Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

EZH2 regulates PD-L1 expression via HIF-1α in non-small cell lung cancer cells

Yuetao Zhao [a, 1], Xin-xin Wang [a, 1], Wei Wu [b], Haixia Long [a], Jiani Huang [a], Zhongyu Wang [a], Tao Li [a], Shu Tang [a], Bo Zhu [a, **], Degao Chen [a, *]

[a] Institute of Cancer, Xinqiao Hospital, Army Medical University, Chongqing, China
[b] Department of Cardiothoracic Surgery, Southwest Hospital, Army Medical University, Chongqing, China

ARTICLE INFO

Article history:
Received 9 July 2019
Accepted 13 July 2019
Available online xxx

Keywords:
EZH2
PD-L1
HIF-1α
lung cancer

ABSTRACT

Lung cancer is the most commonly diagnosed cancer and accounts for most cancer-related mortalities worldwide. The high expression of programmed death ligand 1 (PD-L1) is an important factor that promotes immune escape of lung cancer, thus aggravates chemotherapy resistance and poor prognosis. Therefore, understanding the regulatory mechanism of PD-L1 in lung cancer is critical for tumor immunotherapy. Enhancer of Zeste homolog2 (EZH2), an epigenetic regulatory molecule with histone methyltransferase activity, promotes the formation of an immunosuppressive microenvironment. This study aimed to investigate the role of EZH2 in PD-L1 expression and in the progression of lung tumors. We found that EZH2 was upregulated in lung cancer tissues and positively correlated with PD-L1 levels and poor prognosis. Further, shRNA-expressing lentivirus mediated EZH2 knockdown suppressed both the mRNA and protein expression level of PD-L1, thus delaying lung cancer progression in vivo by enhancing anti-tumor immune responses. Moreover, the regulatory effect of EZH2 on PD-L1 depended on HIF-1α. The present results indicate that EZH2 regulates the immunosuppressive molecule PD-L1 expression via HIF-1α in non-small cell lung cancer cells.

1. Introduction

Lung cancer is the most commonly diagnosed cancer worldwide and accounts for the most cancer-related mortalities [1,2]. Despite recent advancements in screening, surgical resection, chemotherapy, radiation therapy, and targeted therapy, the cure rate of lung cancer, and especially non-small cell lung cancer (NSCLC), has not been significantly improved [3]. In lung cancer, upregulation of programmed death ligand 1 (PD-L1) is an important factor promoting immune evasion, thus exacerbating chemotherapeutic resistance and yielding a poor prognosis [4–7]. Accordingly, immune checkpoint therapy, based on blocking programmed death 1 (PD-1)/PD-L1 has yielded promising outcomes for cancer treatment [7–9]. Unfortunately, the objective response rate of monotherapy using PD-1/PD-L1 pathway inhibitors is generally low, averaging at approximately 20% [10,11]. Moreover, the costs of such treatment are high. Further understanding of the mechanisms underlying PD-L1-induced immune evasion in lung cancer would help improve immunotherapy.

PD-L1 is regulated by specific cancer microenvironments [12]. In hypoxic environments, upregulated hypoxia-inducible factor alpha (HIF-1α) can regulate PD-L1 by binding a hypoxia response element of the *PDL1* promoter to activate its transcription [13–15]. However, the mechanism underlying PD-L1 regulation in lung cancer is unclear.

Enhancer of Zeste homolog2 (EZH2) serves as an important epigenetic regulator with histone methyltransferase activity, which can catalyze lysine trimethylation of histone H3 at lysine 27 (H3K27) and elicit gene silencing [16–18]. EZH2 is overexpressed in various tumor tissues including prostate cancer [19], breast cancer [20,21], cutaneous melanoma [22], and lung carcinoma tissue [23,24]. Moreover, EZH2 inhibitors have yielded promising

Abbreviations: EZH2, enhancer of Zeste homolog2; H3K27, lysine trimethylation of histone H3 at lysine 27; HIF-1α, hypoxia-inducible factor alpha; LLC, Lewis lung carcinoma; NC, negative control; MFI, mean fluorescence intensity; NSCLC, non-small cell lung cancer; PD-1, programmed death1; PD-L1, programmed death ligand 1; TIL, tumor infiltrating $CD8^+$ T cells; TH1, T helper 1.

* Corresponding author.
** Corresponding author.
E-mail addresses: bo.zhu@tmmu.edu.cn (B. Zhu), degaochen1008@163.com (D. Chen).

