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Original Article

HECW2 knockdown suppresses the development of infantile hemangioma by inhibiting ALKBH5/LDHA axis-mediated glycolysis

Kun Peng, Renpeng Xia, Fan Zhao, Yong Xiao, Tidong Ma, Ming Li, Yong Feng, Chonggao Zhou

Department of Fetal and Neonatal Surgery, The Affiliated Children's Hospital of Xiangya School of Medicine, Central South University (Hunan Children's Hospital), Changsha 410007, Hunan, China

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Abstract: HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 (HECW2), a member of E3 ubiquitin ligase family, was identified as a hub gene in infantile hemangioma (IH). This study investigated the roles and mechanisms of HECW2 in IH development. Our investigation revealed that HECW2 was up-regulated in proliferative and involuting IH tissues compared with normal adjacent tissues. Hemangioma endothelial cells (HemECs) were isolated and transfected with over-expressed HECW2 or knockdown plasmids. Functional studies demonstrated that HECW2 over-expression facilitated proliferation, migration, invasion as well as inhibited apoptosis in HemECs. Furthermore, over-expressed HECW2 markedly promoted glycolysis in HemECs, as evidenced by increased glucose uptake, lactate production, and adenosine triphosphate (ATP) generation. In contrast, HECW2 knockdown showed the opposite results. Mechanistically, HECW2 regulated the ubiquitination of AlkB homolog 5 (ALKBH5), subsequently enhancing the expression of lactate dehydrogenase A (LDHA) through ALKBH5-mediated m6A demethylation of LDHA mRNA. HECW2 knockdown suppressed glycolysis and tumor-like cellular behaviors in HemECs, which were abrogated by LDHA over-expression. Additionally, in vivo validation using an IH xenograft mouse model demonstrated that HECW2 knockdown significantly suppressed tumor growth. These findings established HECW2 as a key regulator in IH progression through the regulation of ALKBH5/LDHA-mediated glycolysis, suggesting its potential as a therapeutic target for IH treatment.

Keywords: Infantile hemangioma (IH), Glycolysis, Hemangioma endothelial cells, HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 (HECW2), AlkB homolog 5 (ALKBH5), Lactate dehydrogenase A (LDHA)

Introduction

Infantile hemangioma (IH) is the most common benign vascular tumor affecting infants and children, with prevalence rates reaching as high as 12% within the first year of life [1]. IH typically progresses through three phases: proliferative (first 6-12 months after birth), involuting (starting around 13 months of age), and involuted (by 4-7 years of age) [2]. The proliferative phase features rapid growth of tumor lesion, followed by gradual shrinkage and fading during the involuting phase. The complete resolution observed in approximately 90% of cases in the final involuted phase. Superficial IH characteristically manifest as erythematous cutaneous patches, whereas deep lesions typically demonstrate bluish subcutaneous discoloration or nodular elevation on the skin [1]. Despite its benign nature, IH can cause significant morbidity due to complications such as ulceration, bleeding, infection, and, in rare cases, vision obstruction, respiratory distress or heart failure [3]. Current clinical management of hemangiomas encompasses a spectrum of interventions, ranging from observation to pharmacological therapies (e.g., β-blockers like propranolol) and surgical and/or laser interventions [4, 5]. Notably, recent advancements in minimally invasive techniques, such as photodynamic therapy, have shown promising results in reducing tumor size with benefits such as minimal trauma and faster recovery times [6]. The etiology of IH remains largely enigmatic, presenting a unique challenges for physicians in the fields of pediatric dermatology

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and oncology. Emerging research suggests that IH may originate from abnormal differentiation of vascular endothelial stem cells, with genetic and epigenetic variations playing a critical role in its pathogenesis [7]. Recent studies have revealed the role of estrogen in IH development, suggesting potential links between maternal hormone levels and IH growth [8]. Furthermore, the updated integration of genomics, transcriptomics and proteomics provides promise for identifying novel biomarkers and therapeutic targets for IH [9]. Although significant progress has been made in understanding and managing IH, unraveling the genetic and epigenetic variations in IH is essential to optimize treatment strategies and improve outcomes for affected infants and children.

HECW2 (HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2), alternatively designated as neural precursor cell expressed developmentally down-regulated 4 (NEDD4)-like E3 ubiquitin-protein ligase 2 (NEDL2), is recognized as a constituent of the NEDD4 family of E3 ubiquitin ligases. NEDD4 E3 ligases are pivotal in the process of recognizing and altering proteins by means of ubiquitination, thereby playing a role in a plethora of cellular pathways including cell proliferation, apoptosis, and inflammatory responses [10]. A growing body of research has highlighted the involvement of HECW2 in the neurodevelopmental disorders [11, 12]. Notably, previous studies have indicated a potential role of HECW2 in regulating tumorigenesis. For instance, HECW2 has been identified as a predictor for the prognosis of prostate cancer [13]. It is also recognized as a novel biomarker for the prognosis of neuroblastoma patients, and is expected to become a promising target for the treatment of high-risk neuroblastoma patients [14]. These findings underscore the importance of HECW2 in cancer biology and its prognostic potential in clinical settings. A recent mechanistic study revealed that HECW2 facilitated the development and chemoresistance of colorectal cancer by regulating the ubiquitination of lamin B1 protein and activating the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathways [15]. Moreover, HECW2 mediated the ubiquitin-proteasome degradation of proliferating cell nuclear antigen (PCNA), which is a critical factor in promoting rapid tumor proliferation [16]. Notably, a bioinformatics analysis identified that HECW2 was a hub up-regulated gene in the protein-protein interaction (PPI) network, exhibiting a strong correlation with IH [17]. While the exact roles and mechanisms of HECW2 in IH remain unclear, the roles of HECW2 in the ubiquitin-proteasome system suggests its involvement in regulating the expressions of tumor development-related genes and key signaling pathways, further driving cancer progression. Moreover, HECW2 may modulate endothelial cell junction stability and angiogenesis [18], which is a crucial pathophysiology of IH. These mechanistic insights provide a foundation for exploring the roles of HECW2 in IH progression.

Glycolysis is a primary metabolic reprogramming pathway in cancer cells. The pronounced increase in aerobic glycolysis, a phenomenon historically noted in cancer biology, is a key feature of cancer cells [19]. This metabolic adaptation enables cancer cells to efficiently convert glucose into pyruvic acid and subsequently lactate, thereby supporting tumor growth and metastasis [20]. Hemangioma-derived endothelial cells (HemECs) play a pivotal role during both the proliferation and involution phases of IH. It has demonstrated that HemECs display an elevated glycolysis metabolism compared to human umbilical vein endothelial cells (HUVECs) [21]. Additionally, propranolol, a first-line drug for IH, has been demonstrated to inhibit glucose metabolism in HemECs by suppressing glycolysis [21]. Recent evidence has illustrated that glycolysis-associated molecules, such as lactate dehydrogenase A (LDHA) and hexokinase 2 (HK2), are highly expressed in IH [22]. Experimental evidence has also suggested that the inhibition of these molecules regulates the proliferation, migration, and angiogenesis of HemECs. However, further investigations are needed to clarify the molecule mechanisms of glycolysis in IH.

This study explored the roles and molecular mechanisms of HECW2 and glycolysis molecule LDHA in IH progression, aiming to provide a potential treatment target for IH.

Materials and methods

Clinical samples

IH tissues and normal adjacent tissues were collected from IH patients (n=40; aged between 3 months old and 5 years old) at Hunan

Children's Hospital. These cases were divided into involuting IH (n=17) and proliferative IH (n=23) according to their histopathological examination results. All tissue samples were harvested during surgical operation, and then frozen in liquid nitrogen for experimental use. This study has been approved by the Ethic Committee of Hunan Children's Hospital and has obtained informed consents from the guardians of patients.

