Fbw7 Inhibits the Progression of Activated B-cell Like Diffuse Large B-cell Lymphoma by Targeting the Positive Feedback Loop of the LDHA/lactate/miR-223 Axis

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Research

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Abstract

Background: F-box and WD repeat domain-containing 7 (Fbw7) is an ubiquitin ligase and tumor suppressor which targets a variety of oncogenic proteins for proteolysis. We previously reported that Fbw7 regulates apoptosis in diffuse large B-cell lymphoma (DLBCL) through Fbw7-mediated ubiquitination of Stat3. However, the mechanism by Fbw7-mediated tumor metabolism remains undefined. We examined the function of Fbw7 for tumor metabolism and progression in DLBCL.

Methods: The effect of Fbw7 overexpression on Lactate Dehydrogenase A (LDHA)-related tumor metabolism was explored in activated B-cell (ABC) like DLBCL. And Fbw7-mediated expression of LDHA in DLBCL was detected by immunoprecipitation for protein interaction, ubiquitination assay, western blotting and mRNA qualitative analyses. In vitro and in vivo studies were done to measure the function of the Fbw7-mediated LDHA/lactate/miR-223 axis in DLBCL progression.

Results: We demonstrated that the ubiquitin-ligase Fbw7 played a key role in LDHA-related metabolism. As LDHA and its catalytic lactate were critical for tumor growth and progression in ABC-DLBCL, our results demonstrated that Fbw7 could interact with LDHA to trigger its ubiquitination and degradation, and lactate negatively regulated Fbw7 via inducing the expression of miR-223, which targeted Fbw7 3'-UTR to inhibit its expression. In vivo and in vitro studies revealed that miR-223 promoted tumor growth and that the effects of miR-223 on tumor growth were primarily related to the inhibition of Fbw7-mediated LDHA's ubiquitination.

Conclusions: Our study uncovers a positive functional loop consisting of a Fbw7-mediated LDHA/lactate/miR-223 axis, which may support the future ABC-DLBCL therapy by targeting LDHA-related inhibition.

Introduction

DLBCL is a heterogeneous malignancy which derived from mature B cells and accounts for 30–35% of all nodal lymphomas [1, 2]. Based on generation sequencing and cell-of-origin, DLBCL could be stratified into two molecular subtypes, the activated B cell (ABC) type and the germinal center B cell (GCB) type. ABC DLBCL is related with tumor aggressive growth and the lower cure rate [3, 4]. It's also characterized by poor prognosis, increased drug resistance and constitutive activation of NF-kB [5]. Clinical drug resistance of ABC-DLBC for CHOP or R-CHOP therapy is a significant problem and the underlying mechanism of ABC-DLBCL-specific resistance remains unclear [6, 7].

The reprogramming of energy metabolism is a critical aspect of tumor development and progression. To make sure a sufficient supply of intermediary metabolites for the ATP generation and synthesis of new biomass, tumors often rewire their metabolism by activating a group of glycolysis-related enzymes [8]. An increased rate of glycolysis enables the diversion of metabolites into pathways that promote the biosynthesis of organelles and macromolecules required for tumor growth and resistance to

chemotherapy [9–11]. Serum lactate dehydrogenase (LDH) is a key marker for aggressive non-Hodgkin lymphoma (NHL) and is one of the factors listed in the International Prognostic index (IPI) [12, 13]. High LDH activity was reported following treatment with hematopoietic growth factors and intensive chemotherapy regimens [14, 15]. Lactate dehydrogenase A (LDHA) catalyzes pyruvate to lactate and is known as a vital checkpoint for tumor anaerobic glycolysis [16]. However, underlying mechanism through which LDHA regulates tumor metabolism remains unclear in DLBCL.

Fbw7 is a ubiquitin ligase that acts an important role in cancer by targeting several key proteins for proteolysis [17–22]. In previous study, we demonstrated that Fbw7 regulates apoptosis in ABC-DLBCL by Fbw7-mediated ubiquitination of Stat3 [23]. However, the underlying mechanism of Fbw7-mediated tumor metabolism is still unknown. Here, we demonstrated that Fbw7 plays a key role in LDHA-related metabolism. Fbw7 interacts with LDHA which results in its ubiquitination and degradation. Moreover, LDHA and its catalytic activity is critical for tumor growth and progression in ABC-DLBCL. Interestingly, we confirmed that lactate negatively regulates Fbw7 by inducing the expression of miR-223. MiR-223 targets the Fbw7 3’-UTR thus inhibiting its expression. In vivo and in vitro studies showed that miR-223 promotes tumor growth and these effects are associated with the negative regulatory effects of Fbw7-mediated ubiquitination of LDHA. This study reveals a positive functional loop, the Fbw7-mediated LDHA/lactate/miR-223 axis, and our findings may lead to a strategy for treating ABC-DLBCL through LDHA inhibition.

Methods

Reagents

Antibodies were used in the study: anti-Fbw7 (for western blots) (ab109617, Abcam, Cambridge, UK), anti-Fbw7 (for IHC) (H00055294-M02, Abnova, Taipei City, Taiwan), anti-LDHA, 3682; anti-phospho-LDHA^{Tyr10}, 8176; anti-HK2, 2867; anti-Glut1, 12939s; anti-PDK1, 5662; anti-Ubiquitin, 3936; anti-Flag Tag, 14793; anti-His Tag, 9991 (Cell Signaling Technology, Beverly, MA, USA), anti-β-actin, 60008-2-Ig (Proteintech, Rosemont, IL, USA). And other chemicals were purchased from Amresco (Dallas, TX, USA) and Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The ABC-DLBCL cell lines SU-DHL-2, OCI-LY-3, OCI-LY-10, U2932 and HEK293 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and maintained in a humidified incubator with 5% CO_2 at 37°C. Cell lines were confirmed by short tandem repeats-polymerase chain reaction (STR-PCR) genotyping and compared with American Type Culture Collection (ATCC) database.

Cell transfection

Plasmids encoding Flag-Fbw7, His-LDHA and HA-Ubiquitin were constructed by cloning PCR amplified into pcDNA3.1. LDHA siRNA target sequence was 5'-GUUCAUCAUUCCCAACAUUTT-3' (siRNA1) and 5'-AAUGUUGGGAAUGAUGAACTT-3' (siRNA2). LDHA siRNA and siRNA-control (siRNA-NC), miR-223 inhibitor and mimic were purchased from RIBOBIO (Guangzhou, China). The constructs were transfected using Lipofectamine 2000 and 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacture.

Metabolism analysis

The Lactate dehydrogenase (LDH) assay kit (C0016, Beyotime Biotechnology) and L-Lactate Assay Kit II (1200051002, Eton Bioscience) were used to measure intracellular LDH and lactate levels, respectively, in DLBCL cells according to the manufacturer's protocols. ATP generation was detected using an ATP detection assay kit (S0026, Beyotime Biotechnology).

Tissue samples and IHC

Thirty-two human DLBCL patients were collected at the Guangdong Provincial People's Hospital. And written informed consent was obtained from all of the patients and this study was approved by the Biomedical Research Ethics Committee of Guangdong Provincial People's Hospital. And patients with detail clinicopathological characteristics are rendered in Supplementary Table S1. For immunohistochemistry (IHC) detection, Paraffin embedded tissues were sectioned at 4-μm thickness, microwaved in Na citrate buffer, pH 6, for 10min and following primary antibody (anti-Fbw7 or anti-LDHA). Secondary antibody was biotinylated, and then sections were developed with diaminobenzidine. Staining results were estimated by two pathologists and the scoring criteria was: 0 (no staining), 1 (weak staining), 2 (intermediate staining), and 3 (strong staining).

Western blot analysis

Cells were homogenized in RIPA lysis buffer on ice for 30 min and then centrifuged at 14000 ×*g* for 30 min. Whole protein extract was measured using the BCA Assay (Pierce, Rockford, IL, USA), 30 μg of whole protein was added onto 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Billerica,MA, USA).

Immunoprecipitation and ubiquitination assay

For protein immunoprecipitation analysis, cells were lysed and 1μg of antibodies (anti-Fbw7 or anti-LDHA) was added, which was incubated with protein A/G beads (Millipore) and eluted in SDS buffer at 95°C for 4 min. The beads were then washed and boiled in SDS loading buffer and immunoprecipitation complexes were detected by western blotting analysis.

For ubiquitylation assay, cells were transfected with indicated plasmids and/or siRNA and then treated with MG132 (20 μM). And then cells were harvested, lysed and followed by immunoprecipitation of His-tag LDHA and western blotting to detect His-LDHA ubiquitination.

Quantification PCR analysis

For mRNA quantification, total RNAs were isolated using RNAiso (Takara, Shiga, Japan) Plus and 1 μg of total RNA used for cDNA synthesis using PrimeScript RT Master Mix (Takara). Real-time PCR reactions were carried out using SYBR Premix Ex Taq (Takara) and run on ABI 7500 PCR system (Applied Biosystems, Carlsbad, CA, USA). The PCR program consisted of 40 cycles at 95°C for 5 s and 60°C for 30 s. Gene expression was determined relative to β-actin (for mRNA) or U6 (for miRNA) and calculated using the $2^{-\Delta\Delta CT}$ method. The miDETECT A Track™ miRNA qPCR Kit (RIBOBIO, Guangzhou, China) was used to examine the expression levels of mature miR-223. The gene-specific primers used for the PCR reaction is listed in Supplementary Table S2.

Animal model

NOD/SCID mice (4 to 6 weeks) were purchased from the Experimental Animal Tech Co. of Weitonglihua (Beijing, China). Mice were injected with 1×10^7 cells in 100 μl PBS in the left flank. The size of the subcutaneous tumor was measured using calipers twice a week. The tumor volume was calculated using the following formula: tumor volume (mm^3) = (width × height × depth)/2. These experiments were assessed and approved by the Committee of Care and Use of Laboratory Animals of Guangdong Provincial People's Hospital.

To investigate whether the production of miR-223 could promote DLBCL tumor growth in vivo, hsa-miR-223 agomir (RIBOBIO, Guangzhou, China) (1 nmol in 100 μl PBS, twice a week for 4 weeks) was administered intraperitoneally at 1 week following SU-DHL-2 cell injection.

Dual luciferase reporter assays

The targeting sites for miR-223 and the Fbw7 3'-UTR were predicted using the TargetScan algorithm (www.targetscan.org). To validate miR-223-predicted targets, a dual luciferase reporter assay was performed according to the manufacturer (Promega, WI, USA). Cells were transiently transfected with Renilla luciferase vector for 3'-UTR of Fbw7(WT) or mutated miR-223 binding sites. The reporter constructs were co-transfected with negative control or miR-223 mimic into the indicated cells for 24 hours. Firefly luciferase was used as an internal control. Cell lysates were prepared and luciferase activity was detected using a Spark multimode microplate reader (TECAN, Mannedorf, Switzerland).

