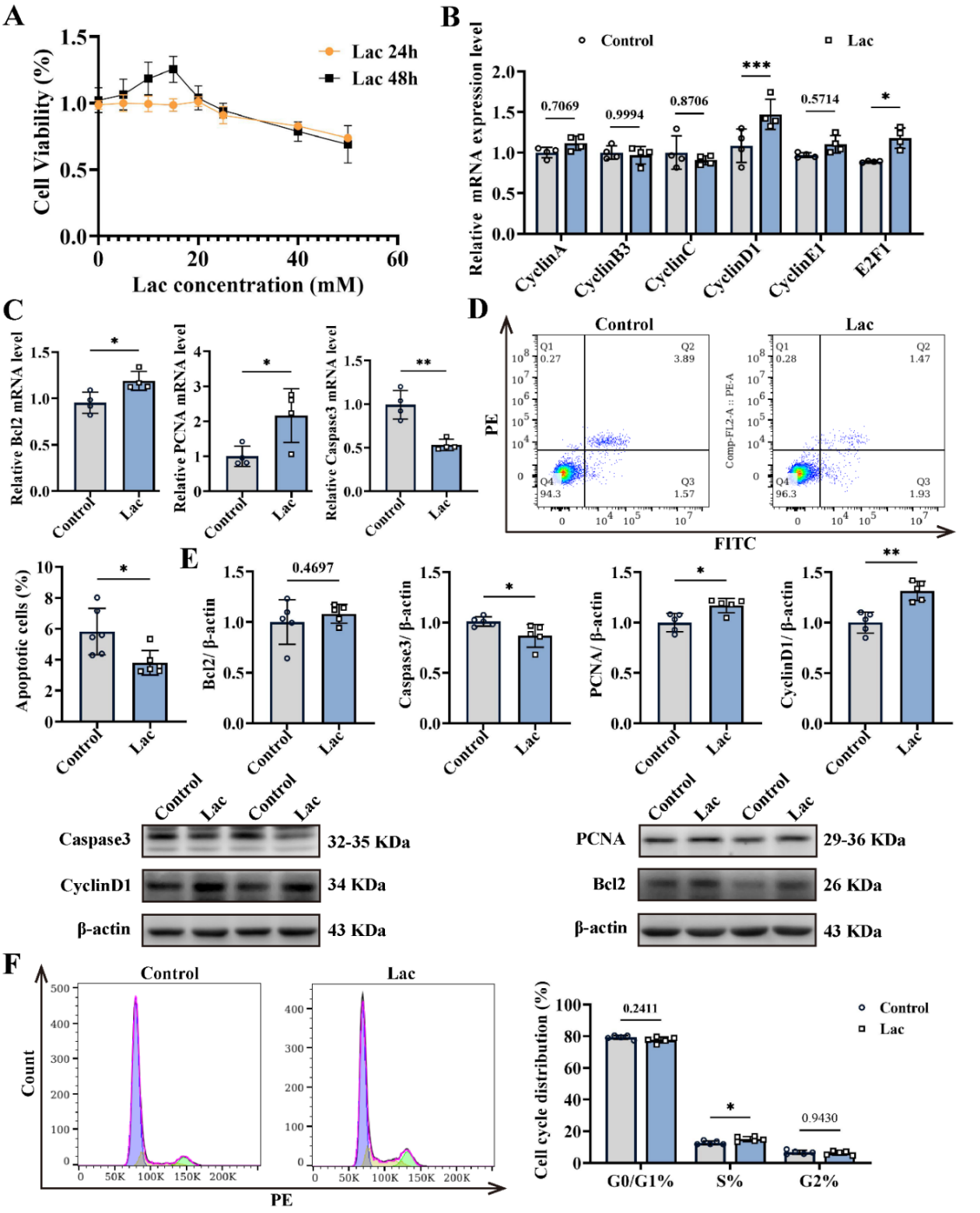

Fig. 5: Effects of lactate on PASMCs proliferation, apoptosis, and cell cycle. (A) Cell viability (CCK-8); (B) mRNA expression of cell cycle-related genes (CyclinA, CyclinB3, CyclinC1, CyclinD1, CyclinE1, E2F1); (C) mRNA expression of Bcl2, Bak, PCNA, and Caspase3; (D) Apoptosis rate (flow cytometry); (E) Western blot and quantification of Caspase3, CyclinD1, PCNA, and Bcl2; (F) Cell cycle distribution (flow cytometry).