Cell isolation and culture

HemECs were isolated from 6 IH tissue samples according to reported methods [23]. HUVECs and mouse hemangioendothelioma cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin solution at 37°C in 5% CO_2.

Cell transfection

All over-expressed plasmids targeting HECW2, AlkB homolog 5 (ALKBH5), and LDHA, as well as short hairpin RNAs (shRNAs) targeting HECW2 and ALKBH5 were obtained from RiboBio (Guangzhou, China). Cell transfection was conducted using Lipofectamine™ 3000 (Invitrogen, USA).

Western blot analysis

Proteins were isolated using a radioimmunoprecipitation assay (RIPA) lysis buffer and then were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were pre-treated with a 5% non-fat milk, and were subsequently exposed to primary antibodies at a constant temperature of 4°C overnight. This was succeeded by the application of secondary antibodies for 2 h. The detection of protein bands was achieved with an enhanced chemiluminescence (ECL) detection kit provided by Beyotime in China. The quantification of these bands was performed using the ImageJ software. The specific primary antibodies (Abcam, UK) utilized in this process are detailed as follows: HECW2 (1:1000, ab92711), ALKBH5 (1:1000, ab195377), LDHA (1:5000, ab52488), and glyceraldehyde-3-phosphate dehydrogenase (1:2500, ab9485).

Immunohistochemistry

The paraffin-embedded IH tissue sections (4 μm in thickness) were subjected to microwave treatment in a sodium citrate solution. The process was halted by immersing the sections in a 3% hydrogen peroxide (H_2O_2) for 10 min. Following this, the sections were treated with goat serum and then exposed to primary antibodies specific to HECW2 at a low temperature of 4°C overnight. The subsequent step involved the application of a secondary antibody at 37°C for 1 h. The staining process was completed using a 3,3'-diaminobenzidine tetrahydrochloride staining kit (Solarbio, Beijing, China). Finally, the stained sections were examined under a light microscope (Olympus, Tokyo, Japan).

Cell counting Kit-8 (CCK-8) assay

After the transfection process, HemECs were distributed into 96-well plates and subjected to incubation at specific times of 0, 24, 48, and 72 hours. At each designated time, 10 μL of CCK-8 regent (Dojindo, Japan) was introduced into each well and further incubating for 2 h. The final step involved measuring the absorbance (450 nm) using a micro-plate reader.

Colony formation assay

HemECs were plated in 6-well plates at 500/well and allowed to grow for two weeks. Then, the colonies were fixed using a 4% paraformaldehyde and then stained with a 0.1% crystal violet solution for 20 min. The number of colonies was determined using Image J software.

Wound healing assay

HemECs were seeded in 6-well plates and allowed to grow until they reached 60-70% confluence. Next, a straight wound was gently introduced on cell surface using a sterile micropipette tip, and any detached cells were cleared by flushing with phosphate buffered saline. The plates were subsequently filled with serum-free medium and incubated for 48 h. Images of cell migration at the wound site were captured at 0 and 24 h using a light microscope, and the migration distance was determined using ImageJ software.

Transwell invasion assay

The Transwell system (Corning, NY, USA), equipped with 8 μm pores, was pre-coated with a layer of Matrigel matrix. A total of 50,000 serum-starved HemECs were placed in the upper chamber, opposite to DMEM enriched with 10% FBS in the lower chamber. Following 24 h of incubation, the cells that had migrated to the lower chamber were stained using a 0.5% crystal violet solution for 30 min. Cell images were obtained using an Olympus microscope from Tokyo, Japan.

Cell apoptosis

HemECs (1×10^6 cells) were stained with Annexin V-FITC and propidium iodide staining solution (Solarbio, Beijing, China) for 15 min in darkness. The apoptotic rate of the cells was subsequently assessed using a flow cytometer (BD Bioscience, USA).

Co-immunoprecipitation (Co-IP)

The protein supernatant of HemECs was incubated with anti-HECW2 antibody or anti-ALKBH5 antibody at 4°C overnight. These were then mixed with 100 μL of protein A/G agarose beads and incubated at 4°C for 4 h. The beads were subsequently isolated and heated in a loading buffer for 5 min to elute the proteins, and the immunoprecipitated proteins were subsequently analyzed via Western blot analysis.

Ubiquitination assay

HemECs were transfected with plasmids encoding for HA-Ub, MYC-HECW2, and Flag-ALKBH5. Cells were treated with 10 μM MG132 for 4 h before lysis. Cell lysate was collected and subjected to immunoprecipitation with an anti-ALKBH5 antibody overnight at 4°C. Ubiquitination level was assessed using Western blot analysis.

Methylated RNA immunoprecipitation quantitative polymerase chain reaction (MeRIP-qPCR)

Total RNAs were extracted from HemECs using TRIzol reagent, and subsequently fragmented into approximately 100-nucleotide pieces using the Magnesium RNA fragmentation Module (NEB, Beijing, China). Magna ChIP Protein A+G Magnetic Beads were pre-bound to an anti-m6A antibody and then used to immunoprecipitate the mRNA fragments at 4°C overnight. The mRNA enriched with m6A modifications was released from the beads and subsequently analyzed via RT-qPCR.

Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNAs were extracted from HemECs using TRIzol reagent (Invitrogen, USA). First-strand cDNA synthesis was conducted with the Prime Script RT reagent Kit (Takara, Dalian, China). RT-qPCR reaction was carried out in triplicate using SYBR Premix Ex Taq II (Takara, Dalian, China). LDHA mRNA level were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase, and relative quantification was performed using the $2^{-\Delta\Delta CT}$ method.

RNA pull-down assay

A biotinylated LDHA probe, sourced from Genechem in Shanghai, China, was mixed with Streptavidin magnetic beads (Invitrogen, USA) and incubated for 2 h. This probe-coated bead mixture was then combined with HTR8/SVneo cell lysate at 4°C overnight. The proteins that interacted with the probe were eluted and further characterized using Western blot analysis.

Glycolysis test

Glucose uptake, lactate production, and adenosine triphosphate (ATP) level were examined using the Glucose Uptake Assay Kit (Abcam, USA), Lactate Assay Kit (Cell Biolabs, USA), and ATP Assay Kit (Beyotime, Dalian, China), respectively, according to manufacturers' instructions. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using a Seahorse XFe96 Analyzer (Seahorse Bioscience). HemECs (1×10^4 cells/well) were seeded on Seahorse XF96 microplates and incubated overnight. ECAR and OCR were detected using a Seahorse XF Glycolysis Stress Test kit (Agilent Technologies) and a Seahorse XF Cell Mito Stress Test kit (Agilent Technologies) according to previously reported methods [24, 25].

Animal xenograft tumor

BALB/c nude mice, aged between 4 and 6 weeks and weighing between 16 and 18 g, were sourced from the animal facility at Hunan

Children's Hospital. These mice were housed in sterile conditions. Ethical approval for all animal procedures was granted by the Ethics Committee of Hunan Children's Hospital. HemECs (5×10^6 cells), either transfected with sh-NC or sh-HECW2, were implanted subcutaneously into the right flank of each mouse (eight mice per group). Tumor growth was monitored weekly using caliper measurements. On day 28 post-injection, the mice were euthanized through intraperitoneally injection of 150 mg/kg pentobarbital sodium solution, and the tumor tissues were collected for subsequent studies.