Statistical analysis

SPSS 23.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for Statistical analyses. All data are showed as the mean ± SD. Experiments were repeated at least three times. One-way analysis of variance or a two-tailed unpaired Student t test was applied to evaluate the data. Differences were known as significant at *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. The relationship between Fbw7 level and LDHA were assessed using the Pearson χ^2 test. Each assay was performed in triplicate.

Results

Fbw7 inhibits LDHA in activated B-cell like diffuse large B-cell lymphoma

The ubiquitin-ligase Fbw7 plays as a tumor suppressor by targeting lots of proto-oncogenes for proteolysis in cancer. In our early study of ABC-DLBCL, we found that Fbw7 significantly regulates apoptosis. To investigate the role of Fbw7 in tumor metabolism, we overexpressed Fbw7 in SU-DHL-2 and OCI-LY-3 cells (Fig.1a). Firstly, we measured intracellular LDH and lactate production which are two basic indicators of the Warburg effect. Results show that Fbw7 reduced intracellular LDH and lactate production, suggesting an inhibitory role in glycolysis (Fig. 1b and 1c). Tumor cells depend on glycolysis for ATP production, which facilitates rapid growth and progression. We analyzed the relationship between Fbw7 expression and ATP production. Data show Fbw7 inhibited ATP production in SU-DHL-2 and OCI-LY-3 cells (Fig. 1d). To further investigate the role of Fbw7 in glycolysis, the expression of key enzymes of the glycolysis, including GLUT1, HK2, LDHA, and PDK1, were examined. Western blotting analysis revealed that the overexpression of Fbw7 primarily decreased LDHA expression compared with other enzymes of glycolysis (Fig. 1e). To investigate the relationship between Fbw7 and LDHA, 32 samples from DLBCL patients was analyzed using IHC. Case 1 and case 2 refer to two representative samples shown by low and high expression of Fbw7 which was compared with high and low expression of LDHA (Fig. 1f). A Spearman rank correlation analysis furtherly verified that Fbw7 was negatively associated with LDHA (Fig. 1g). The correlation between Fbw7, LDHA and other clinicopathological features, such as patient DLBCL subtype, age, sex, EB virus status were showed in Supplementary Table S1. Taken together, these results show that Fbw7 acts an important role in ABC-DLBCL glycolysis by regulating LDHA expression and activity.

Fbw7 inhibits the stability of LDHA

Previous studies have shown that the Fbw7 ubiquitin-ligase influences LDHA in ABC-DLBCL, prompting us to confirm if Fbw7 regulates LDHA activity or stability. DLBCL cells were transfected with Flag-Fbw7 plasmids and western blotting analysis prompted that Fbw7 reduced LDHA protein expression (Fig. 2a and 2b). After 24 hours post-transfection, SU-DHL-2 cells added with cycloheximide (CHX, an inhibitor of protein synthesis) and the stability of LDHA was detected. The half-life of LDHA reduced from 8 h to approximately 4 h following Fbw7 upregulation (Fig. 3c and 3d). Similarly, upregulation of Fbw7 significantly decreased LDHA levels n OCI-LY-3 cells (Fig. 3e and 3f). These results indicate that Fbw7 reduced LDHA stability by accelerating its degradation.

Fbw7 interacts with and ubiquitinates LDHA

We next explored the mechanism by which Fbw7 alters LDHA stability and investigated Fbw7 to interact with LDHA. Immunoprecipitation analysis demonstrated that Fbw7 interacted with LDHA using an anti-Fbw7 antibody (Fig. 3a, top). The immunoprecipitation of LDHA with an anti-LDHA antibody resulted in co-immunoprecipitation of Fbw7 (Fig. 3b, bottom). Similarly, co-immunoprecipitation of Fbw7 and LDHA were demonstrated in OCI-LY-3 and OCI-LY-10 (Fig. 3b and 3c). By mass spectrometry of Fbw7 co-

immunoprecipitation samples, a key phosphorylated tyrosine residue of LDHA phosphorylation sites (Y10) was detected, which was harvested and analyzed by reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. S1). These results show that Fbw7 directly interacts with LDHA.

As lots of substrate are reported for Fbw7 to recognize and ubiquitination, we furtherly explored whether Fbw7 directly regulate the stability of LDHA. To investigate this possibility, SU-DHL-2 and U2932 cells were transfected with a plasmid encoding His-LDHA and HA-ubiquitin along with and without Flag-Fbw7. Immunoblotting revealed that the level of ubiquitination's LDHA significantly increased after upregulation of Fbw7 with or without of MG132, an inhibitor of the 26S proteasome (Fig. 3d and 3e). Our results imply that Fbw7 interacts with LDHA and targets it for ubiquitination.

Inhibition of LDHA suppresses tumor progression in ABC-DLBCL

It has been reported that LDHA was pivotal for cell viability and tumor cell proliferation and elevated serum LDH expression is associated with poor prognosis in DLBCL patients [16, 24]. To explore the exact function of LDHA in DLBCL, SU-DHL-2 and OCI-LY-10 cell lines were transfected with normal control or siRNA against LDHA. Quantitative PCR confirmed that LDHA expression was significantly reduced following transfection (Fig.4a). We also measured intracellular LDH and lactate production, two basic indicators of the Warburg effect. Consistently, downregulation of LDHA decreased intracellular LDH and lactate production (Fig. 4b and 4c). Tumor cells rely on glycolysis for energy production which match cell demands for rapid growth and metastasis. We furtherly detected the impact of LDHA on ATP production. As expected, downregulation of LDHA reduced ATP production in cell lines of SU-DHL-2 and OCI-LY-3 cells (Fig. 4d). Cell proliferation was confirmed using the Cell Counting Kit-8 (CCK-8) assay and the results indicated that silencing LDHA expression significantly inhibited the proliferation of DLBCL cells. These results suggest that LDHA promotes cell growth by enhancing tumor metabolism.

Lactate as a metabolite inhibited Fbw7 expression in ABC-DLBCL

Lactate as the end product of glycolysis, has normally been deemed to metabolic waste rather than a fuel for tumor cells [25]. Interestingly, published in *Cell* had described the metabolism of lactate in human lung tumors *in vivo*, which shows exogenous lactate was consumed and used as a respiratory fuel , predominantly over glucose [26]. Therefore, we investigated the role of lactate in DLBCL and its impact on the expression of Fbw7 and LDHA. Lactate was added to SUDHL-2 and OCI-LY-10 cells over a range of concentrations and Fbw7, LDHA, and pY10-LDHA levels were determined by western blotting analysis. The results showed that lactate inhibited the expression of Fbw7 and enhanced LDHA and pY10-LDHA expression with a positive feedback effect (Fig. 5a and 5b). Next, we measured intracellular LDH and lactate production in DLBCL cells. Consistently, lactate increased intracellular LDH and lactate production (Fig. 4c and 4d). To investigate the effect of lactate in the Fbw7-mediated reduction of LDHA, we treated cells with lactate to upregulate the expression of Fbw7 in SUDHL-2 and OCI-LY-10 cells. Western blotting analysis showed that the addition of lactate significantly increased the expression of LDHA and pY10-

LDHA and reversed the Fbw7-mediated inhibition of LDHA (Fig. 5e and 5f). Taken together, our data indicate that lactate promotes DLBCL progression by suppressing Fbw7 and through positive feedback of LDHA.

Increased miR-223 level induced by lactate further suppressed Fbw7 expression and promoted DLBCL proliferation

As stated above, we found that lactate could inhibit the expression of Fbw7 and promotes the expression of LDHA and pY10-LDHA in SUDHL-2 and OCI-LY-10 cells. However, the underlying mechanism by which lactate suppresses Fbw7 in DLBCL is unknown. Under hypoxic conditions, cancer cells may exhibit an increased dependency on glycolysis compared with normal tissues. LDHA catalyzes pyruvate to lactate resulting in the production of large molecular compounds that are required for a wide range of cellular processes including ATP production. It was previously reported that the miR-223/Fbw7 axis plays an important role in lung cancer cells under hypoxic conditions and miR-223 has been shown to target Fbw7 in various cancers [27-32]. Hence, we further investigated whether lactate inhibits Fbw7 by regulating the expression of miR-223. Lactate was added to cells in concentrations ranging from 0 to 20 mM. Quantitative PCR analysis revealed that the expression of miR-223 significantly increased after lactate treatment (Fig.6a). To further elucidate the relationship between miR-223 and Fbw7 in DLBCL, we treated DLBCL cells with a miR-223 inhibitor. Western blotting analysis revealed a significant increase in Fbw7 expression and decreased LDHA expression among the glycolysis-related enzymes (Fig.6b). To investigate the role of miR-223 in DLBCL progression in vivo, NOD-SCID mice were injected with SU-DHL-2 cells and then treated with a miR-223 mimic twice a week. In this vivo model, treatment with miR-223 mimic promoted tumor growth (Fig.6c). Moreover, three conserved binding sites for miR-223 and the Fbw7 3’-UTR were identified by the TargetScan algorithm (Fig. 6d). To confirm the effect of miR-223 targeting of Fbw7 in DLBCL, we measured the luciferase activity of the 3’-UTR region (190-197) of Fbw7 (WT-3’-UTR) and its mutant. The wild type 3’-UTR reporter was significantly decreased compared with the mutant 3’-UTR reporter (Fig. 6e). And the metabolic analysis revealed that Fbw7 and miR-223 inhibitor similarly reduced intracellular LDH and ATP production (Fig. 6f and 6g). In conclusion, our data confirmed that lactate-induced miR-223 targets Fbw7 and promotes DLBCL proliferation.

The positive feedback loop of LDHA/lactate/miR-223 in ABC-DLBCL.

To investigate the effect of lactate in the Fbw7-medicated reduction of LDHA, SUDHL-2 and OCI-LY-10 cells were treated with lactate and the miR-223 inhibitor. Western blot analysis results revealed that the addition of lactate significantly increased the expression of LDHA and pY10-LDHA and reversed miR-223-mediated inhibition of LDHA (Fig. 7a and 7b). We previously demonstrate that Fbw7 targeted LDHA for ubiquitination and degradation. Therefore, we hypothesized that miR-223 may indirectly regulate LDHA degradation. To test this concept, His-LDHA and HA-ubiquitin were co-expressed with and without the miR-223 inhibitor in SU-DHL-2 and OCI-LY-10 cells. Immunoblotting revealed that the ubiquitination of LDHA increased significantly after treatment with the miR-223 inhibitor in the presence or absence of

MG132 (Fig. 7c and 7d). These results conclusively indicate that miR-223 regulated LDHA through a Fbw7-mediated mechanism.

Discussion

As an ubiquitin ligase, Fbw7 plays a key role in malignant tumors by targeting several key proteins for proteolysis. We previously reported that Fbw7 regulates apoptosis in DLBCL. However, the underlying mechanism through which Fbw7 regulates tumor metabolism remains unknown. Here, we explored the role of Fbw7 in regulating tumor metabolism in ABC-DLBCL and revealed that Fbw7 targets LDHA for ubiquitylation and degradation. We also identified a positive functional loop consisting of a Fbw7-mediated LDHA/lactate/miR-223 axis that affects DLBCL progression.