PDK4 mediates PASMCs proliferation by regulating the glycolytic pathway: To investigate the functional role of PDK4 in PVR of AS broilers, a PDK4 overexpression plasmid was constructed, and the PDK inhibitor DCA (10mM) was used. RT-qPCR and Western blot analyses showed that both PDK4 mRNA and protein expression levels in the PDK4-overexpressing group (Ad-PDK4) were significantly higher than those in the empty vector-transfected group (Vector; $P<0.05$), indicating successful construction of the PDK4 overexpression plasmid (Fig. 6G and J).

PDK4 is a key regulator of glucose metabolism. Overexpression of PDK4 promoted glycolysis in PASMCs, as evidenced by upregulated mRNA expression of key glycolytic enzymes (GLUT1, PKM2, LDHA, MCT1; Fig. 6K; $P<0.05$), increased lactate production (Fig. 6E;

P<0.05), enhanced glucose consumption (Fig. 6I; P<0.001), and elevated intracellular LDH levels (Fig. 6D; P<0.01). Conversely, inhibition of PDK4 under hypoxia yielded opposite results: downregulated mRNA expression of glycolytic enzymes (LDHA, GLUT1, HK2) and lactate production comparable to that in normoxia-cultured PASMCs (Fig. 6J and F). PDK4 overexpression alone promoted PASMCs proliferation and reduced PASMCs apoptosis (Fig. 6A, B, C and G). While DCA treatment significantly suppressed hypoxia-induced cell proliferation (Fig. 6H; P<0.05) and increased PASMC apoptosis (Fig. 6L; P<0.001). These results indicate that PDK4 is involved in PVR in AS broilers, through regulation of glycolysis and PASMC proliferation.