Statistical analysis

Results from three independent experiments were averaged and displayed as mean ± standard deviation (SD). The statistical analysis was conducted using SPSS version 22.0. The normal distribution of data was verified by the Shapiro-Wilk test. Parametric tests were applied only if the data conformed to a normal distribution. Specifically, two-group comparisons in the study were carried out using Student's t-test, while one-way analysis of variance (ANOVA) followed by Tukey-Kramer correction was utilized for analyzing multiple groups. Non-parametric tests (Kruskal-Wallis test/Mann-Whitney test) were used if data were not normally distributed. For longitudinal measurements involving repeated time points, repeated-measures ANOVA was utilized. $P<0.05$ was considered statistically significant.

Results

HECW2 was up-regulated in IH tissues and cells

A bioinformatics analysis identified that HECW2 was up-regulated and functioned as a hub gene in the PPI network, exhibiting a strong correlation with IH [17]. We first measured HECW2 protein levels in clinical human IH tissues. Western blot analysis results illustrated that HECW2 was up-regulated in proliferative and involuting IH tissues compared with normal adjacent tissues (**Figure 1A**, **1B**). Consistently, IH tissues exhibited higher protein level than normal adjacent tissues, as measured by immunohistochemistry (**Figure 1C**). Furthermore, our evidence also indicated that HECW2 was up-regulated in HemECs and mouse hemangioendothelioma cells compared with HUVECs (**Figure 1D**, **1E**). Consequently, we speculated that HECW2 could play a significant role in the advancement of IH.

Over-expressed HECW2 facilitated proliferation, migration, invasion and inhibited apoptosis in HemECs

Subsequently, we intended to elucidate the impact of HECW2 on IH progression by introducing oe-HECW2 or sh-HECW2 into HemECs. We firstly showed that HECW2 protein level was prominently increased after oe-HECW2 transfection, and were decreased after sh-HECW2 transfection (**Figure 2A**, **2B**). The over-expression of HECW2 was observed to facilitate cell proliferation (**Figure 2C**) and colony formation (**Figure 2D**, **2E**) in HemECs. Conversely, the reduction of HECW2 suppressed these processes. Furthermore, we illustrated that the over-expression of HECW2 promoted cell migration (**Figure 2F**, **2G**) and invasion (**Figure 2H**, **2I**), whereas the converse results were observed following HECW2 knockdown. Additionally, we discovered that the over-expression of HECW2 resulted in a decrease in cell apoptosis, imposing an opposite effect in HemECs in comparison to the cellular level prior to the decrease (**Figure 2J**, **2K**).

HECW2 increased ALKBH5 stability through ubiquitination regulation

HECW2 is known as a E3 ubiquitin ligase regulating protein stability. Results generated from the GeneMANIA platform (https://genemania.org/) suggested a potential interaction between HECW2 and AlkB homolog 5 (ALKBH5) (**Figure 3A**). Further, Co-IP experiments confirmed this interaction in HemECs (**Figure 3B**, **3C**), and their co-localization fluorescence staining in HemECs were presented in **Figure 3D**. Then, we found that the over-expression of HECW2 significantly increased the expression level of ALKBH5 protein in HemECs. Conversely, the down-regulation of HECW2 resulted in a lower expression of ALKBH5 (**Figure 3E**, **3F**). Subsequently, we investigated whether HECW2 regulated the expression level of ALKBH5 through ubiquitination. Our results revealed that HECW2 induced ALKBH5 ubiquitination in HemECs (**Figure 3G**), and HECW2 knockdown decreased the stability of ALKBH5 protein (**Figure 3H**, **3I**). Collectively, our results revealed that HECW2

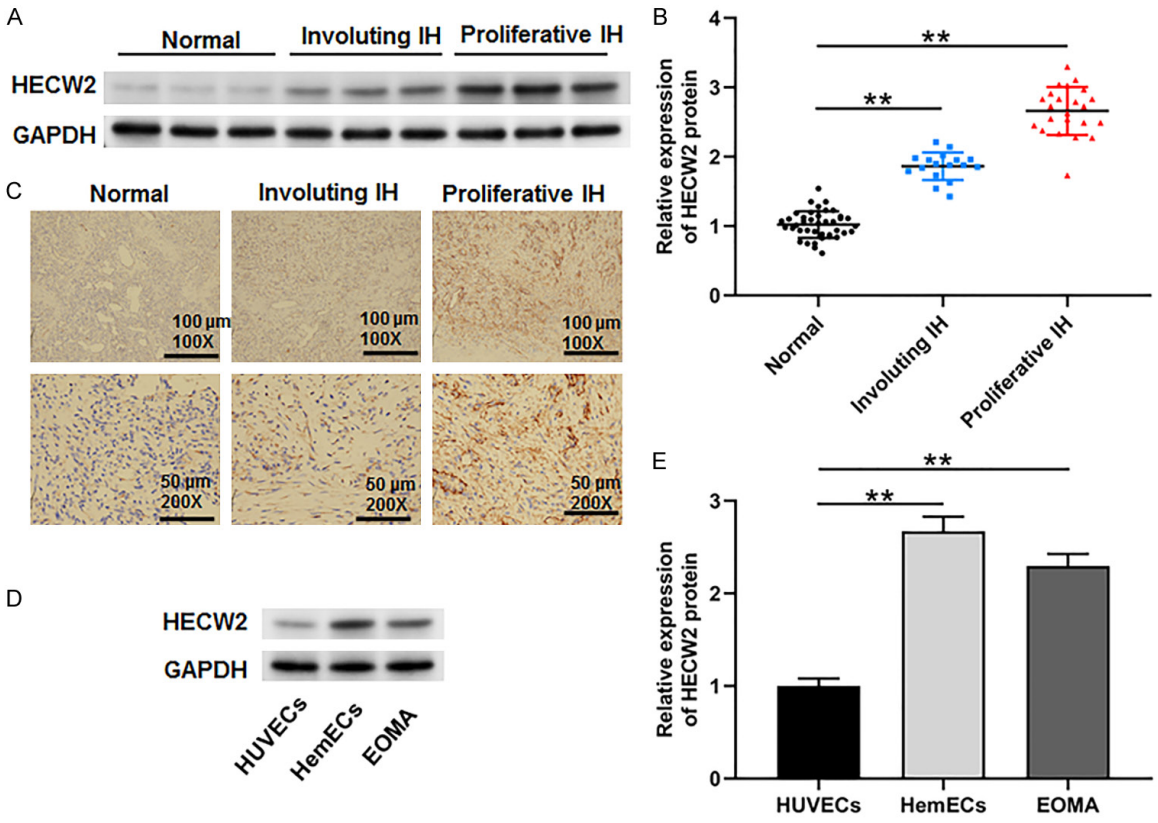

Figure 1. HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2 (HECW2) is up-regulated in infantile hemangioma (IH) tissues and cells. HECW2 protein levels in IH tissues and normal adjacent tissues were examined using (A, B) Western blot analysis and (C) immunohistochemistry. (D, E) HECW2 protein levels in hemangioma endothelial cells (HemECs), mouse hemangioendothelioma cells (EOMA), and human vascular endothelial cells (HUVECs) were determined using Western blot analysis. Results are expressed as mean ± standard deviation (SD). $^{**}P<0.01$.

increased ALKBH5 stability through ubiquitination regulation.

HECW2 enhanced LDHA expression through regulating ALKBH5-mediated LDHA m6A demethylation

The Sequence-based RNA Adenosine Methylation Prediction database showed the N6-methyladenosine (m6A) methylation sites in LDHA (**Figure 4A**). We found that the over-expressed ALKBH5 reduced LDHA m6A methylation level, while ALKBH5 knockdown showed the opposite results (**Figure 4B**). Subsequently, we explored whether HECW2 regulated ALKBH5-mediated LDHA m6A demethylation. RNA pull-down results illustrated that the over-expression of HECW2 led to an increase of the enrichment of ALKBH5 in TRIM37 mRNA (**Figure 4C**, **4D**). Furthermore, the over-expression of HECW2 decreased LDHA m6A methylation, whereas its knockdown produced the reverse effect (**Figure 4E**). HECW2 knockdown reduced the stability of LDHA mRNA (**Figure 4F**). Additionally, LDHA mRNA and protein levels were elevated upon the over-expression of HECW2, and conversely, they were diminished when SPTBN1 was suppressed (**Figure 4G-I**). Consequently, we suggested that HECW2 enhanced LDHA expression through regulating ALKBH5-mediated LDHA m6A demethylation.