[1] These authors contributed equally.

https://doi.org/10.1016/j.bbrc.2019.07.039
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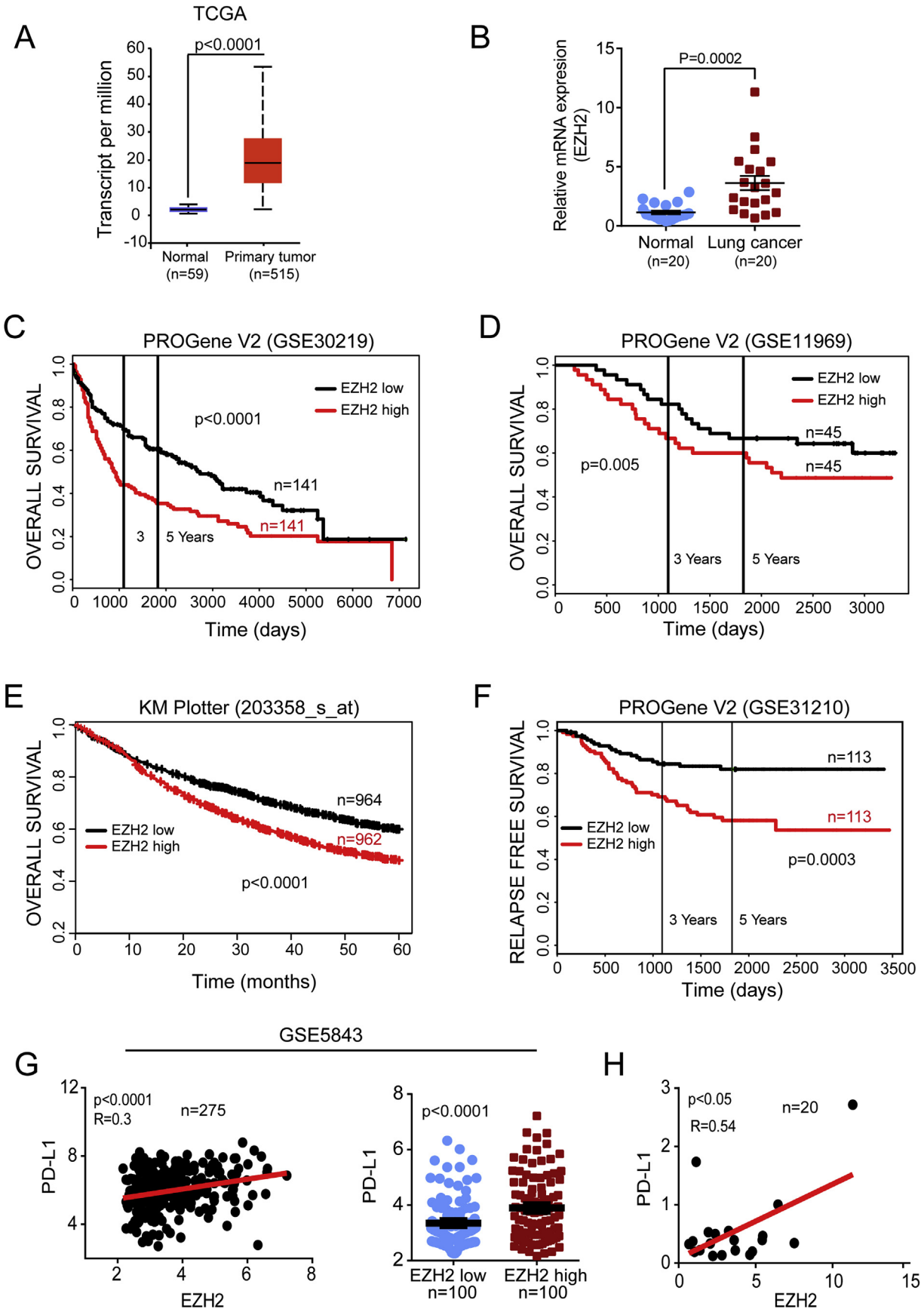

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outcomes in early clinical trials [25–27], suggesting this protein as a potential therapeutic target. Furthermore, EZH2 represses tumor-specific production of T helper 1 (TH1)-type chemokines CXCL9 and CXCL10, thus establishing an immunosuppressive microenvironment in human ovarian cancer [28]. Therefore, is thus worth investigating whether EZH2 is involved in the formation of the immunosuppressive microenvironment in lung cancer. In this study, we investigated the role of EZH2 in PD-L1 expression and in the progression of lung tumors.

2. Materials and methods

2.1. Patients and clinical samples

Twenty human lung cancer tissues and normal adjacent lung tissues were obtained from newly diagnosed lung cancer patients at the Southwest Hospital (Chongqing, China) from 2017 to 2018. The samples were frozen in liquid nitrogen immediately. All patient signed the informed consent before operation. The study was approved by the research ethics boards at the Southwest Hospital.

2.2. Cell culture

Lewis lung carcinoma (LLC) cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA)

supplemented with 10% FBS (Gibco, Carlsbad, CA, USA), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Beyotime Biotechnology, Jiangsu, China). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Viral transduction

GFP-expressing shEZH2 lentivirus were constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China). Empty GFP-expression lentivirus was used as the negative control (NC). LLC cells were cultured in 24-well plates at 60,000 cells per well. Cells were transduced with the shEZH2 or empty lentivirus at a multiplicity of infection of 50 in accordance with the manufacturer's instructions. Puromycin (5 μg/mL) was used to select stably transfected cells. Quantitative reverse transcription PCR (qRT-PCR) and western blotting were carried out to verify knockdown efficiency.