The reprogramming of energy metabolism is a pivotal step of tumor disease [33]. Warburg effect was known as an important feature of the metabolism in tumor and a method of ATP generation through oxidative phosphorylation occurring even under normal oxygen concentrations [34]. The key role of Fbw7 for tumor metabolism has recently received increased attention. Fbw7 may be through regulating a broad range of metabolic pathways by targeting lots of pivotal substrates including HIF-1alpha, c-Myc, SREBP, mTOR, PGC-1α, and REV-ERBα [35]. To explore the role of Fbw7 for tumor metabolism of DLBCL, we overexpressed Fbw7 in cell lines of SU-DHL-2 and OCI-LY-3. We found that Fbw7 plays a vital role in ABC-DLBCL cell glycolysis by regulating LDHA. Consistently, overexpression of Fbw7 reduced intracellular LDH, lactate, and ATP production, thus demonstrating its inhibitory role in glycolysis. Fbw7 expression primarily decreased LDHA expression compared with other glycolysis-related enzymes. Furthermore, IHC staining results revealed that high level of Fbw7 was associated with low expression of LDHA in Case 1. Conversely, low level of Fbw7 was associated with high expression of LDHA in Case 2. And the analysis results of Spearman rank correlation further demonstrated that the expression of Fbw7 was negatively associated with LDHA.

LDHA catalyzes pyruvate to lactate and is known as a vital checkpoint of anaerobic glycolysis. Our results showed that Fbw7 targets LDHA for ubiquitylation. First, we demonstrated that Fbw7 reduced the stability of LDHA in a dose-dependent manner and the half-life of LDHA was decreased. Moreover, co-immunoprecipitation experiments showed that Fbw7 interacts with LDHA and the ubiquitination of LDHA was significantly increased following Fbw7 expression. Fbw7 is capable of governing a lot of metabolic pathways by facilitating the degradation of key substrates including HIF-1alpha and c-Myc. It has been reported that LDHA gene expression is upregulated by both HIF-1alpha and Myc in cancer cells to achieve promoted lactate production. Thus, the regulation of LDHA via a Fbw7-mediated mechanism may contribute in a direct and indirect manner.

Tumors often exhibit increased dependency on glycolysis compared with normal tissues as evidenced by their enhanced glucose uptake and preferential use of glycolysis as opposed to oxidative phosphorylation for ATP production [36]. Elevated LDHA expression is also associated with poor outcome in tumor patients. Our data showed that downregulation of LDHA decreases intracellular LDH, lactate,

and ATP production in DLBCL. Silencing LDHA expression significantly reduced the proliferation of DLBCL cells as measured by the Cell Counting Kit-8 (CCK-8) assay.

Traditionally, lactate has been considered to be metabolic waste rather than an energy source for tumor cells. Interestingly, we found that lactate promotes the expression of LDH and ATP production. Furthermore, we demonstrated that lactate inhibits the expression of Fbw7 and promotes LDHA with a positive feedback effect. However, the relationship between lactate and Fbw7 is unclear. Under hypoxic conditions, tumors frequently exhibit an increased dependency on glycolysis and the level of lactate. It was reported that the miR-223/Fbw7 axis plays an important role in lung cancer cells under hypoxic conditions. We confirmed that lactate inhibits Fbw7 by regulating the expression of miR-223. As expected, qPCR analysis revealed that the expression of miR-223 significantly increased after lactate treatment in DLBCL. Finally, we discovered that miR-223 targets the 3'-UTR region of Fbw7 to inhibit its expression. Collectively, these results indicated that miR-223, which is induced by lactate, targets Fbw7 and promotes DLBCL proliferation.

In this study, we revealed a key role for Fbw7 in restraining the positive feedback of the LDHA/lactate/miR-223 axis. As a tumor suppressor in human cancer, ubiquitin-ligase Fbw7 targets many of proto-oncogenes which are tagged and ubiquitylated for degradation. Deletion of Fbw7 by genetic edition results in developmental defects or genetic instability and Fbw7 inactivation by loss of function or mutation is related to tumor growth and progression [37–39]. In DLBCL, we found that the miR-223 inhibitor displays an ability to resist deregulation by Fbw7. The miR-223 inhibitor stabilizes the expression of Fbw7 through a competitive binding mechanism and inhibits the positive feedback from the LDHA/lactate/miR-223 axis. In conclusion, Fbw7 plays a key role in inhibiting the progression of ABC-DLBCL by restricting the positive feedback loop of the LDHA/lactate/miR-223 axis.

Conclusions

Collectively, our findings uncovered a positive functional loop consisting of a Fbw7-mediated LDHA/lactate/miR-223 axis, which promotes DLBCL tumor growth and progression may. Moreover, miR-223 targets 3'-UTR region of Fbw7 and Fbw7 targets LDHA for ubiquitilation and degradation, suggesting that the future ABC-DLBCL therapy by targeting LDHA-related inhibition of Fbw7.

Abbreviations

Fbw7: F-box and WD repeat domain-containing 7; DLBCL, diffuse large B-cell lymphoma; LDHA, Lactate Dehydrogenase A; ABC, activated B-cell; GCB, germinal center B cell; LDH, lactate dehydrogenase; IPI, International Prognostic index; STR, short tandem repeats; ATCC, American Type Culture Collection; IHC, immunohistochemistry; CCK8, Cell Counting Kit-8

Declarations

Ethics declaration

This study was approved by the Research Ethics Committee of Guangdong

Provincial Hospital and written informed consent was obtained from all patients.

Consent for publication

The authors confirm that they have obtained written consent from each patient to publish the manuscript.

Availability data

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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Authors' contributions

Y-HL and SY carried out the conception and design. T-RG, FZ, CY, W-DC, F-PX, D-LL, X-LL and ZL participated in the acquisition of data (acquired and managed patients, provided facilities etc.). Cell and molecule experiments: SY, T-R G and D-YL carried out the cell and molecule experiments. T-RG carried out the animal experiments. SY and T-RG carried out the analysis and interpretation of data (statistical analysis, biostatistics, computational analysis). Y-HL drafted the manuscript. Study supervision: Y-HL and SY. All authors read and approved the final manuscript.

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Figures

Fig.1

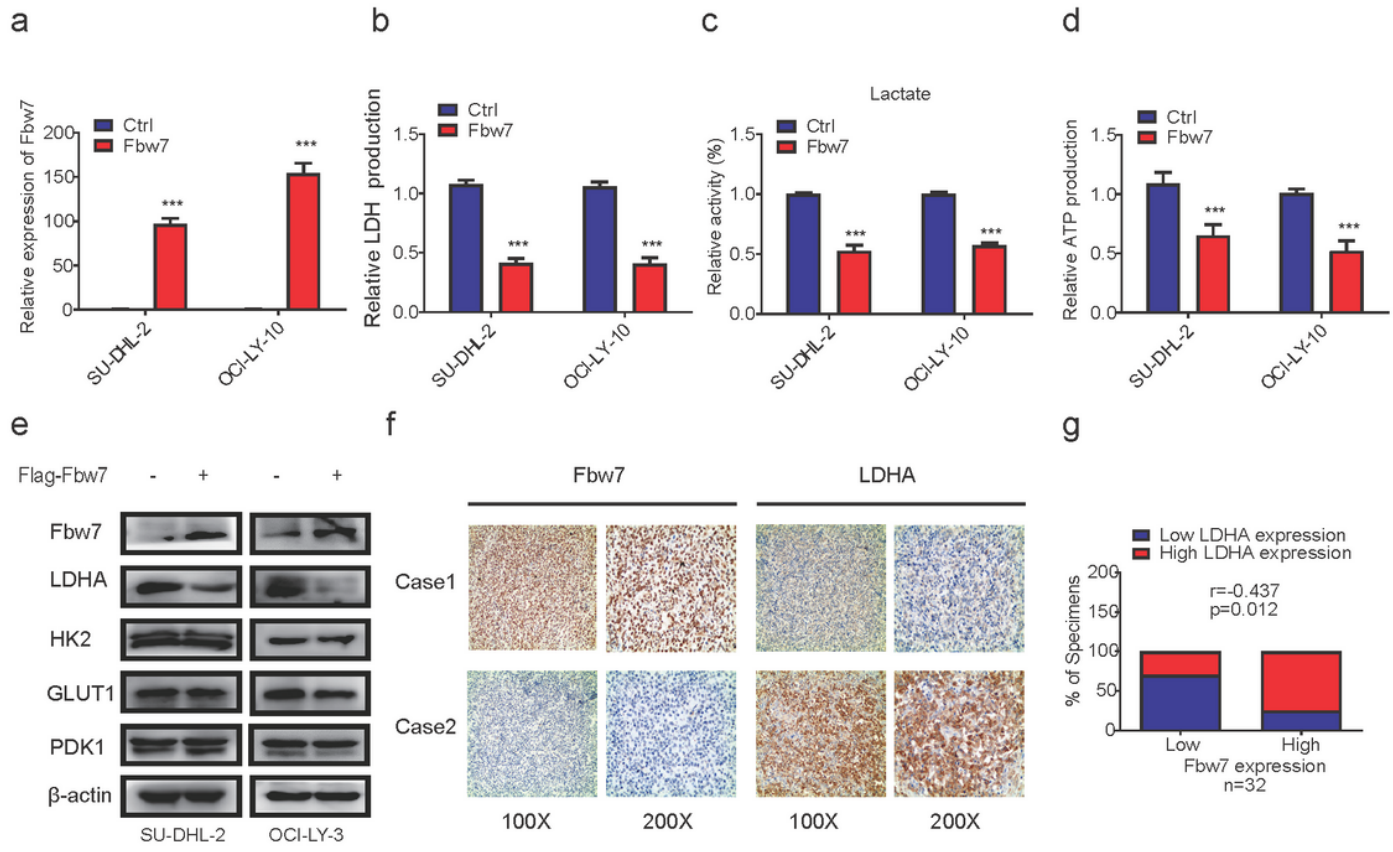

Figure 1

Fbw7 regulates LDHA in activated B-cell like diffuse large B-cell lymphoma. a, QPCR analysis of the relative mRNA expression in SU-DHL-2 and OCI-LY-3 cell lines. Cells were transfected with Flag-Fbw7 and vector control to detect Fbw7 expression. The mean ± SD is shown for three independent experiments. ***P <0.001. b, Fbw7 inhibited intracellular LDH in ABC-DLBCL cells. The mean ± SD is shown for five independent experiments. ***P <0.001. c, Fbw7 reduced intracellular lactate production in ABC-DLBCL cells. Results are presented as mean ± SD. ***P <0.001. d, Fbw7 reduced ATP production. Results are presented as mean ± SD. ***P <0.001. e, Western blotting results showed that overexpression of Fbw7 mainly inhibited LDHA expression in Glycolysis relative proteins. And the expression of Fbw7, LDHA, HK2, GLUT1, PDK1 were shown. f, IHC staining results of Fbw7 and LDHA were shown with representative images from the same tumor case. g, Spearman correlation analysis between Fbw7 and LDHA in 32 cases of DLBCL tissues.