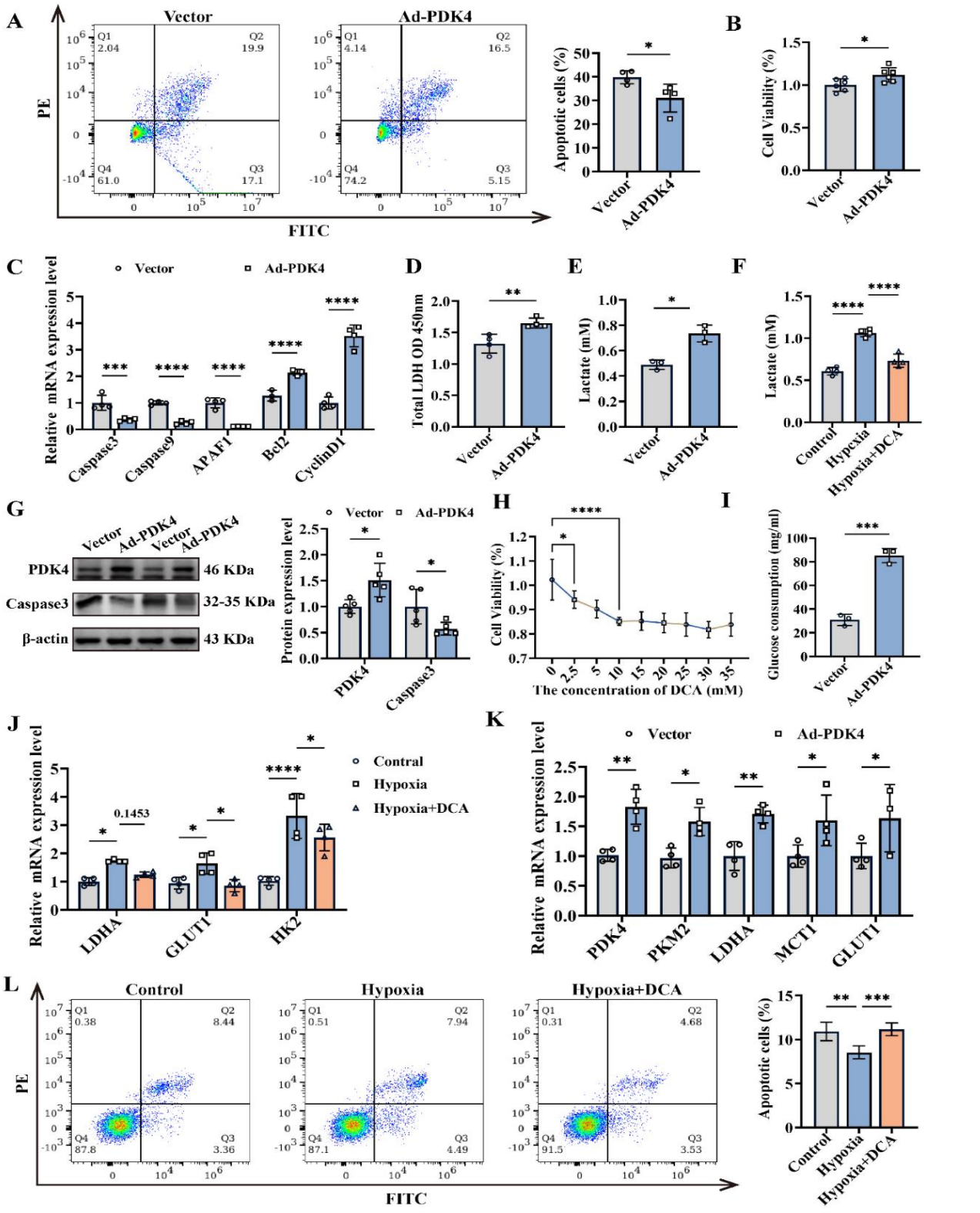

Fig. 6: Effects of PDK4 on glycolysis, proliferation, and apoptosis of PASMCs. (A) Apoptosis (flow cytometry); (B) Cell viability (CCK-8); (C) mRNA expression of apoptosis- and proliferation-related genes; (D) Intracellular LDH; (E–F) Lactate production; (G) Western blot of PDK4 and Caspase3; (H) Cell viability under DCA treatment (CCK-8); (I) Glucose consumption; (J) mRNA expression of LDHA, GLUT1, and HK2; (K) mRNA expression of glycolysis-related genes (PDK4, PKM2, LDHA, MCT1, GLUT1); (L) Apoptosis under DCA treatment (flow cytometry).

DISCUSSION

The high metabolic rate of the broilers also makes them more sensitive to hypoxia because the broilers are fast growing and thus demand more oxygen. As a result, an increase in the cellular oxygen consumption rate exceeding a body oxygen supply capacity produces the cardiopulmonary system compensatory adaptations to increase oxygen uptake and delivery. Nevertheless, in broilers with AS, such a compensatory effect leads to irreversible pathologic effects including a high ratio of RV/TV, right ventricular dilation and PVR (Guo *et al.*, 2023). In the present study, venous PO_2 levels in AS broilers were significantly lower than those in healthy controls. As fast-growing animals, broilers exhibit an inherent imbalance between pulmonary vascular development and the metabolic demands of rapidly growing muscle, rendering them more susceptible to hypoxic stress. This physiological mismatch may partly explain the high incidence of pulmonary hypertension syndrome (PHS) under intensive production conditions. The damage that occurs in broilers due to hypoxia is presumed to be multi-organ in nature (Khajali, 2022). Against the past results, myocardial injury was supported in AS broilers due to hypoxia, and the additional organs (liver and lungs) were also damaged (Zhang *et al.*, 2024). This is a multi-organ impairment that can be as a result of oxidative stress related to high altitude and high energy diets in broilers but this was not investigated in the study (Peng *et al.*, 2013).

PVR represents a key pathological endpoint in the progression of pulmonary hypertension and has been widely reported in various animal models, including rats, mice, and broilers (Li *et al.*, 2022; Awada *et al.*, 2023; Li *et al.*, 2024). In the present study, examination of lung and pulmonary artery tissues from naturally occurring AS broilers revealed significant increases in mMTPA and WA/TA%, accompanied by elevated expression of proliferation-related proteins such as PCNA in the vascular wall. These findings are consistent with previous reports on pulmonary vascular remodeling in PHS and further support the notion that excessive proliferation of PASMCs is a primary cellular basis for vascular wall thickening and lumen narrowing (Gomez *et al.*, 2020). It is well established that hypoxia disrupts the balance between proliferation and apoptosis in PASMCs, promoting abnormal cell growth, migration, and accumulation within the vascular wall, thereby driving vascular remodeling (Wang *et al.*, 2022). Oxygen sensing in vascular endothelial and smooth muscle cells is mediated by multiple signaling systems, including hypoxia-inducible factors (HIFs), NADPH oxidases, and endothelial nitric oxide synthase (eNOS), which coordinate cellular responses to changes in oxygen availability (Ullah *et al.*, 2021). Vascular cells of the wall react to changes in oxygen concentration with the stress calls of endothelial-mesenchymal transition (EndMT), angiogenesis, cell growth, and cell migration (Jing *et al.*, 2018； Tang *et al.*, 2023). Furthermore, under hypoxic conditions, aberrant extracellular signal transduction pathways, such as AKT, ERK1/2, and NF-κB, lead to the activation of anti-apoptotic signaling. This disrupts the balance of apoptosis-related genes and proteins, suppresses apoptosis-related pathways in PASMCs, and allows abnormally proliferating PASMCs to survive and accumulate, thereby promoting pulmonary vascular remodeling (Li *et al.*, 2024).