2.4. Mice and tumor challenge experiments

6–8 weeks female C57BL/6 mice were purchased from the Animal Institute of the Academy of Medical Science (Beijing, China). All animal experiments were approved by the ethics committee of the army Medical University. Two million LLC cells successfully transduced with the shEZH2 or NC lentivirus in 100 μL PBS were injected subcutaneously in the right flank. Tumor tissues were harvested 15 d after injection.

2.5. Treatments

For hypoxia induction, LLC cells were incubated in a hypoxia chamber with 1% oxygen for 24 h. HIF-1α prolylhydroxylase-2 inhibitor IOX2 (Selleck Chemicals, Houston, TX, USA) was supplemented in the culture medium, and cells were further incubated for 24 h.

For in vivo treatment studies, GSK343 (Selleck Chemicals, Houston, TX, USA) was administered (i.v.) at 50 mg/kg body weight and an equivalent amount of ethanol was injected in the control group.

2.6. RNA isolation and qRT-PCR analysis

Total RNA was extracted from lung cancer tissues or cells, using RNAiso Plus (TaKaRa Bio, Otsu, Japan) in accordance with the manufacturer's instructions. Thereafter, 1 μg of total RNA was used for cDNA synthesis with the ReverTra Ace® qPCR RT Master Mix (TOYOBO) in accordance with the manufacturer's instructions. qPCR was performed using the SYBR® Green Realtime PCR Master Mix Plus (Toyobo CO.,LTD. OSAKA JANPAN) and the ABI 7500 Prism Sequence Detection System (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. β-actin was considered the internal control for normalization of relative mRNA expression levels. The following primers were used: human EZH2, (forward) 5′-AATCAGAGTACATGCGACTGAGA-3′ and (reverse) 5′- GCTGTATCCTTCGCTGTTTCC-3′; human Actin, (forward) 5′-AAGGAGCCCCACGAGAAAAAT-3′ and (reverse) 5′-ACCGAACTTGCATTGATTCCAG-3′; mouse EZH2, (forward) 5′-AGTGACTTGGATTTTCCAGCAC-3′ and (reverse) 5′-AATTCTGTTGTAAGGGCGACC-3′; mouse HIF-1α, (forward) 5′-ACCTTCATCGGAAACTCCAAAG-3′ and (reverse) 5′-CTGTTAGGCTGGGAAAAGTTAGG-3′; mouse PD-L1, (forward) 5′-GCTCCAAAGGACTTGTACGTG-3′ and (reverse) 5′-TGATCTGAAGGGCAGCATTTC-3′; mouse Actin, (forward) 5′-ATGACCCAAGCCGAGAAGG-3′ and (reverse) 5′-CGGCCAAGTCTTAGAGTTGTTG-3′.

2.7. Western blot analysis

Cells were lysed on ice with RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China) containing protease inhibitors. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, 30 μg of protein was separated via vertical SDS-PAGE and protein bands were electro-transferred on to polyvinylidene fluoride membranes (Hoffman-La Roche Ltd., Basel, Switzerland). Further, 5% BSA (Boster Biological Technology Co. Ltd., Wuhan, China) was used to block the membranes for 1 h at room temperature. The membranes were incubated overnight with mouse polyclonal anti-EZH2 (dilution 1:1000, Cell Signaling Technology, Beverly,MA,USA), rabbit anti-beta-Actin (dilution 1:1000; Bioss, China), and mouse monoclonal anti-PD-L1 (dilution 1:1000, Proteintech, Chicago, IL) at 4 °C overnight. After three washes with tris-buffered saline with tween (TBST), membranes were probed with the secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Bands were detected using BeyoECL Plus (Beyotime, Shanghai,China). GAPDH was used as a loading control.

2.8. Flow cytometry

Tumor tissues were harvested 15 d after injection and digested with I Collagenase (Sigma, USA) and 1 mg/mL DNase I (Roche, USA) in RPMI for 40 min at 37 °C. Thereafter, the cell suspension was

Fig. 1. EZH2 upregulation leads to a poor prognosis among lung cancer patients and is positively correlated with PD-L1 expression. (A) EZH2 is over-expressed in lung cancer tissues in comparison with normal lung tissues. mRNA expression data were obtained using UALCAN. (B) Quantitative reverse transcription PCR (qRT-PCR) analysis was performed to validate the expression of EZH2 in human lung cancer tissues (n = 20). (C–D) Survival analysis of EZH2 with OS in lung cancer patients from PROGene V2 website. (E) Survival analysis of EZH2 with OS in lung cancer patients from KM Plotter website. (F) Survival analysis of EZH2 in lung cancer patients with relapse-free survival from PROGene V2 website. (G) The correlation between EZH2 and PD-L1 mRNA expression in lung cancer samples was analyzed from the available RNA-seq dataset obtained from Gene Expression Omnibus ($R = 0.3$, $P < 0.0001$). (H) The correlation between EZH2 and PD-L1 mRNA expression in 20 lung cancer samples.