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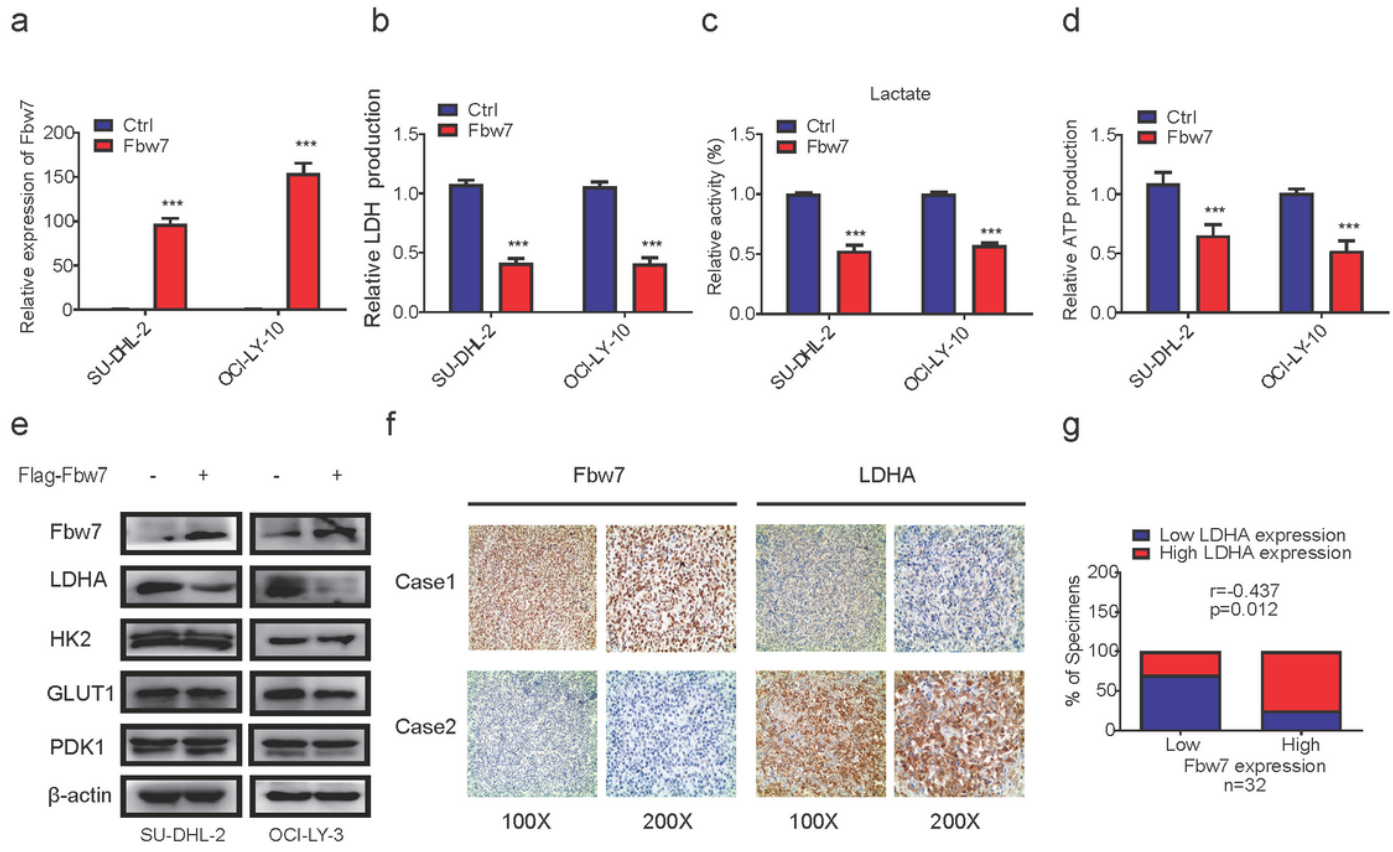

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Fig.2

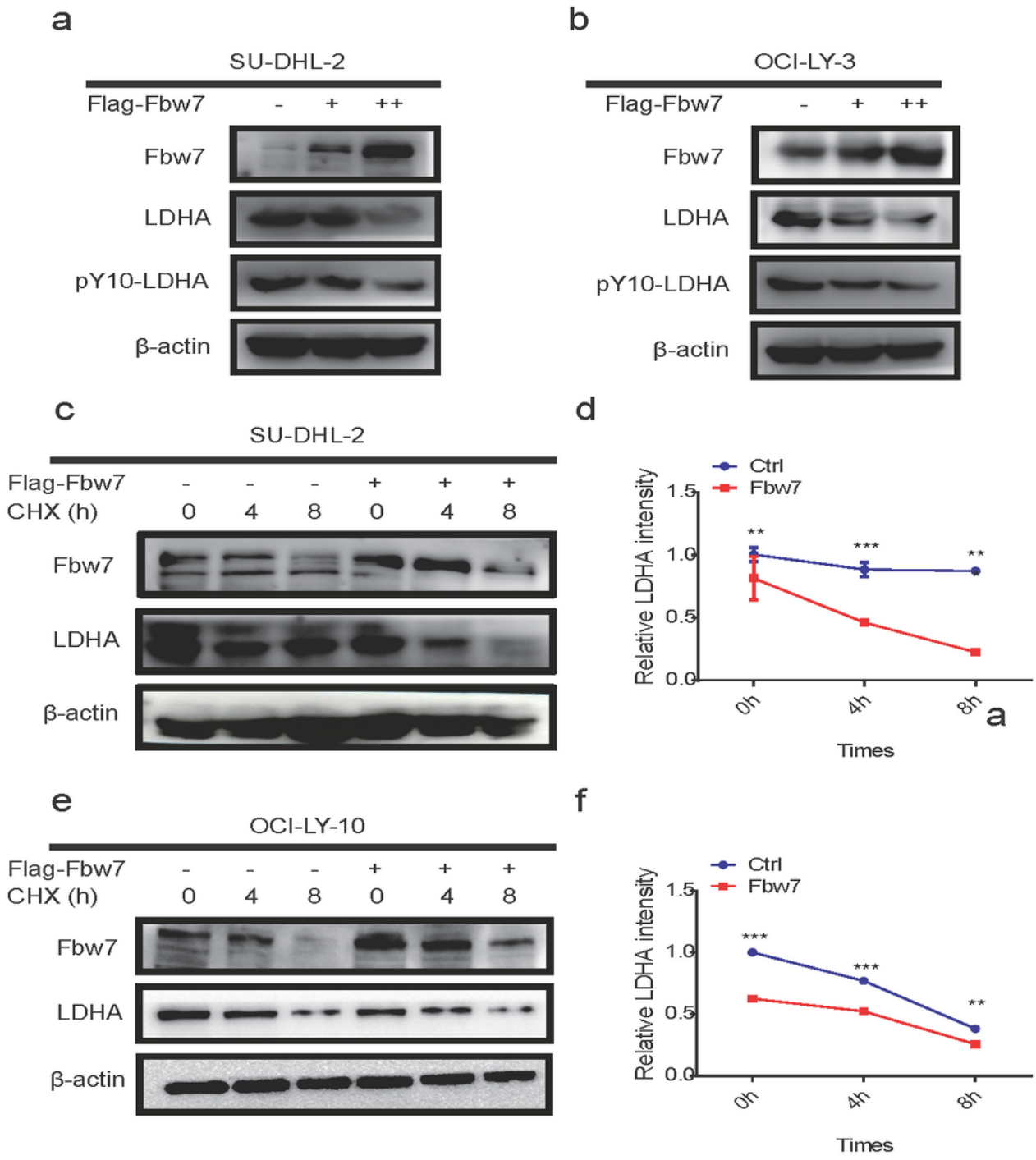

Figure 2

Fbw7 decreased the stability of LDHA. a-b, Western blotting analysis SUDHL-2 and OCI-LY-3 cells transfected with Flag-Fbw7 to detect the expression of LDHA and pY10-LDHA. c-d, SUDHL-2 cells were transfected with Flag-Fbw7 and treated with cycloheximide (CHX; 10 mg/ml) for the indicated time intervals to examine the stability of LDHA. Western blotting results were shown(c) and the mean ratios of the indicated proteins was quantified through densitometry in line graph(d). e-f, The similar results were

shown in OCI-LY-10 cells. Results are displayed as mean ± SD in the line graph for three independent experiments using two-tailed unpaired Student t test. **, P<0.01; ***, P < 0.001.

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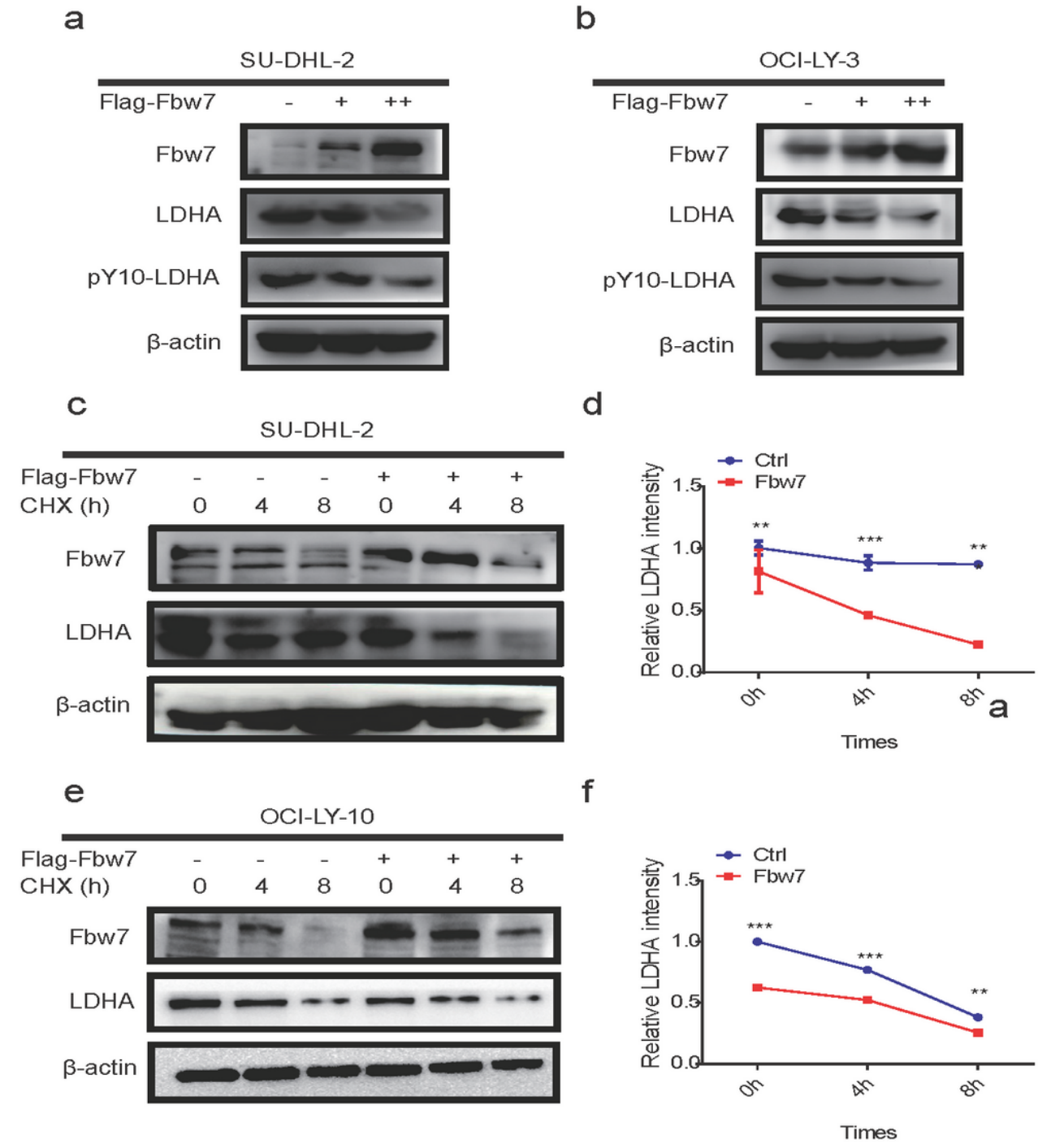