The over-expressed HECW2 promoted glycolysis in HemECs

Given the role of LDHA in glycolysis, we next explored the role of HECW2 in glycolysis during IH progression. Our results implicated that HECW2 over-expression increased glucose uptake (**Figure 5A**), lactate production (**Figure 5B**), and ATP level (**Figure 5C**) in HemECs, while HECW2 knockdown had the opposite effects. Moreover, ECAR and OCR were assessed to reflect aerobic glycolysis and oxidative phos-

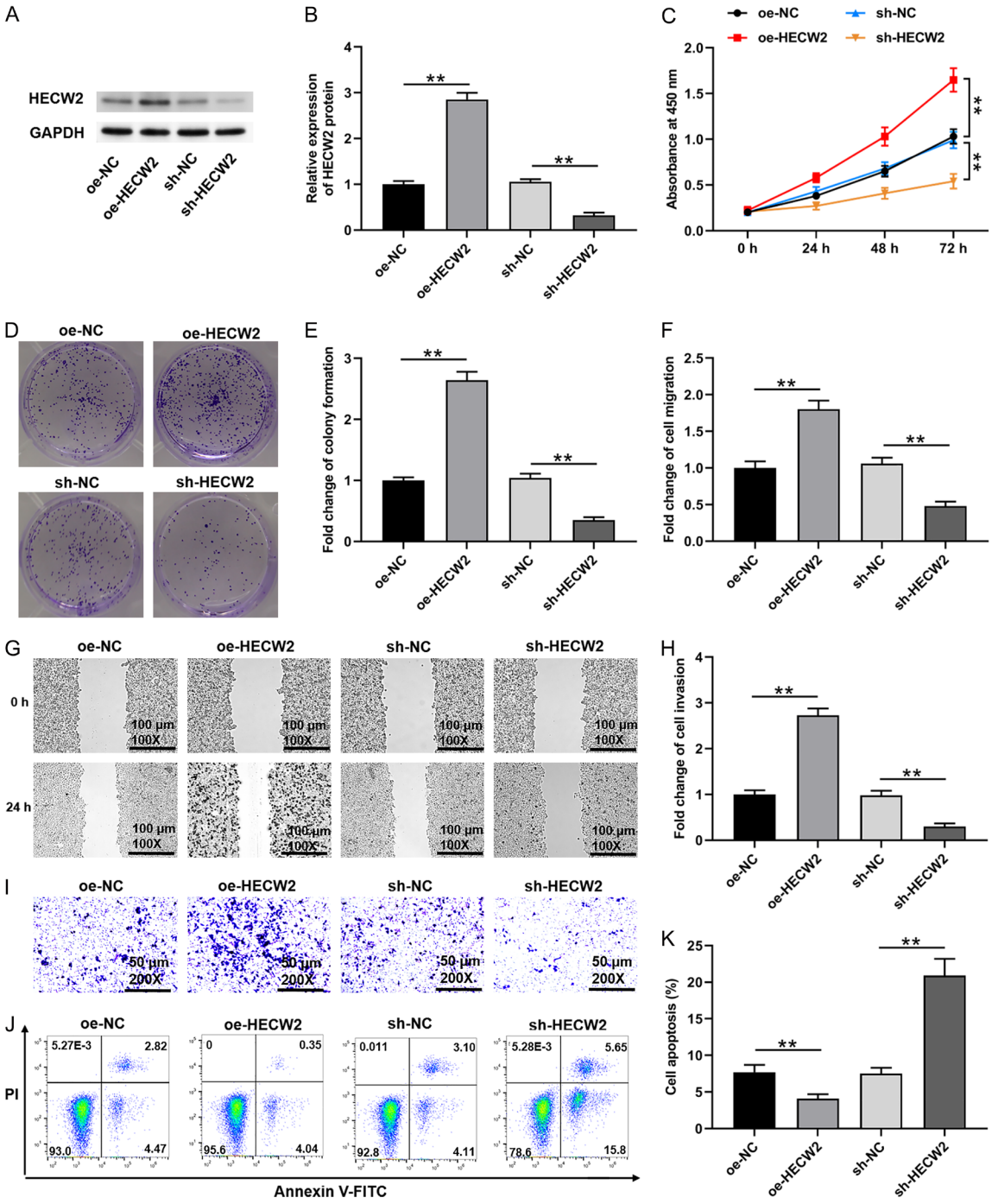

Figure 2. Over-expressed HECW2 facilitated proliferation, migration, invasion and inhibited apoptosis in HemECs. HemECs were transfected with HECW2 over-expression plasmids (oe-HECW2) or HECW2 shRNA (sh-HECW2). (A, B) Western blot analysis was utilized to assess HECW2 protein levels. Cell proliferation was examined using (C) Cell Counting Kit-8 (CCK-8) assay and (D, E) clone formation assay. (F, G) Wound healing assay was employed to evaluate cell migration. (H, I) Transwell assays quantified cell invasion. (J, K) Cell apoptosis was analyzed using flow cytometry. Results are expressed as mean ± SD. $^{**}P<0.01$.

phorylation, respectively. In addition, we showed that the over-expressed HECW2 increased glycolysis and glycolytic capacity (**Figure 5D**) and decreased oxidative phosphorylation (**Figure 5E**) in HemECs, whereas the converse results were observed after the knockdown of