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filtered through a 100-μm micron filter. Single-cell suspensions were stained with percp/Cyanine5.5 anti-mouse CD8 (clone 53−6.7, Biolegend), PE/Cy7 anti-mouse CD3 (clone 145-2C11), and APC anti-mouse PD-1 (clone EH12.2H7, Biolegend) for 30 min at a 1:100 dilution on ice, in accordance with the manufacturer's instructions. To analyze the function of CD8 T cells, single cells were stimulated with eBioscience™ Cell Stimulation Cocktail (plus protein plus transport inhibitors) (Thermo Fisher Scientific, Waltham, MA, USA) for 5 h in a humidified atmosphere with 5% CO_2 at 37 °C. Surface staining was performed after stimulation using FITC anti-mouse IFN-γ (clone XMG1.2, Biolegend), percp/Cyanine5.5 anti-mouse CD8 (clone 53−6.7, Biolegend), and PE/Cy7 anti-mouse CD3 (clone 145-2C11). After surface staining, intracellular cytokine staining was performed with the Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences, USA) in accordance with the manufacturer's instructions. Samples were harvested using a Gallios flow cytometry system (Beckman coulter, USA) and analyzed using FlowJo software (TreeStar).

2.9. Analysis of The Cancer Genome Atlas (TCGA) database

We analyzed differences in *EZH2* mRNA expression between normal lung tissues and lung cancer tissues, using UALCAN (http://ualcan.path.uab.EdU./analysis.html) [29], followed by Kaplan−Meier plotter (http://kmplot.com/analysis) [30,31] and PROGgeneV2 prognostic Database (http://www.abren.net/PrognoScan/) [32] to obtain the survival data of NSCLC patients. The cohort was classified on the basis of high and low EZH2 expression levels and overall survival was analyzed with an auto-select best cutoff. Data on EZH2 and PD-L1 expression were obtained from Gene Expression Omnibus (GEO) datasets and used for correlation analysis.

2.10. Statistical analyses

GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used for data analysis. All statistical data are expressed as mean ± SEM values. Statistical significance was evaluated with Student's *t*-test. The correlation between PD-L1 and EZH2 expression was assessed using Pearson's correlation coefficients, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. EZH2 overexpression yields a poor prognosis among lung cancer patients and is positively correlated with expression of the immunosuppressive molecule PD-L1

EZH2 was upregulated in lung cancer tissues rather than in normal lung tissues, as revealed via bioinformatics analysis using UALCAN (Fig. 1A). Furthermore, qRT-PCR analysis to validate EZH2 expression in 20 paired lung cancer tissues and normal lung tissues revealed that *EZH2* mRNA was upregulated in lung cancer tissues rather than in normal lung tissues, concurrent with the findings of bioinformatics analysis (Fig. 1B).

Furthermore, TCGA was analyzed to assess the association between EZH2 expression and lung cancer patient prognosis, using PROGgene V2 and Kaplan-Meier Plotter. EZH2 upregulation was significantly associated with a poor overall survival (Fig. 1C−E) and predicted a poor relapse-free survival (Fig. 1F).

EZH2 regulates anti-tumor immune responses and PD-L1 overexpression is an important mechanism underlying immune evasion among tumors. To investigate the associations among these proteins, correlation analysis was performed considering mRNA expression data ($n = 275$) of lung cancer patients from GEO datasets. As shown in Fig. 1G, EZH2 expression is associated with PD-L1 upregulation ($R = 0.3$, $p < 0.0001$). Furthermore, qRT-PCR analysis revealed that EZH2 expression levels are positively correlated with PD-L1 expression (Fig. 1H). These results indicate that EZH2 is upregulated in lung cancer tissues and closely associated with a poor patient prognosis. Furthermore, EZH2 expression levels are positively correlated with those of PD-L1, a negative immunoregulator, indicating that EZH2 potentially regulates PD-L1 and plays a role in anti-tumor immunity.

3.2. EZH2 knockdown in lung cancer cells leads to PD-L1 downregulation

To elucidate the regulatory effect of EZH2 on PD-L1 expression, we knocked down EZH2 expression in LLC cells with shEZH2 lentivirus. As shown in Fig. 2A−C, the mRNA and protein expression level of EZH2 were downregulated 24 h after transduction, especially in the shEZH2-2 group. Subsequently, we examined changes in PD-L1 expression levels upon qRT-PCR, western blotting, and flow cytometry analyses. Both PD-L1 mRNA and protein were markedly downregulated upon shEZH2 knockdown (Fig. 2D−G). Further, LLC cells were treated with the EZH2 inhibitor GSK343 at different concentrations; consequently, the average fluorescence intensity of PD-L1 decreased after 24 h of GSK343 treatment. These results indicate that PD-L1 is regulated by EZH2 in mouse lung cancer cells.