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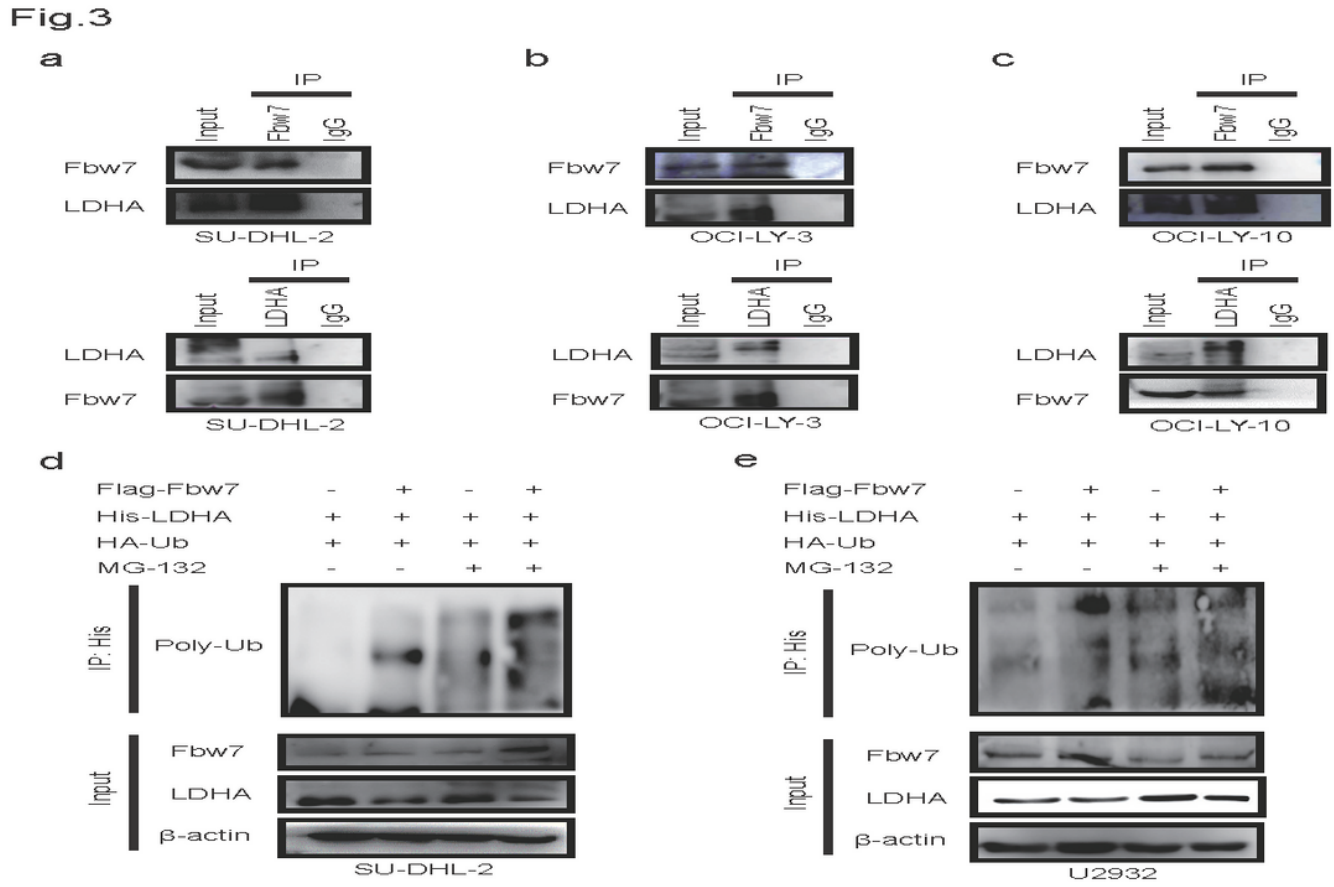

Figure 3

Fbw7 interacts with and ubiquitinates LDHA. a-c, Interaction between endogenous Fbw7 and LDHA in ABC-DLBCL cell lines. Cell lysates from SU-DHL-2, OCI-LY-3 and OCI-LY-10 cells were immunoprecipitated with anti-Fbw7 or anti-LDHA antibody and the original results were analyzed by western blotting. IgG was used as a control. d and e, SU-DHL-2 and U2932 cells were transfected with the indicated plasmids. Cells were treated with or without 10 mM MG132 for 6 after 24 h post transfection, the Fbw7-mediated polyubiquitination status of LDHA was examined by immunoblotting.