Consistent with previous findings reported by Wang *et al.* (2022) hypoxia was shown to accelerate cell cycle progression in PASMCs and promote excessive proliferation. Notably, our results further suggest that this process is closely associated with increased lactate production derived from enhanced glycolytic activity.

In the present study, both hypoxia and PDK4 overexpression were associated with enhanced glycolytic activity and increased lactate production. Enhanced glycolysis under hypoxic conditions may provide a rapid source of energy and abundant biosynthetic intermediates, thereby supporting the metabolic demands of cell proliferation(Wertheim *et al.*, 2023). Emerging evidence suggests that lactate is not merely a metabolic byproduct, but also functions as a signaling molecule that participates in the regulation of cell cycle progression, abnormal cell proliferation, and tissue remodeling through mechanisms such as lactylation (Liu *et al.*, 2023; Peng *et al.*, 2025). However, the specific role of lactate in pulmonary vascular remodeling remains incompletely understood. In this study, lactate was found to promote cell cycle progression from the G1 to S phase, providing a potential explanation for its pro-proliferative effect on PASMCs. Notably, this finding is consistent with the elevated blood lactate levels observed in AS broilers.

Our results show that PDK4 expression is upregulated in the lungs, pulmonary arteries of AS broilers, and hypoxia-cultured PASMCs, and confirm that PDK4 can promote PASMCs proliferation. Consistent with this, studies on various metabolic diseases have also reported that high PDK4 expression enhances cell proliferative capacity (Liu *et al.*, 2021; Li *et al.*, 2023; Ma *et al.*, 2023)**.** PDK4 has also been observed to be upregulated in multiple cell lines with high glycolytic rates in response to hypoxic stimulation, indicating its involvement in glucose metabolism (Li *et al.*, 2023; Ma *et al.*, 2023). We similarly verified the function in the current study. Combined with the upregulated PDK4 expression in the pulmonary vasculature of AS broilers, we hypothesize that it may participate in AS-related PVR through two potential pathways: First, PDK4 may compensatorily enhance PASMCs' glycolytic dependence by inhibiting PDH activity, providing energy and intermediate metabolites for abnormal cell proliferation. (Chen *et al.*, 2024). Our study confirmed that this process promotes glycolysis-mediated excessive lactate production, which supplies energy and metabolic intermediates for aberrant PASMC proliferation. However, due to experimental design limitations, we did not explore the roles of other glycolytic intermediates (e.g., phosphoenolpyruvate, ribose-5-phosphate) in this process, which is an area that needs further investigation in the future. In addition to its role in glucose metabolism, PDK4 has been reported to regulate fatty acid oxidation and metabolic substrate utilization, suggesting a potential involvement in lipid metabolic balance. Such metabolic alterations may influence vascular cell function and structural stability, thereby contributing to vascular remodeling(Song *et al.*, 2021). To date, no relevant studies have reported this mechanism, and its feasibility and specific molecular pathways require further validation.

Conclusions: The current study results show that AS broilers develop hypoxemia and acidosis, accompanied by pulmonary vascular remodeling characterized by vascular wall thickening, enhanced proliferation, and reduced apoptosis of PASMCs. PDK4 expression was significantly upregulated in pulmonary tissues and hypoxia-treated PASMCs, along with activation of glycolysis-related pathways. Further experiments demonstrated that hypoxia and PDK4 overexpression increased glycolytic activity and lactate production, and promoted PASMCs proliferation, whereas inhibition of PDK4 produced opposite effects. In addition, lactate promoted cell cycle progression from G1 to S phase, thereby enhancing PASMCs proliferation. These findings suggest that PDK4 contributes to pulmonary vascular remodeling in AS broilers by enhancing glycolysis and promoting PASMCs proliferation.

Conflict of interest: All contributing authors affirm that they have no conflicts of interest to report.

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Ethical statement: All animal experiments obtained review and approval from the Animal Ethics Committee of South China Agricultural University (approval number: 2025F377), and were implemented in adherence to the institution's guidelines on laboratory animal care and use.

Authorship contributions: PW and YL: Writing - original draft, Formal analysis, Data curation, Conceptualization, Investigation, Visualization. BC, XH and LF: Investigation, Methodology. MIT.: Writing - review & editing. CL, ZT, YL and YW: Validation, Supervision, Conceptualization, Writing - review & editing, Funding acquisition.

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