EZH2 inhibition delays lung cancer progression in vivo and enhances anti-tumor immune responses through PD-L1 downregulation.

To determine whether EZH2 inhibition affects tumor progression, we subcutaneously injected LLC cells and those transduced with shEZH2 into C57BL/6 mice. Assessments of both tumor volume and weight revealed that EZH2 inhibition markedly limited lung cancer progression (Fig. 3A and B). Furthermore, we investigated the effect of EZH2 knockdown on PD-L1 expression in lung cancer cells in vivo. As shown in Fig. 2C, the mean fluorescence intensity (MFI) of PD-L1 decreased significantly in the shEZH2 group rather than the vector control group, as determined via flow cytometry analysis. Since it is difficult to achieve a transduction efficiency of 100%, we also detected PD-L1 expression in GFP-positive and GFP-negative cells in the same tumor tissues, which represented transfected and non-transfected cells, respectively. Consistently, the MFI of PD-L1 in the GFP-positive group was also significantly lower than that of GFP-negative cells in the paired assay (Fig. 2D). These results indicate that EZH2 suppression can result in PD-L1 downregulation in vivo.

Since the PD-1/PD-L1 signaling axis mediates an inhibitory immune response, we subsequently investigated whether EZH2 knockdown in lung cancer cells would downregulate PD-L1 to promote an anti-tumor immune response. The proportion of tumor-infiltrating $CD8^+$ T cells (TILs) was significantly greater in the shEZH2 group than in the control group, along with a significant increase in the proportion of the functional IFN-γ secreted by TILs (Fig. 3E and F).

3.3. EZH2 downregulates PD-L1 via HIF-1α

The tumor microenvironment is hypoxic and is associated with HIF-1α overexpression. This protein can directly bind the enhancer of *PD-L1* to promote its expression. This led us to speculate whether EZH2-mediated PD-L1 regulation is achieved by modulating HIF-1α. Hence, we examined HIF-1α expression in lung cancer cells 24 h after shEZH2 lentivirus transduction. qRT-PCR and flow cytometry analyses revealed that HIF-1α mRNA and protein were significantly downregulated in the shEZH2 group rather than the control group

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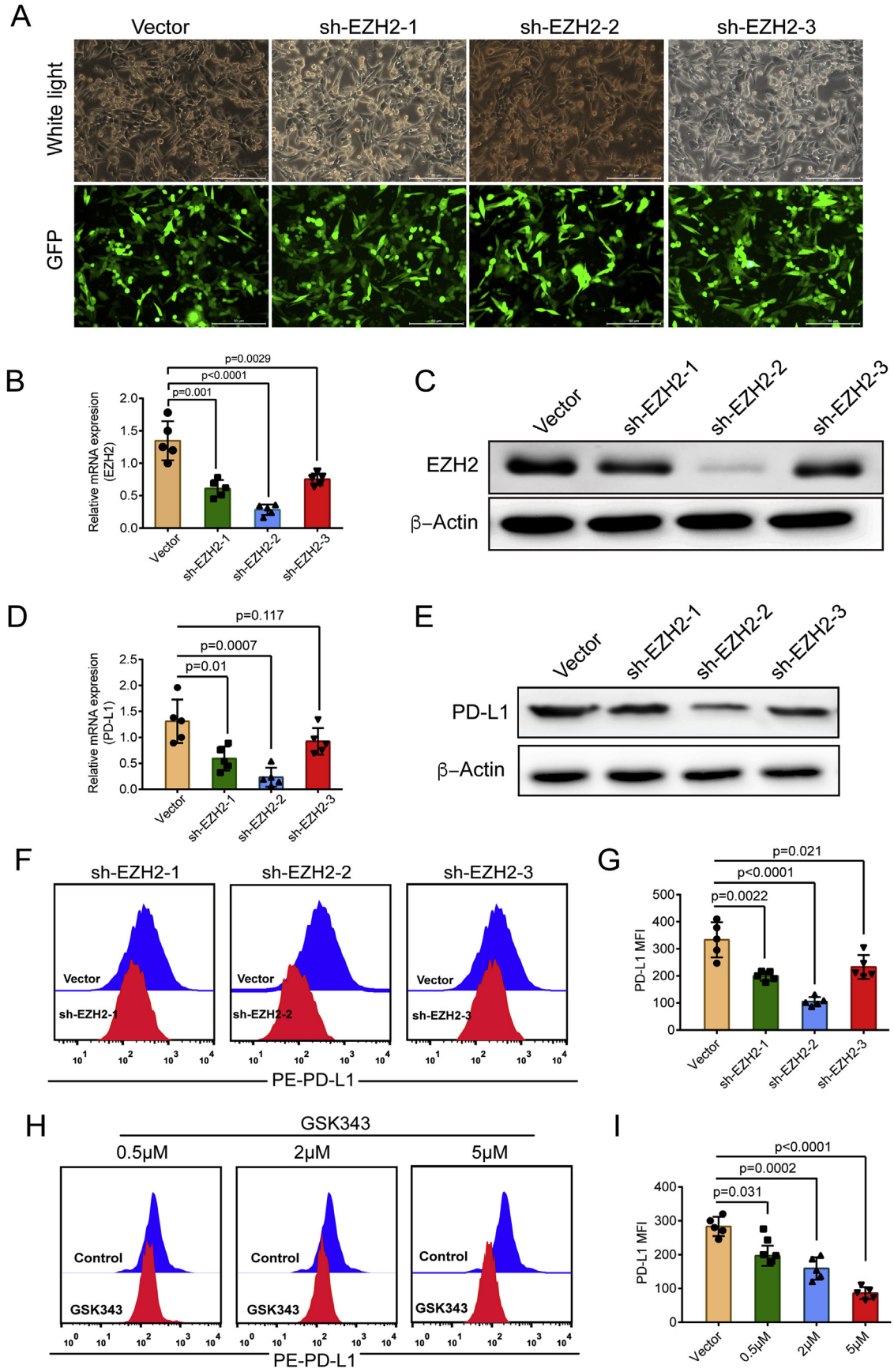