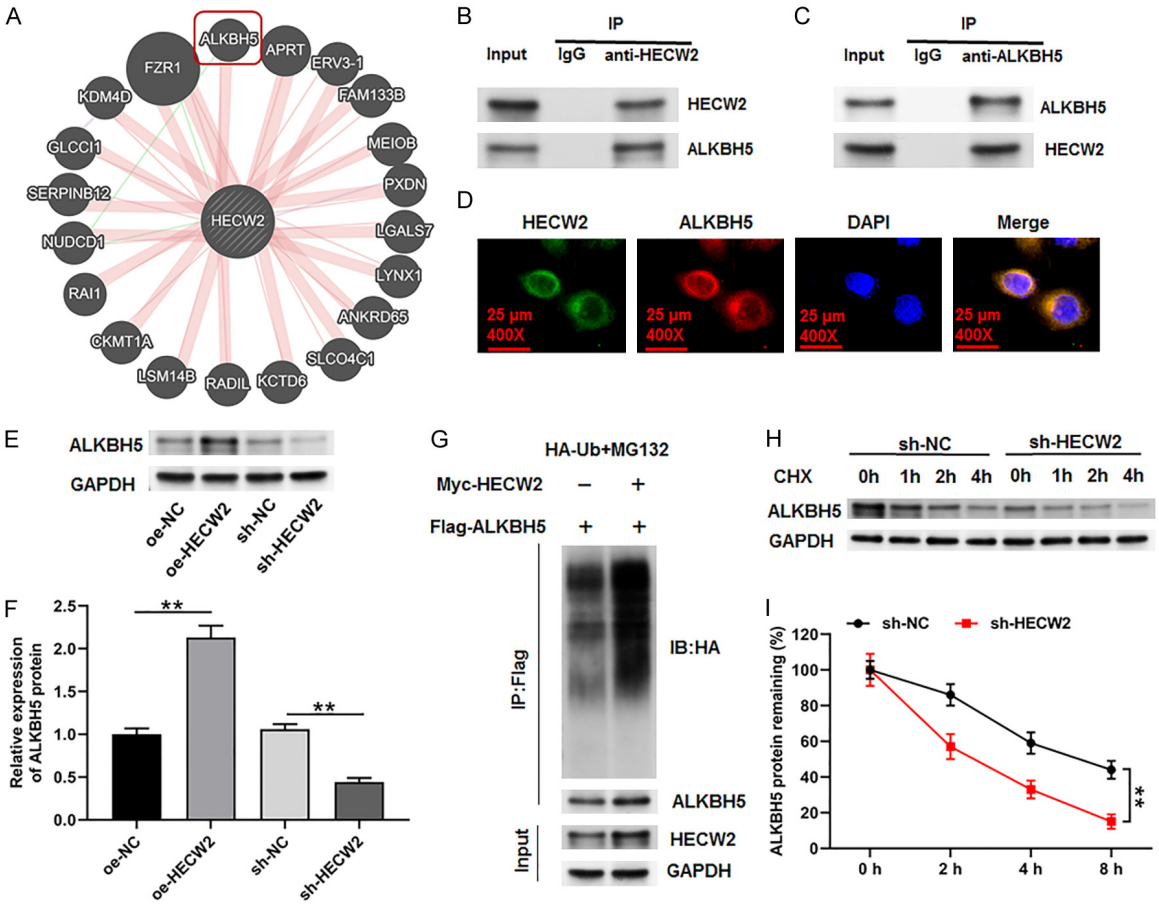

Figure 3. HECW2 increased ALKBH5 stability through ubiquitination regulation. A. The GeneMANIA platform (https://genemania.org/) suggested a potential interaction between HECW2 and AlkB homolog 5 (ALKBH5). B, C. Co-immunoprecipitation (Co-IP) experiments confirmed the interaction between HECW2 and ALKBH5 in HemECs. D. The co-localization fluorescence staining of HECW2 and ALKBH5 in HemECs. E, F. ALKBH5 protein expression was assessed using Western blot analysis. G. HECW2 induced ALKBH5 ubiquitination in HemECs. H, I. ALKBH5 protein stability was evaluated after HECW2 knockdown. Results are expressed as mean ± SD. $^{**}P<0.01$.

HECW2 (**Figure 5F**, **5G**). Hence, we revealed that the over-expression of HECW2 promoted glycolysis in HemECs.

HECW2 knockdown suppressed proliferation, migration and invasion in HemECs by decreasing LDHA expression

To further investigate the role of HECW2/LDHA axis in IH progression, HemECs were transfected with sh-HECW2 and oe-LDHA. Western blot analysis results illustrated that LDHA protein level was decreased after HECW2 knockdown, followed by an increase after the transfection of oe-LDHA (**Figure 6A**, **6B**). HECW2 knockdown suppressed cell proliferation (**Figure 6C**) and colony formation (**Figure 6D**, **6E**) in HemECs, whereas the over-expression of LDHA reversed these changes. Moreover, HECW2 knockdown was found to decrease cell migration (**Figure 6F**, **6G**) and invasion (**Figure 6H**, **6I**), which were then abrogated by the over-expression of LDHA. Besides, HECW2 knockdown promoted the cellular apoptosis process, while the over-expression of LDHA abolished this effect (**Figure 6J**, **6K**).

HECW2 knockdown inhibited glycolysis by decreasing LDHA expression in HemECs

Apart from the aforementioned results, it was demonstrated as well that HECW2 knockdown reduced glucose uptake (**Figure 7A**), lactate production (**Figure 7B**), and ATP level (**Figure 7C**) in HemECs, while these changes were reversed by LDHA over-expression. Subsequen-

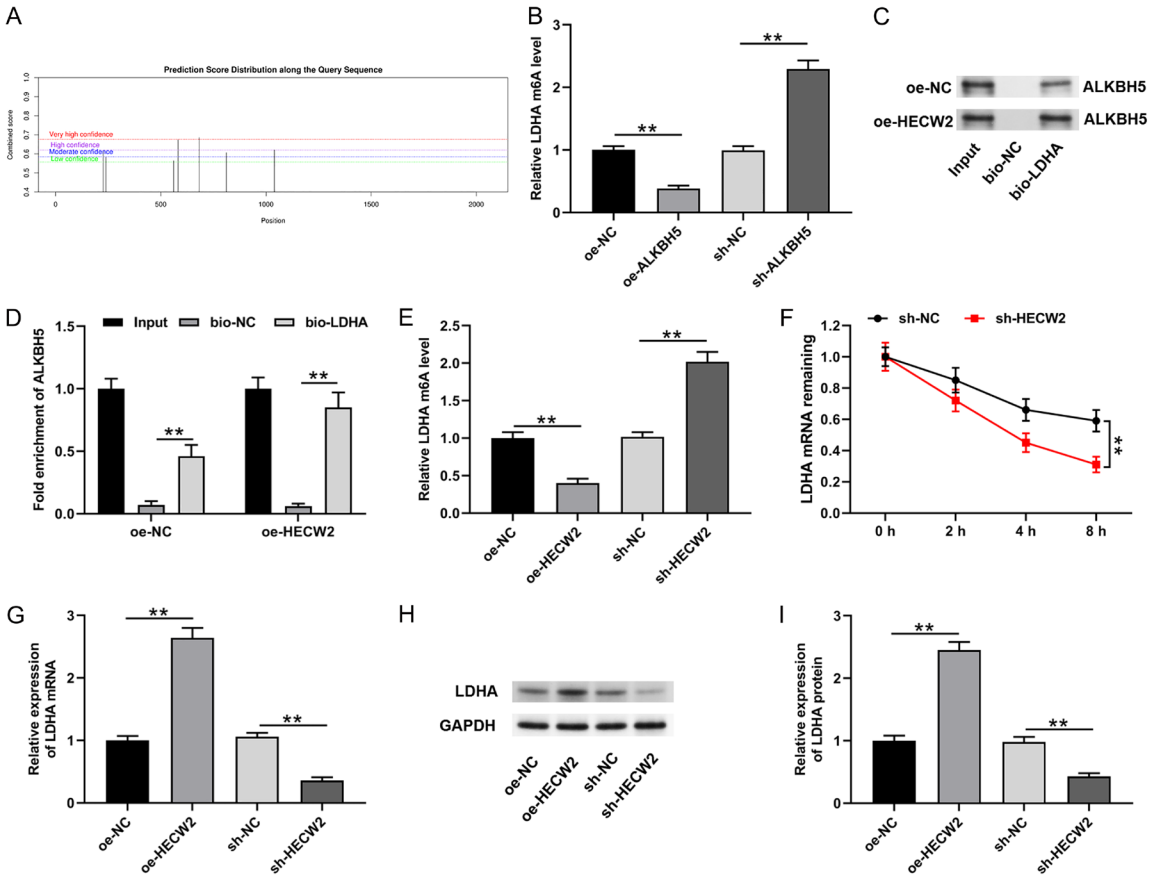

Figure 4. HECW2 enhanced the expression of lactate dehydrogenase A (LDHA) through regulating ALKBH5-mediated LDHA m6A demethylation. A. The Sequence-based RNA Adenosine Methylation Prediction (SRAMP) database (http://www.cuilab.cn/sramp) showed the m6A methylation sites in LDHA. B. LDHA m6A methylation level was assessed using the Methylated RNA Immunoprecipitation Quantitative Polymerase Chain Reaction (MeRIP-qPCR) after ALKBH5 over-expression or knockdown. C, D. RNA pull-down assay detected the enrichment of ALKBH5 in LDHA mRNA. E. LDHA m6A methylation level was assessed using MeRIP-qPCR after HECW2 over-expression or knockdown. F. LDHA mRNA stability was evaluated after HECW2 knockdown. G. LDHA mRNA was evaluated using Real-time Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). G-I. LDHA protein level was assessed using Western blot analysis. Results are expressed as mean ± SD. $^{**}P<0.01$.

tly, we observed that HECW2 knockdown decreased the glycolysis and glycolytic capacity (**Figure 7D**) and oxidative phosphorylation (**Figure 7E**) in HemECs, whereas the over-expression of LDHA reversed the levels of ECAR and OCR in HemECs. Therefore, we indicated that HECW2 knockdown inhibited glycolysis by decreasing LDHA expression in HemECs.