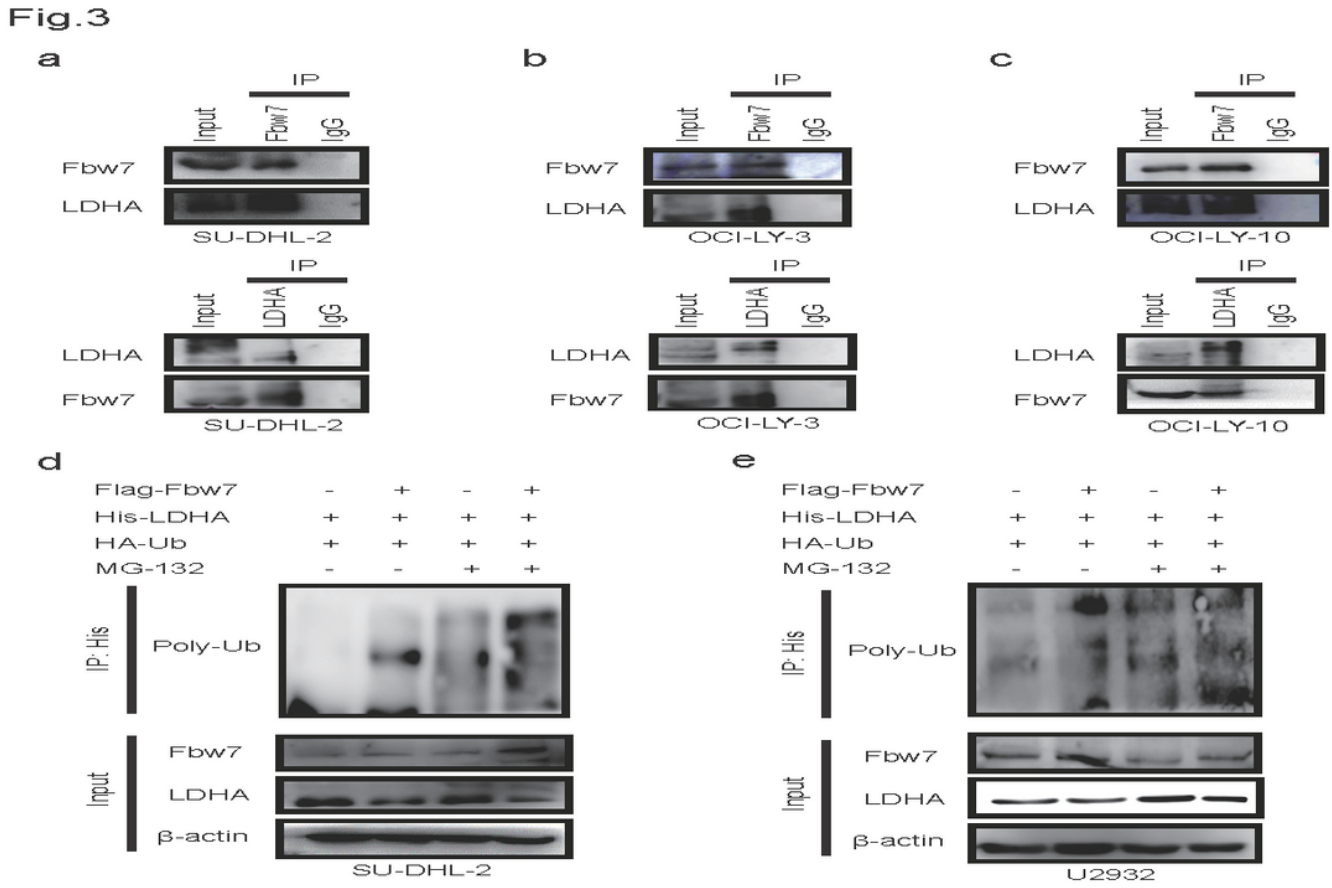

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Fig.4

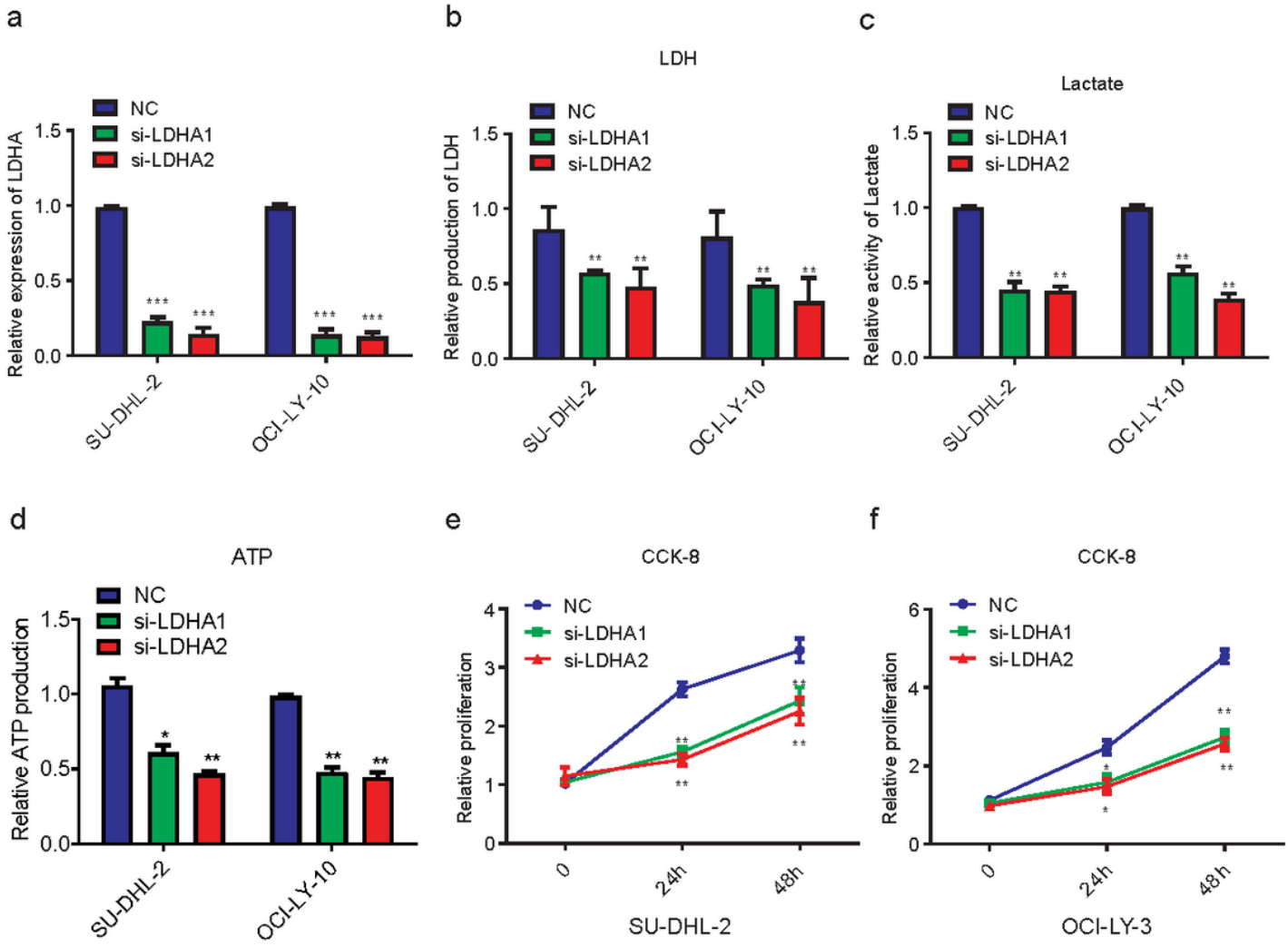

Figure 4

Inhibition of LDHA suppresses tumor progression in ABC-DLBCL a, QPCR analysis of LDHA mRNA expression in SU-DHL-2 and OCI-LY-10 cell lines. LDHA expression was detected after transfecting with siRNA of LDHA. b, Downregulation of LDHA inhibits intracellular LDH in SU-DHL-2 and OCI-LY-10 cells. c, Inhibition of LDHA reduced intracellular lactate production in SU-DHL-2 and OCI-LY-10 cells. d, Inhibition of LDHA reduced ATP production in SU-DHL-2 and OCI-LY-10 cells. e-f, CCK8 proliferation assays were examined to determine cell proliferation of in SU-DHL-2 and OCI-LY-10 cells after transfecting with siRNA of LDHA. Statistical significance was determined by a two-tailed unpaired Student t test and data are shown as mean ± SD in the bar graph for three independent experiments. *, P<0.05; **, P<0.01; ***, P < 0.001.

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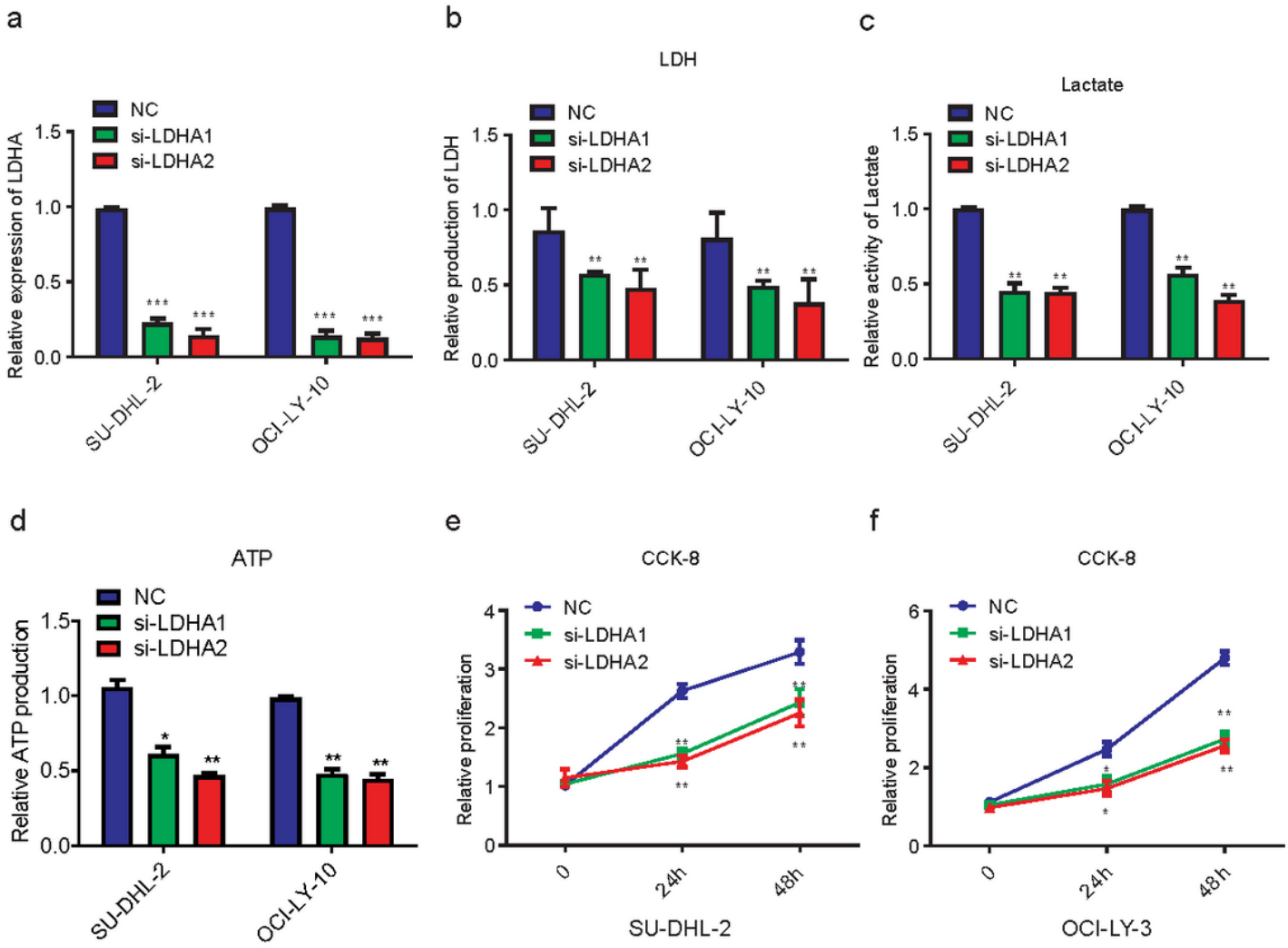

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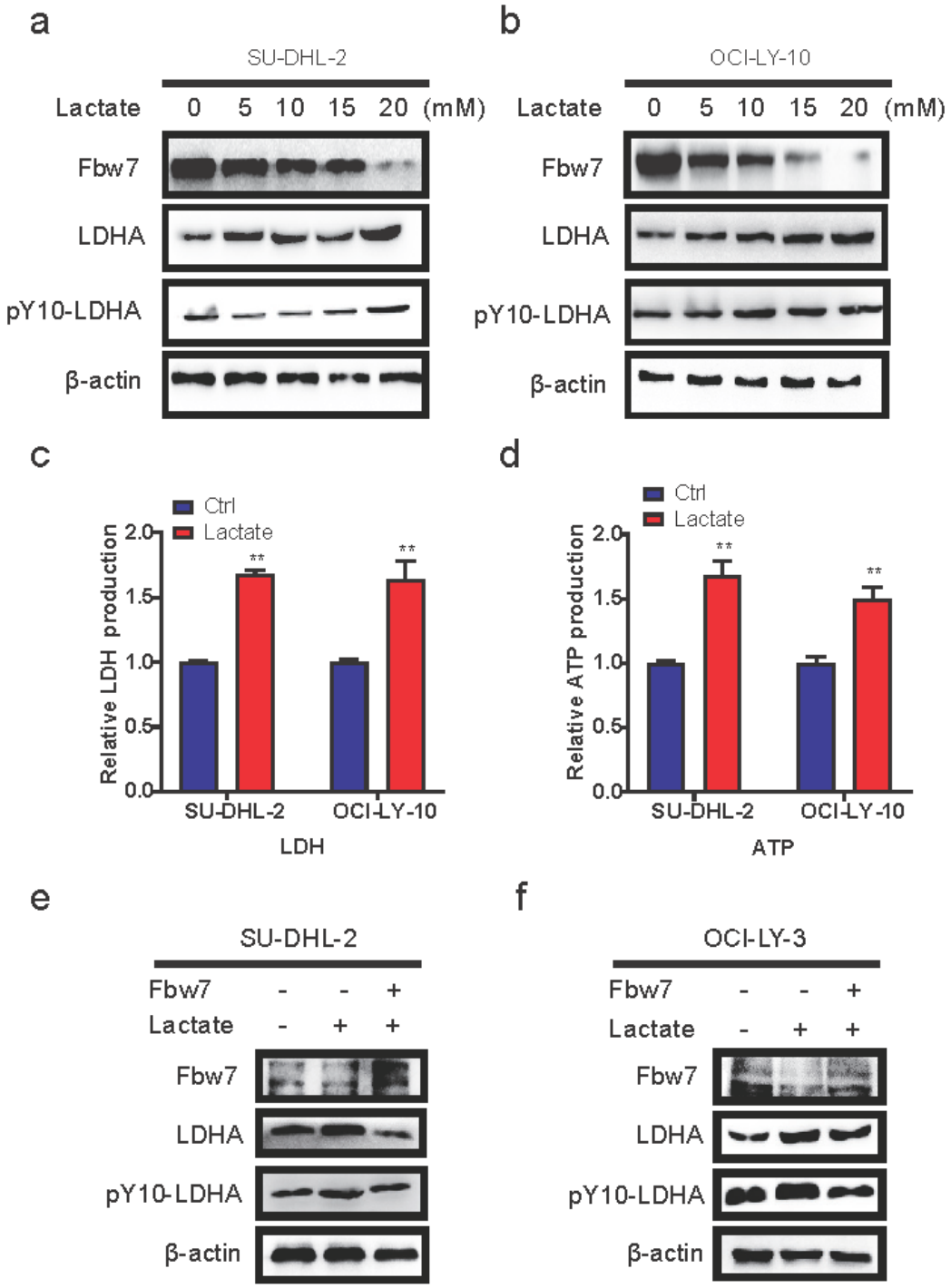