Fig. 2. EZH2 knockdown down-regulated PD-L1 in lung cancer cells. (A) GFP-expressing shEZH2 lentivirus transduction. (B) Verification of the knockdown efficiency of shEZH2 lentivirus via real-time quantitative PCR analysis. (C) Western blotting was performed to analyze the protein expression level of EZH2 24 h after shEZH2 lentivirus transduction. (D) PD-L1 mRNA levels decreased in the shEZH2 group compared to those in the negative control group in LLC cells 24 h after transduction. (E) PD-L1 protein levels decreased in the shEZH2 group compared to those in the negative control group in LLC cells 24 h after transduction. (F–G) Flow cytometry analysis was performed to detect PD-L1 expression levels 24 h after shEZH2 lentiviral transduction in LLC cells. (H–I)PD-L1 protein levels decreased in the GSK343 treatment group compared to those in the control group in LLC cells.

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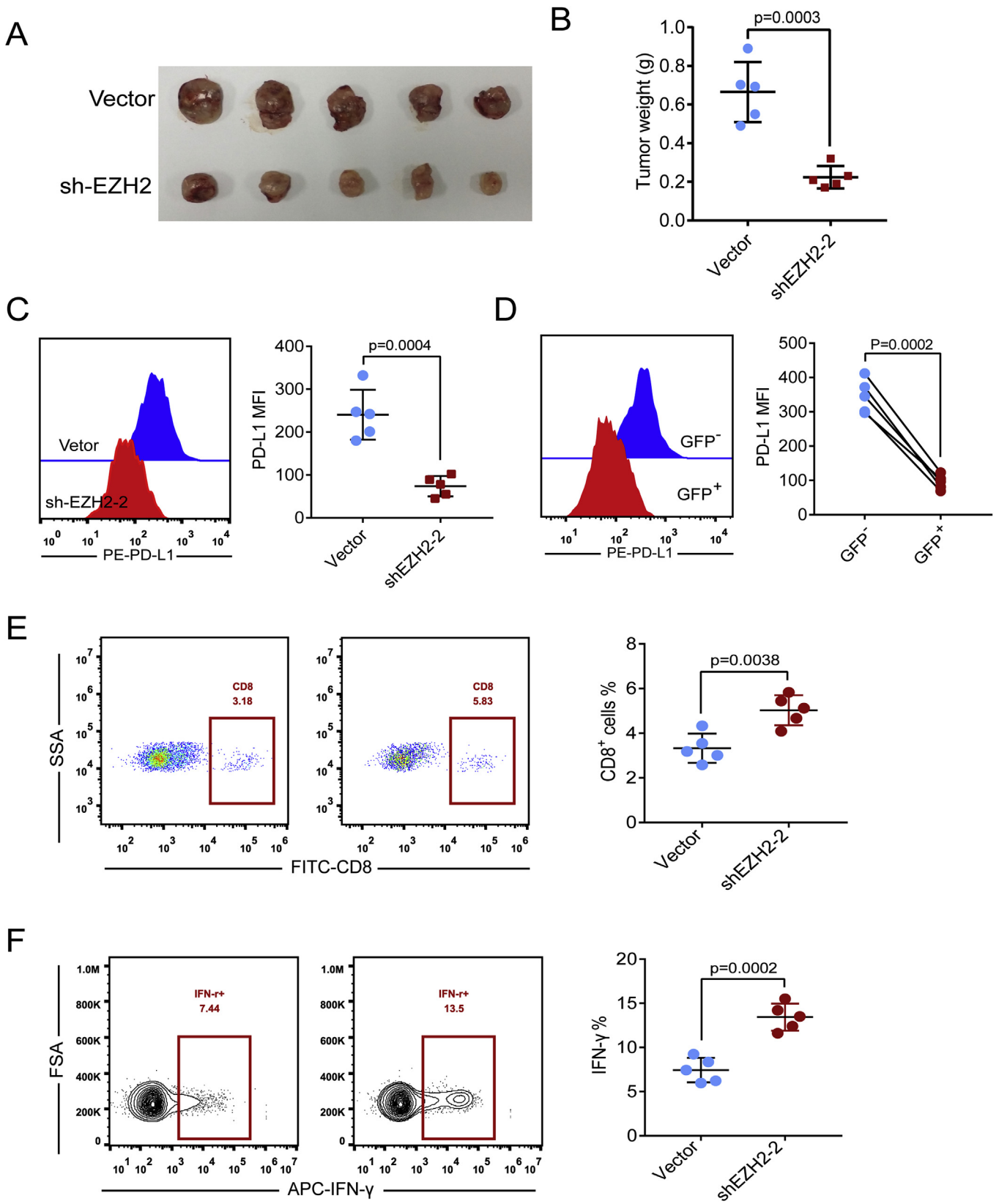