HECW2 knockdown suppressed tumor growth in IH xenograft mice

Finally, we investigated the role of HECW2 in an IH xenograft mouse model. HemECs transfected with sh-HECW2 or negative control were injected into mice. We discovered that the knockdown of HECW2 significantly impeded the progression of tumor growth, reflected by the decreased tumor volume and weight (**Figure 8A-C**). In addition, the administration of sh-HECW2 in xenograft mice resulted in a decrease in HECW2 levels, accompanied by a decrease in the levels of ALKBH5 and LDHA within the tumor tissues (**Figure 8D**, **8E**).

Discussion

As a E3 ubiquitin ligase, HECW2 regulates protein ubiquitination and influences cellular pathways including cell proliferation, apoptosis and inflammatory responses. HECW2 has been found to be involved in regulating tumorigenesis [13, 15, 26]. In hematologic malignancies, comprehensive analysis of gene expression datasets demonstrates that HECW2 is lowly expressed in both acute myeloid leukemia and

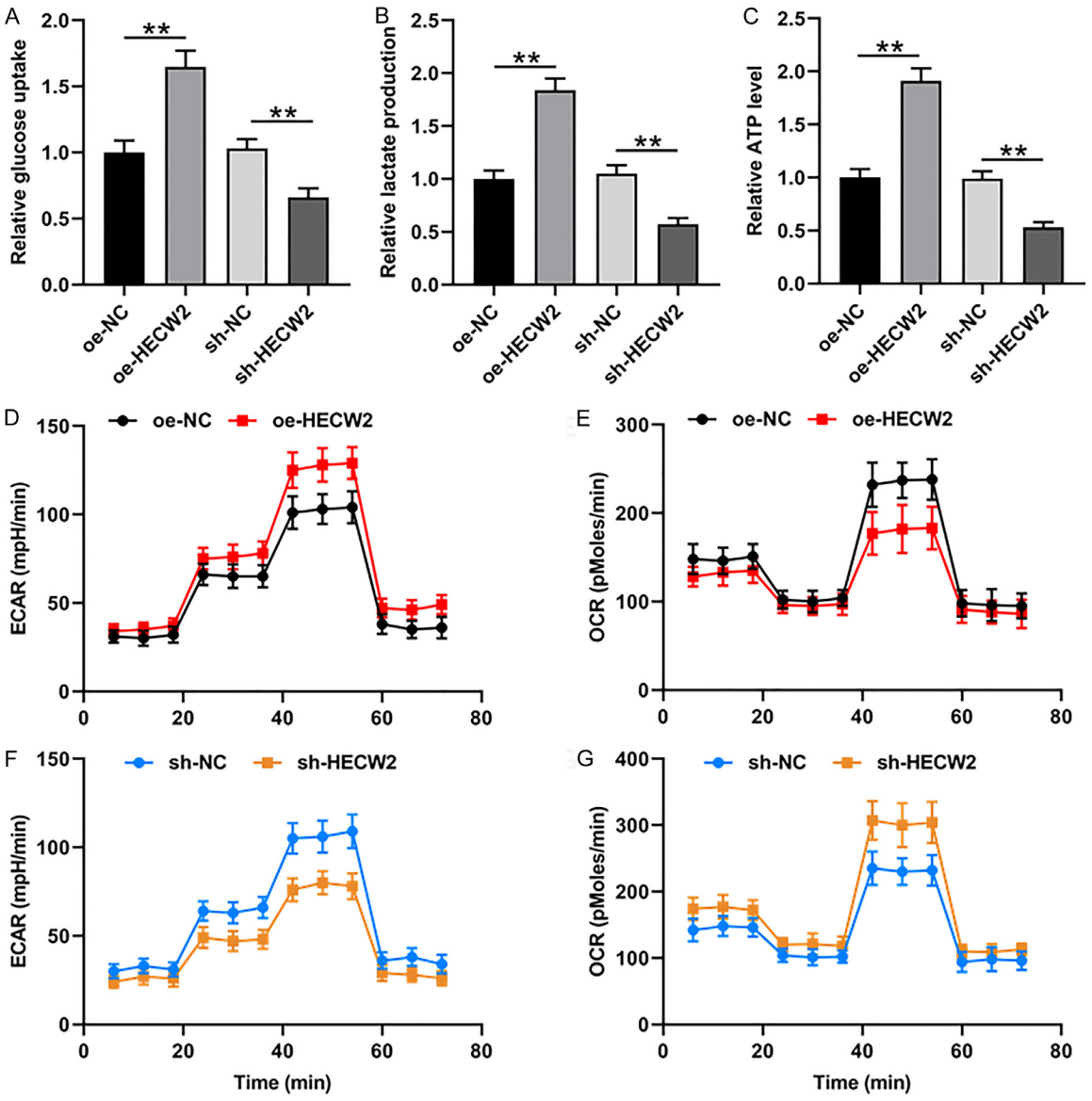

Figure 5. HECW2 over-expression promoted glycolysis in HemECs. HemECs were transfected with either oe-HECW2 or sh-HECW2. The relative (A) glucose uptake, (B) lactate production, and (C) Adenosine triphosphate (ATP) level in HemECs. (D, F) Extracellular acidification rate (ECAR) assay and (E, G) oxygen consumption rate (OCR) assay in HemECs were measured by the Seahorse XF96 extracellular Flux analyzer. Results are expressed as mean ± SD. $^{**}P<0.01$.

diffuse large B cell lymphoma, with its low expression correlating with poor prognosis in patients [26]. In contrast, a recent bioinformatics analysis revealed that HECW2 was up-regulated in IH and functions as a pivotal hub gene in the PPI network, suggesting its strong correlation with IH pathogenesis [17]. Moreover, we noticed that HECW2 stabilized AMOT-like 1 protein expression through ubiquitination regulation, thereby activating angiogenesis in endothelial cells [18], which is a crucial pathogenesis in IH. Interestingly, the functional consequences of HECW2 modulation appear to be cell type-specific, as evidenced by the observation that circ_HECW2 knockdown enhanced proliferation and angiogenesis in human cardiovascular endothelial cells [27]. Based on these diverse findings and considering the potential tissue-specific functions of HECW2, we systematically investigated the roles of HECW2 in HemECs in IH progression. Our results suggested that HECW2 was up-regulat-