Figure 5

Lactate as a metabolite inhibited Fbw7 expression in ABC-DLBCL. a-b, Lactate inhibits Fbw7 and promotes the expression of LDHA and pY10-LDHA. Lactate was treated into SUDHL-2 and OCI-LY-10 cells. After treating cells with Lactate for the indicated concentration gradient, Fbw7, LDHA and pY10-LDHA levels were examined by western blotting. c, Lactate promotes intracellular LDH in SUDHL-2 and OCI-LY-10 cells. The mean ± SD is shown for five independent experiments. **, P <0.01. d, Lactate

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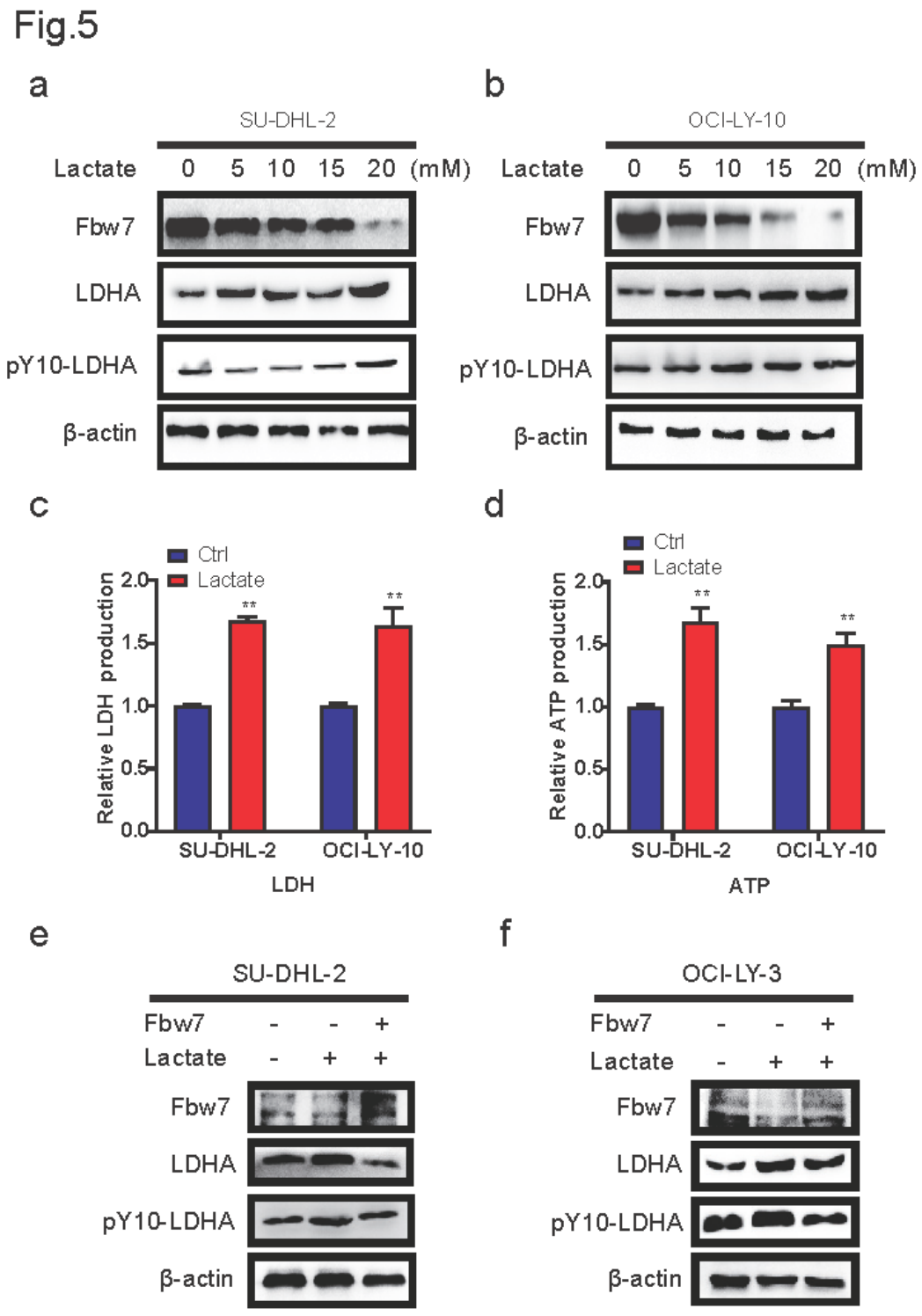

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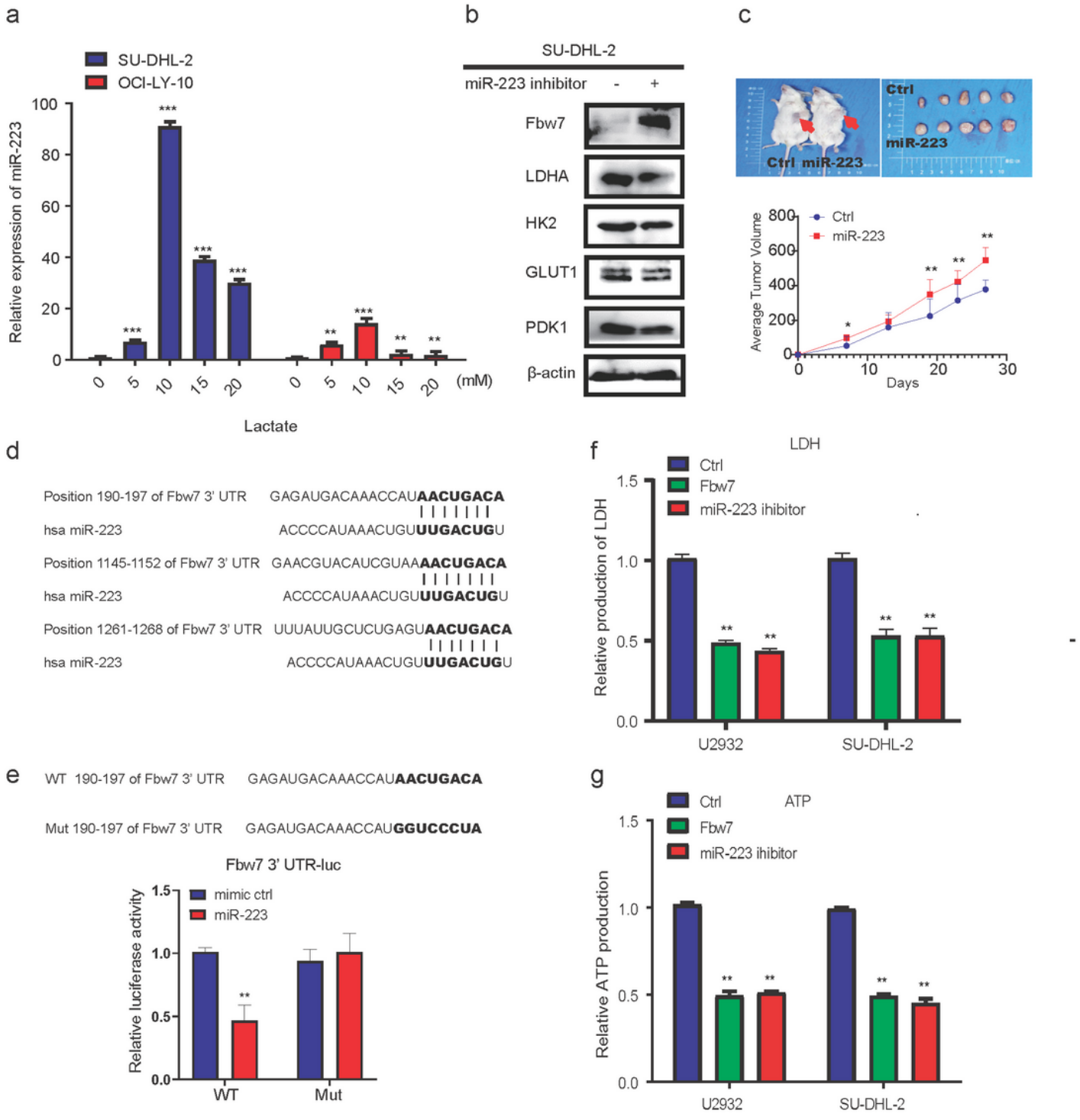

Figure 6

Lactate as a metabolite inhibited Fbw7 expression in ABC-DLBCL a, QPCR analysis of the relative miR-223 expression in SU-DHL-2 and OCI-LY-3 cell lines. Lactate was treated into cells with the indicated concentration gradient. The mean ± SD is shown for five independent experiments. **, P <0.01. ***, P <0.001. b, Western blots showed miR-223 inhibitor increases Fbw7 expression and mainly reduces LDHA expression in Glycolysis relative proteins. And the expression of Fbw7, LDHA, HK2, GLUT1, PDK1 were shown. c, MiR-223 mimic promotes SU-DHL-2 cell proliferation in vivo. Representative images of tumors proliferation in NOD-SCID mice. Mice were implanted with 1 × 107 cells into the flanks of mice (n = 5). Here, mice were treated with miR-223 agomir and agomir of normal control twice a week. 27 days after tumor implantation, mice were sacrificed and tumor volume was measured. 1 cm for Scale bar. **P < 0.01. d, Binding sites of miR-223 and the 3'-UTR of Fbw7 were shown for three conserved sites which was determined by the TargetScan algorithm. e, Reporter constructs containing a position of Fbw7 190-197 3'-UTR region (wild-type, WT) or Fbw7 (MUT) with mutated miR-223 binding site of were co-transfected with a control oligo (mimic ctrl) or miR-223 mimic oligo into HEK293 cells. Luciferase activity was detected 24 hours after transfection. Data are shown as mean ± SD; n=5. **, P <0.01. f, Fbw7 and miR-223 inhibitor reduces intracellular LDH in U2932 and SU-DHL-2 cells. The mean ± SD is shown for five independent experiments. **, P <0.01. g, Fbw7 and miR-223 inhibitor reduced ATP production in U2932 and SU-DHL-2 cells. The mean ± SD is shown for five independent experiments. **, P <0.01.