Fig. 3. EZH2 knockdown delays lung cancer progression in vivo. (A–B) EZH2 inhibition markedly decreased the tumor burden. (C) Flow cytometry analysis revealed that the mean fluorescence intensity (MFI) of PD-L1 was significantly decreased in the shEZH2 group compared to that in the vector group. (D) The MFI of PD-L1 in the GFP-positive group was significantly lower than that of GFP-negative cells in the paired assay. (E) The infiltration of $CD8^+$ T cells was significantly increased in the shEZH2 group compared with the control group. (F) $CD8^+$ T cells in the shEZH2 group have a higher potential to secrete IFN-γ.

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(Fig. 4A and B), indicating that EZH2 downregulation affects HIF-1α expression. Furthermore, hypoxia-induced HIF-1α upregulation in lung cancer cells led to PD-L1 upregulation (Fig. 4C). In addition, IOX2-mediated HIF-1α inhibition resulted in PD-L1 downregulation (Fig. 4D and E). These results suggest that EZH2 potentially affects PD-L1 expression by affecting HIF-1α expression. To further confirm this hypothesis, we overexpressed HIF-1a in lung cancer cells by inducing hypoxia and simultaneously knocked down EZH2. This combinatorial treatment significantly downregulated PD-L1 in comparison with hypoxia treatment alone (Fig. 4F). These results show that EZH2 regulates PD-L1 via HIF-1α in lung cancer cells.

4. Discussion

Harnessing an anti-tumor immune response has been a fundamental strategy for cancer immunotherapy [33]. In the last decade,