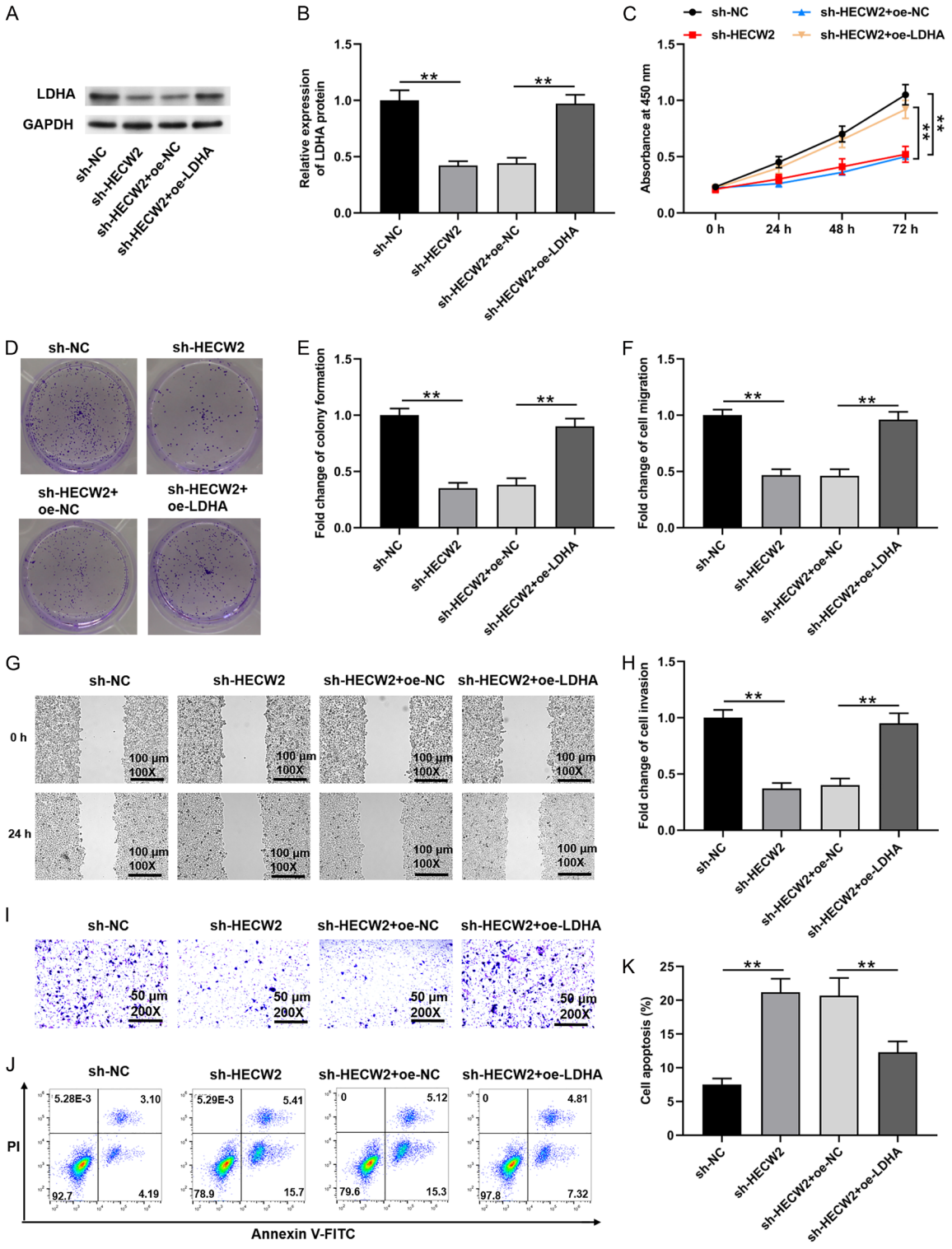

Figure 6. HECW2 knockdown suppressed proliferation, migration and invasion in HemECs by decreasing the expression of LDHA. HemECs were transfected with sh-HECW2 and LDHA over-expression plasmids (oe-LDHA). (A, B) Western blot analysis was utilized to assess the levels of HECW2 protein. Cell proliferation was examined using (C) CCK-8 assay and (D, E) clone formation assay. (F, G) Wound healing assay was employed to evaluate cell migration. (H, I) Transwell assays quantified cell invasion. (J, K) Cell apoptosis was analyzed using flow cytometry. Results are expressed as mean ± SD. $^{**}P<0.01$.

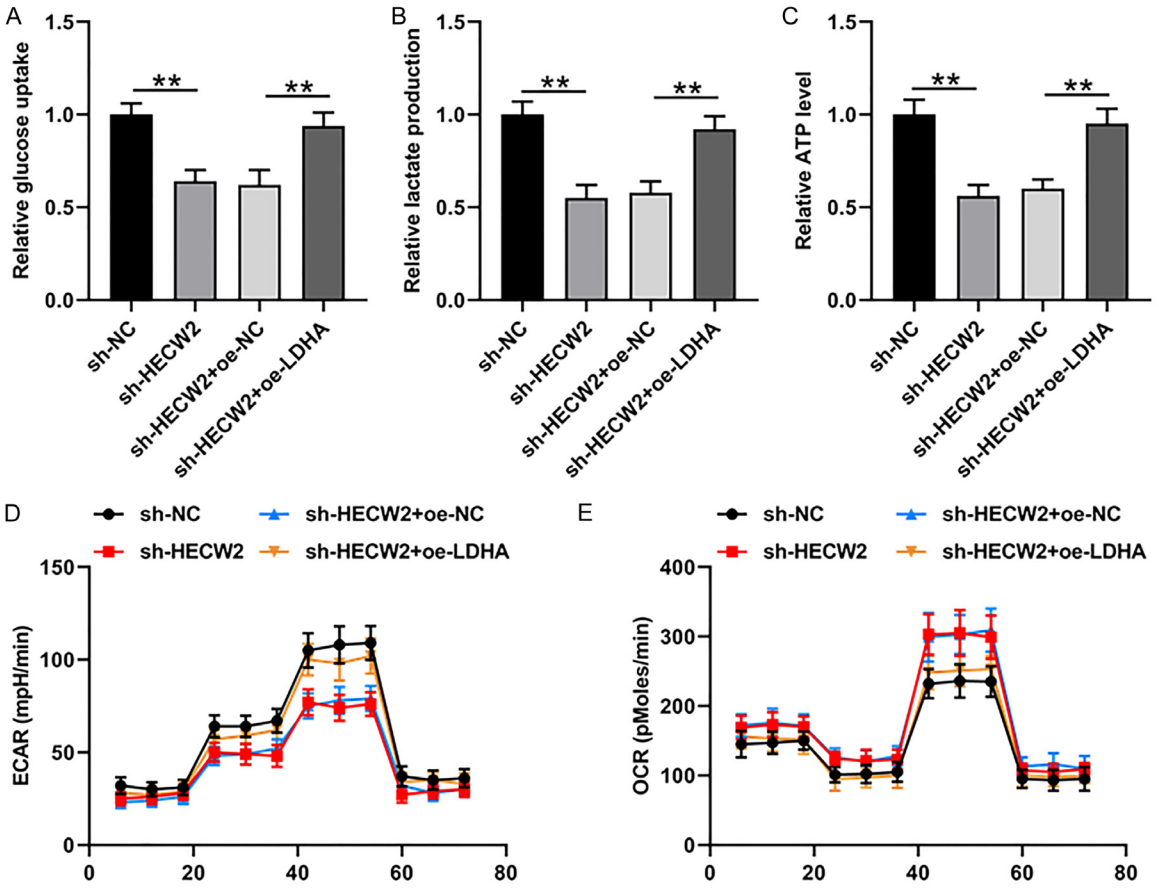

Figure 7. HECW2 knockdown inhibited glycolysis by decreasing LDHA expression in HemECs. HemECs were transfected with sh-HECW2 and oe-LDHA. The relative (A) glucose uptake, (B) lactate production, and (C) ATP level in HemECs. (D) ECAR assay and (E) OCR assay in HemECs were measured by the Seahorse XF96 extracellular Flux analyzer. Results are expressed as mean ± SD. $^{**}P<0.01$.

ed in IH tissues and HemECs. The over-expression of HECW2 facilitated proliferation, migration, invasion and inhibited apoptosis in HemECs, while HECW2 knockdown produced contrary results. Moreover, HECW2 over-expression promoted glycolysis in HemECs. Mechanistically, HECW2 increased ALKBH5 stability through ubiquitination regulation, and then enhanced LDHA expression through regulating ALKBH5-mediated LDHA m6A demethylation. Additionally, our *in-vivo* experiments suggested that HECW2 knockdown suppressed tumor growth in IH xenograft mice.