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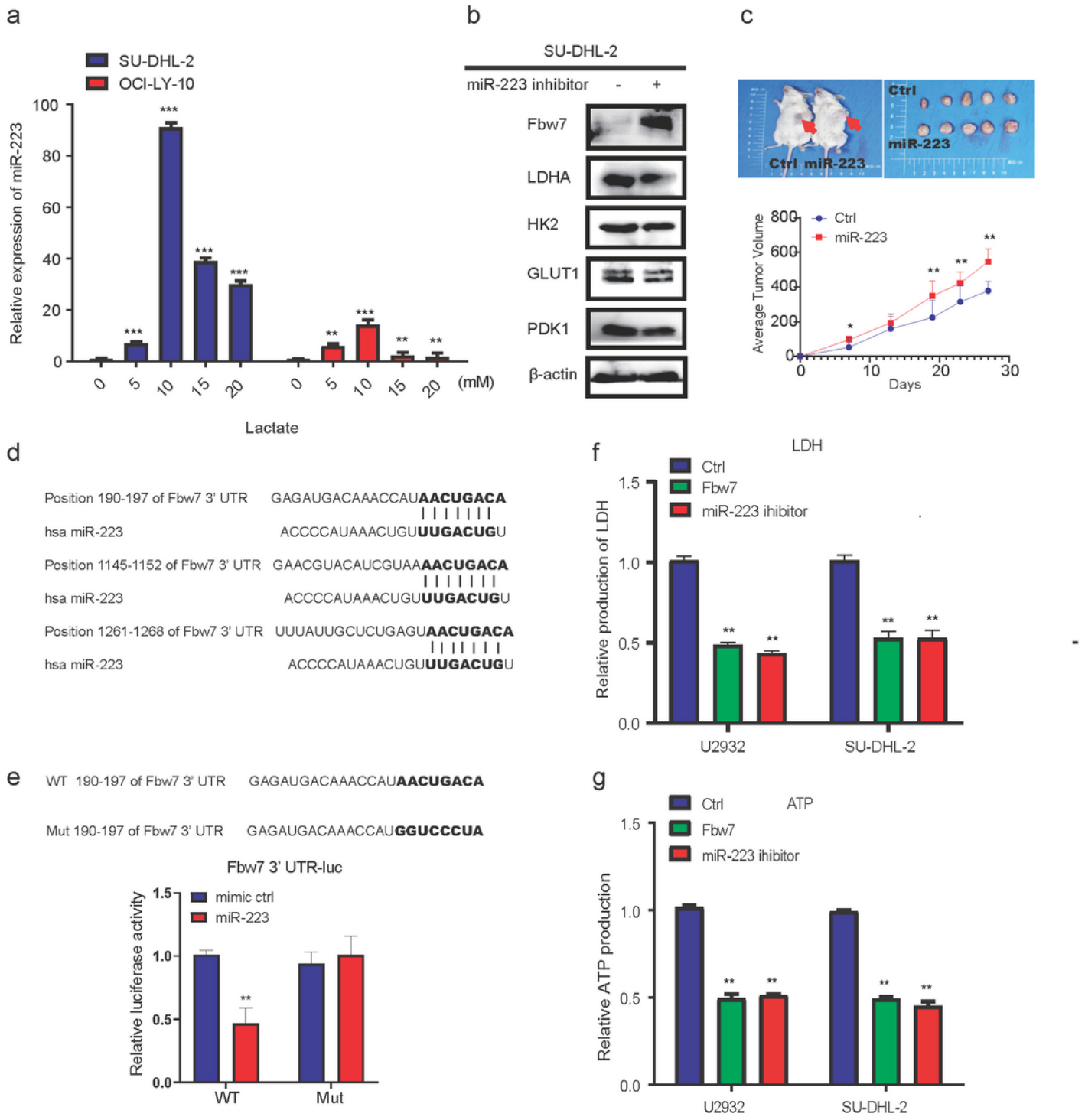

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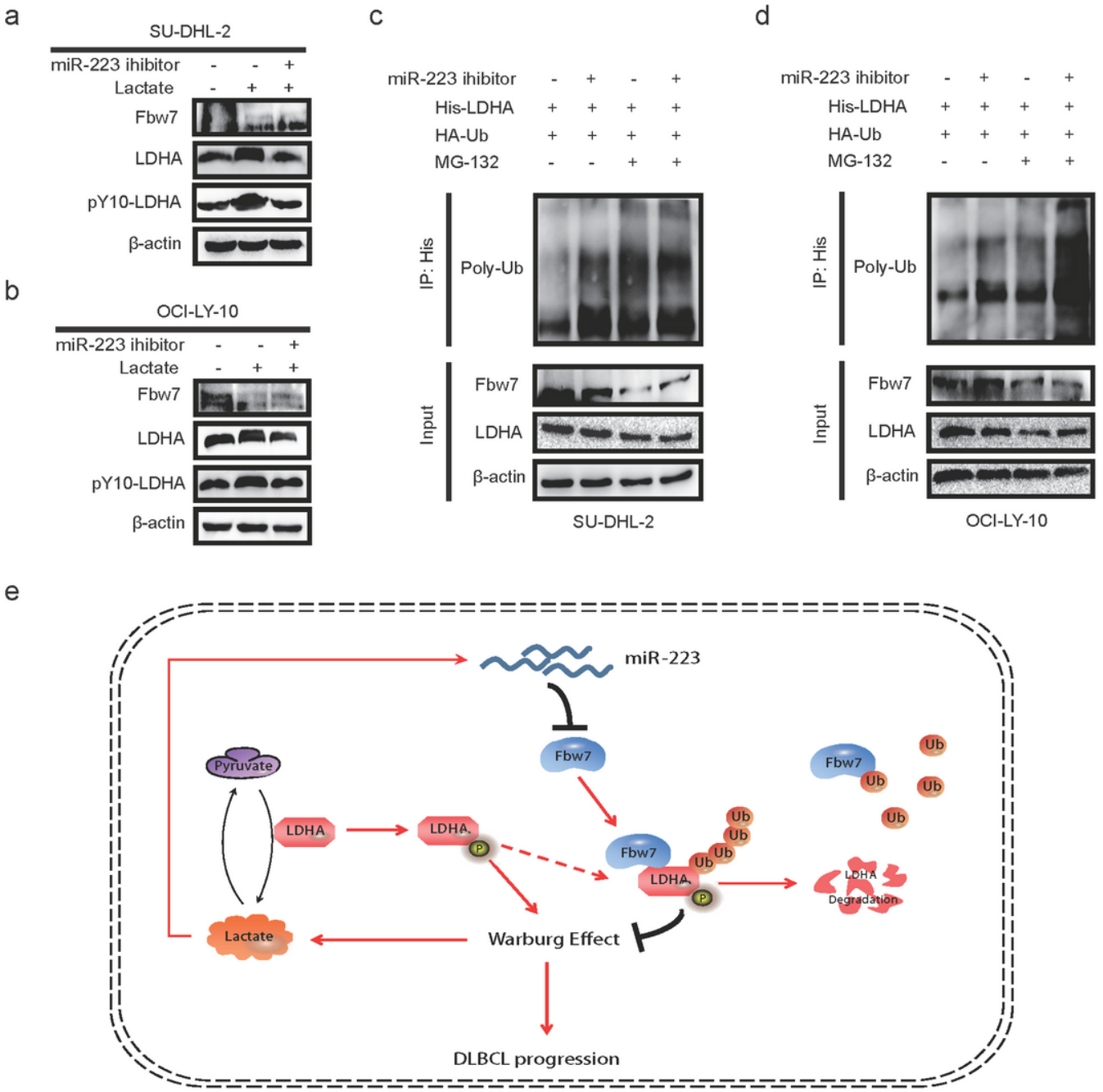

Figure 7

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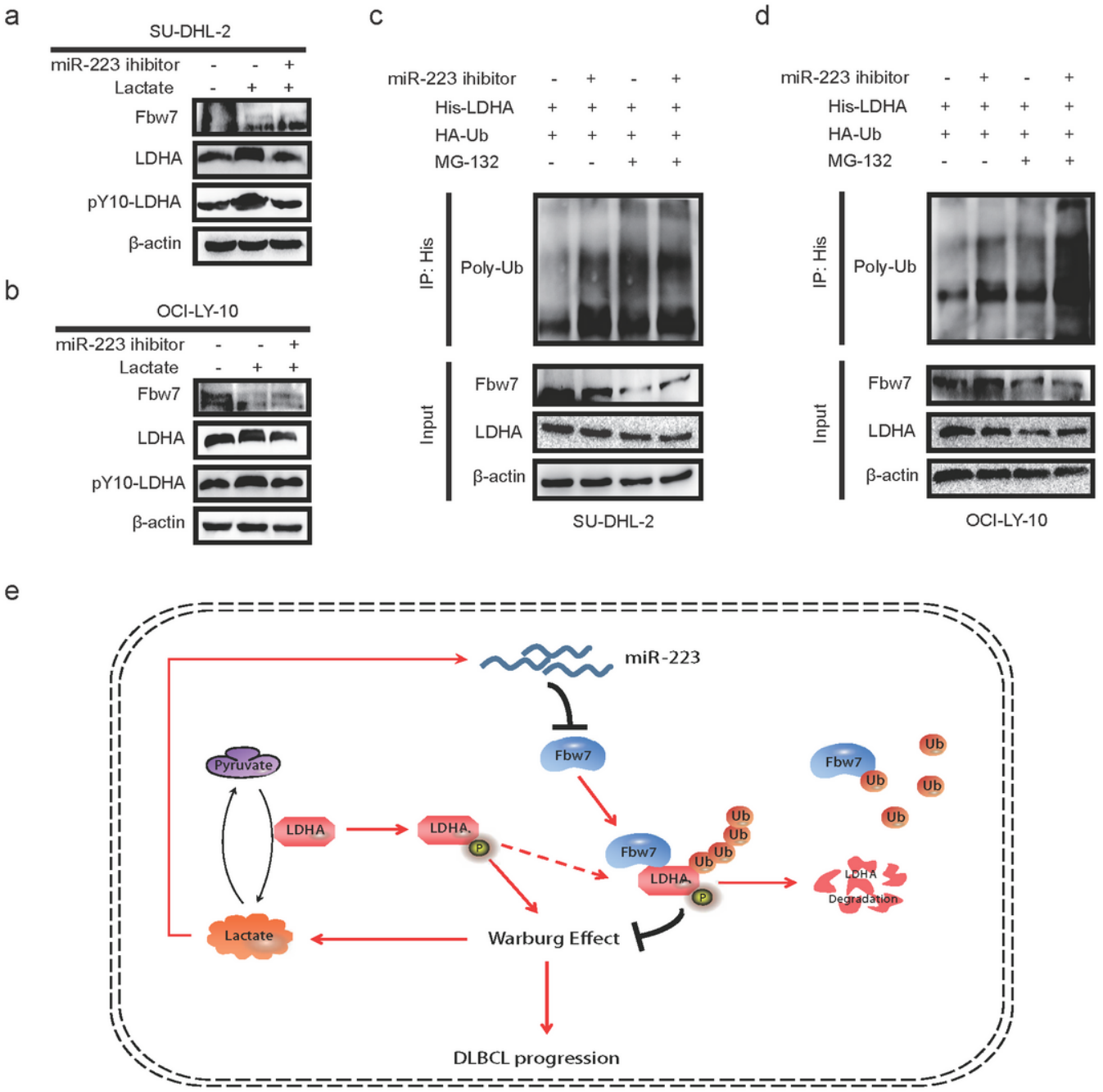

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Supplementary Files

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