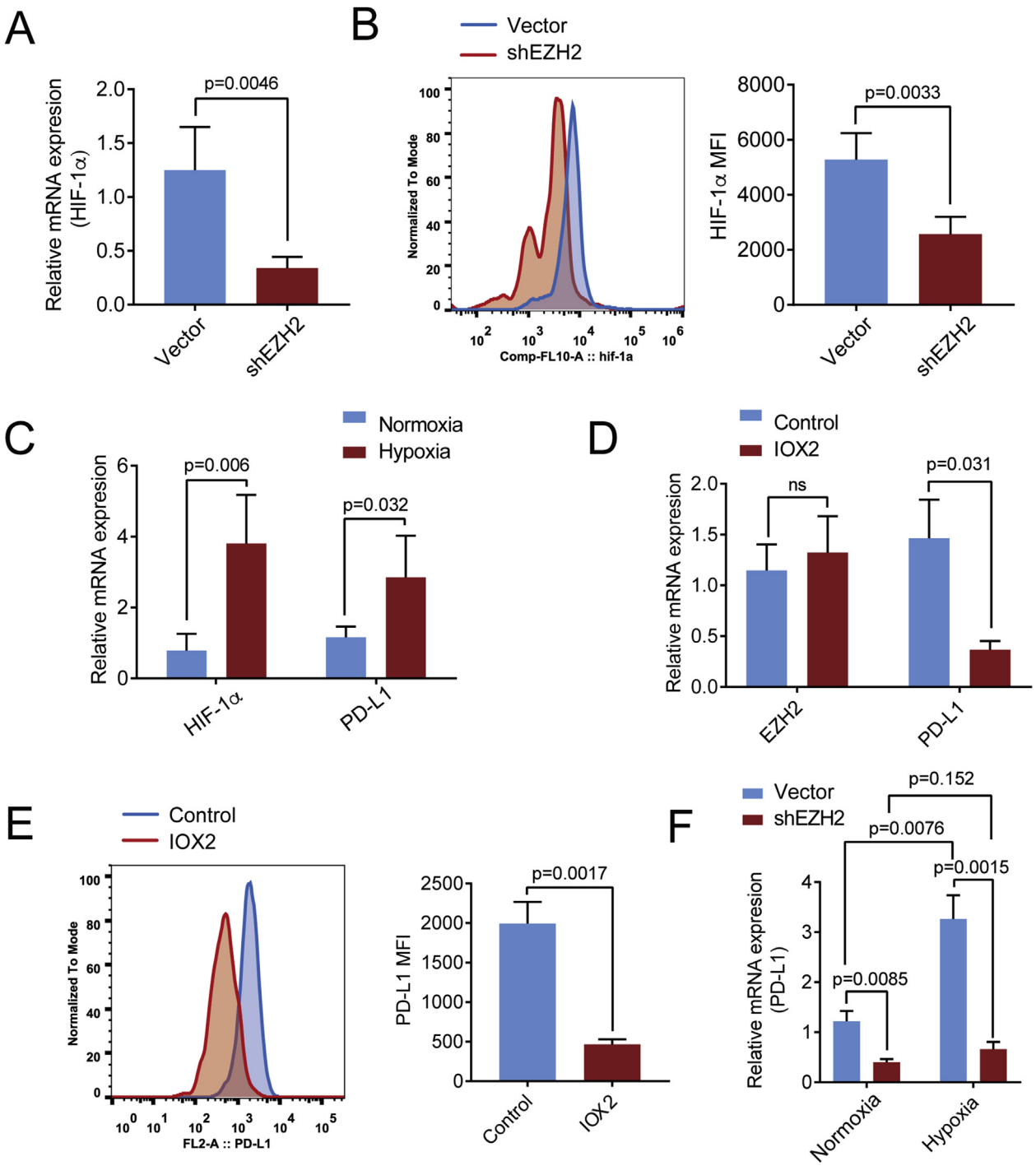

Fig. 4. EZH2 downregulates PD-L1 via HIF-1α. (A) Real-time quantitative PCR analysis was performed to detect the mRNA expression level of HIF-1α in LLC cells after shEZH2 transduction. (B) Flow cytometry analysis revealed that HIF-1α levels were decreased in the shEZH2 group. (C) HIF-1α and PD-L1 mRNAs were upregulated upon hypoxia induction in LLC cells. (D) EZH2 and PD-L1 mRNAs in LLC cells 24h after IOX2 treatment. (E)Flow cytometry results revealed that PD-L1 protein was downregulated in LLC cells 24 h after IOX2 treatment. (F) *PDL1* mRNA was significantly downregulated in the shEZH2-transduced group and the hypoxia group in comparison with the shEZH2 transduction only group.

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drugs targeting the PD-1/PD-L1 pathway have been developed and have achieved highly objective response rates in patients with malignant melanoma and lymphoma [7,8,33]. PD-L1 is upregulated in lung cancer and contributes to chemotherapeutic resistance and poor prognosis by inducing immune evasion; therefore, the PD-1/PD-L1 pathway is a critical immunotherapeutic target for this disease. To elucidate novel PD-L1-regulatory mechanisms, we analyzed gene chip results from GEO datasets and found that EZH2 is upregulated in lung cancer tissues rather than in normal lung tissues; furthermore, patients with higher EZH2 levels had a reduced survival rate. In addition, a strong positive correlation was observed between EZH2 and PD-L1 expression levels, consistent with the literature [34]. shRNA-expressing lentivirus mediated EZH2 knockdown in lung cancer cells led to a concomitant PD-L1 downregulation. Similar results were observed using GSK343, an EZH2 inhibitor. Therefore, EZH2 potentially regulates PD-L1 expression in lung cancer. Moreover, we found that the number of $CD8^+$ T cells in tumor tissues was increased, accompanied by an increase in IFN-γ levels, indicating an enhanced $CD8^+$ T-cell mediated anti-tumor immune response. These results suggest that EZH2 promotes lung cancer progression by upregulating PD-L1, thus inducing tumor immune evasion.

EZH2 promotes the establishment of an immunosuppressive microenvironment in breast cancer [28], and our results suggest that EZH2 promotes immune evasion in lung cancer by upregulating PD-L1, thus rendering it a potential therapeutic target. However, the precise underlying mechanism is unclear. Under hypoxia, PD-L1 is upregulated via direct interaction of HIF-1α with a hypoxia-response element in the *PD-L1* proximal promoter to activate transcription in myeloid-derived suppressor cells, macrophages, dendritic cells, and prostate and breast cancer cell lines [13–15]. Herein, our initial assessment of the associations among EZH2, HIF-1α, and PD-L1 in lung cancer revealed that EZH2 promotes HIF-1α expression, which was correlated with PD-L1 levels in hypoxic environments. Interestingly, EZH2 silencing alleviated the effects of hypoxia on PD-L1 expression, indicating that EZH2 is upstream of HIF-1α-induced PD-L1 expression. Our results show that EZH2 regulates PD-L1 through HIF-1α upregulation.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China [grant number 31700681].

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.07.039.

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Please cite this article as: Y. Zhao et al., EZH2 regulates PD-L1 expression via HIF-1α in non-small cell lung cancer cells, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2019.07.039