RNA demethylase ALKBH5 is involved in the regulation of the m6A methylation and influences a range of cellular progresses. There is a growing body of evidence that suggests ALKBH5 is crucial in numerous types of human cancer, primarily by modulating the post-transcriptional expression of genes in an m6A-dependent manner [28]. For instance, ALKBH5 suppresses the metastasis of gastric cancer by modifying the m6A methylation of protein kinase membrane associated tyrosine/threonine 1 [29]. ALKBH5 inhibits tumor growth in pancreatic cancer by transcriptionally activating period circadian regulator 1 [30]. More importantly, current evidence has illustrated that ALKBH5 is a crucial factor that promotes the development of IH. For instance, ALKBH5 enhances the proliferation, migration and invasion of IH cells by regulating the nuclear paraspeckle assembly transcript 1/miR-378b/Fos-like antigen 1 axis [31]. Moreover, ALKBH5 facilitates IH tumor-like cellular behaviors by regulating the m6A methylation of forkhead box protein f1 [32]. Importantly, ALKBH5 has been found to regulate glycolysis in various cancer types. ALKBH5 enhances glycolysis in resistant

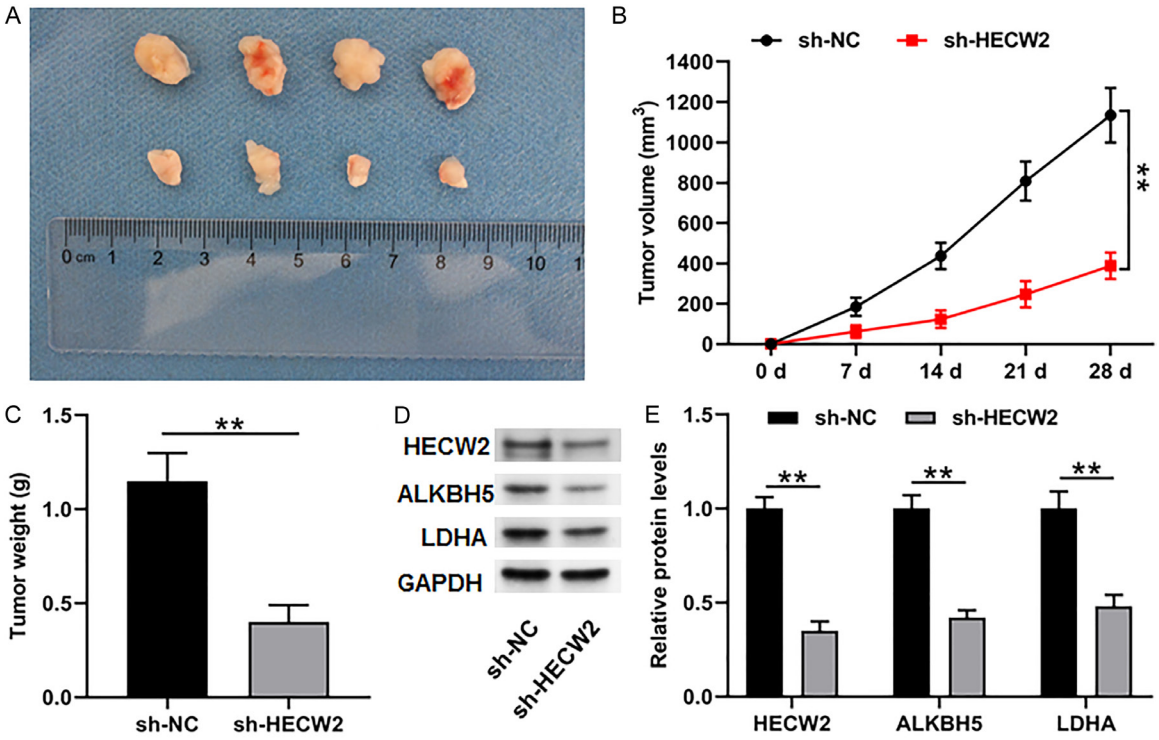

Figure 8. HECW2 knockdown suppressed tumor growth in IH xenograft mice. HemECs transfected with either sh-HECW2 or negative control were injected into mice (n=8 each group). (A) Tumor images, (B) tumor volume, and (C) tumor weight was recorded. (D, E) HECW2, ALKBH5 and LDHA levels were detected using Western blot analysis. Results are expressed as mean ± SD. $^{**}P<0.01$.

breast cancer cells by stabilizing glucose transporters type 4 mRNA via m6A demethylation [33]. ALKBH5 strengthens the stability of LDHA mRNA through m6A demethylation, resulting in an up-regulation of aerobic glycolysis in breast cancer cells [34]. In this study, we revealed that ALKBH5 was stabilized by HECW2, which increased the stability of LDHA through m6A demethylation. Therefore, HECW2 promoted glycolysis and tumor-like cellular behaviors in HemECs via regulating the ALKBH5/LDHA axis.

LDHA is a key enzyme converting pyruvate into lactate in the glycolysis process, which is the final step of the glycolytic pathway [35]. The over-expression of LDHA is usually associated with the rapid proliferation of cancer cells. Over-expressed LDHA in cancer cells can enhance glycolysis and quickly provide energy, assisting in the proliferation and metastasis of cancer cells [35]. In terms of clinical application, the expression level of LDHA is related to the prognosis of various cancers, making it a potential therapeutic target and prognostic predictor of the disease [36]. Moreover, extensive literature data confirm that the inhibition of LDHA suppresses the progression of various cancers, such as pancreatic cancer, lung cancer, and cervical cancer [37-39]. It was reported that the level of LDHA was up-regulated in IH, while the inhibition of LDHA suppressed the migration of HemECs [22]. Consistently, our results proposed that LDHA was up-regulated by the HECW2/ALKBH5 axis in IH. HECW2 knockdown suppressed glycolysis and tumor-like cellular behaviors in HemECs, which were abrogated by the over-expression of LDHA.

In summary, we proposed that HECW2 was up-regulated in IH tissues and HemECs. HECW2 knockdown inhibited proliferation, migration, invasion and facilitated apoptosis in HemECs. In addition, HECW2 knockdown suppressed tumor growth in IH xenograft mice. Mechanistically, HECW2 promoted glycolysis in HemECs via regulating the ALKBH5/LDHA axis.

While this study provides valuable insights into the role of HECW2 in IH progression, some limitations should be acknowledged. First, the

study primarily focused on the ALKBH5/LDHA axis as a downstream mechanism of HECW2, but other potential targets or pathways regulated by HECW2 in IH remain unexplored. Second, the clinical sample size was limited, and the heterogeneity of IH in different stages and locations was not fully addressed. Additionally, the xenograft mouse model may not fully recapitulate the complexity of human IH, particularly in terms of angiogenesis and immune microenvironment. Future research should expand on the clinical cohorts to validate findings across different IH stages and locations, and validate the findings in more clinically relevant models, such as patient-derived xenografts or genetic animal models. Furthermore, integrating multi-omics data and exploring additional downstream targets and pathways is beneficial for expanding the HECW2 regulatory network in IH.

Disclosure of conflict of interest

None.

Address correspondence to: Chonggao Zhou, Department of Fetal and Neonatal Surgery, The Affiliated Children's Hospital of Xiangya School of Medicine, Central South University (Hunan Children's Hospital), No. 86, Ziyuan Road, Yuhua District, Changsha 410007, Hunan, China. E-mail: zhouchonggao6310@163.com

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