Original Research

Increased expression of PD-L1 in endometrial cancer stem-like cells is regulated by hypoxia

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Abstract

Background: The expression levels of the programmed cell death ligand 1 (PD-L1), known as an immune-inhibitory molecule, are closely associated with cancer stem cell (CSCs) immune escape. Recently, PD-L1 has also been reported to be able to regulate the self-renewal of cancer stem cells. However, The expression and intrinsic role of PD-L1 in endometrial cancer stem-like cell (ECSC) maintenance and its underlying mechanism of action remain unclear. **Methods**: Using flow cytometry and western blot assays, we have demonstrated that PD-L1 expression is higher in ECSCs derived from endometrial cancer than in nonstem-like cancer cells. Using mouse xenograft assays for ECSC tumorigenicity. Using gene reporter assay for uncovering the regulation mechanism of PD-L1 in the hypoxia. **Results**: We revealed the high expression levels of PD-L1 in ECSCs and its correlation with self-renewal. We further found that PD-L1 knockdown reduced expression of several pluripotency-related genes (aldehyde dehydrogenase 1 (ALDH1), CD133, OCT4, SOX2, NANOG), impaired ECSC proliferation and undifferentiated colonies and decreased the number of CD133 positive ECSCs and the number of stem-like spheres. Furthermore, we found that PD-L1 knockdown inhibited ECSC tumorigenicity and the PD-L1 induced self-renewal capability of ECSCs was dependent upon hypoxia HIF-1α and HIF-2α activation. **Conclusions**: These data link ECSC maintenance to PD-L1 expression through hypoxia and suggest a promising target for PD1/PD-L1 immunotherapy.

Keywords: Programmed cell death ligand 1; Endometrial cancer stem-like cells; PD1/PD-L1; Hypoxia

1. Introduction

Endometrial carcinoma (EC) is one of the three common malignant tumors of female reproductive system and the most common gynecological genital tract swelling. In recent years, the incidence and mortality of endometrial cancer are gradually increasing, and the trend is getting younger [1–3]. Studies have shown that cancer stem-like cells are a small population of tumor cells with self-renewal and pluripotency capabilities [4], and endometrial carcinoma stem-like cell (ECSC) homeostasis plays an important role in the occurrence, invasion, drug resistance and metastasis of endometrial carcinomas [5,6].

Cancer immunotherapy based on immune checkpoint molecules such as PD-1/PD-L1 can achieve better outcomes for advanced cancers including those with high PD-L1 expression. PD-1/PD-L1 represents a pair of immunocostimulatory factors [7,8] involved in the immunomodulatory processes of the body, such as autoimmunity, transplantation immunity and tumor immunity. Blockade of the PD-1/PD-L1 signaling pathway with targeted antibodies can enhance their endogenous anti-tumor immune effect [9].

PD-L1 is not only highly expressed in tumors and involved in their immune escape [10], but also has been found to be highly expressed in some tumor stem cells where it can regulate their self-renewal [11,12]. It has been reported that PD-L1 is highly expressed in breast cancer stem cells, where the PI3K/Akt signaling pathway is activated to promote the expression of stemness factors OCT4 and NANOG, and thus maintain the characteristics of breast cancer stem cells. There is also a strong correlation between PD-L1 expression and stemness in breast cancer patients [13]. Furthermore, tumor stem cells with higher PD-L1 can promote their immune escape [14]. Therefore, it is of great value to study the role of PD-L/PD-L1 signal transduction mechanisms in tumor stem cells, and to elucidate the molecular mechanism between PD-L1 and tumor stem cell self-renewal, with an aim of inhibiting this signaling pathway for tumor immunotherapy. Ultimately, this may represent a novel approach for the development of tumor therapies. However, the relationship between PD-L1 and ECSC maintenance and its underlying mechanism of action remains elusive. Therefore, our study aims to determine the relationship between the expression of PD-L1 and the accumulation of stemness-related genes, and thus dissect the potential mechanism involved in the maintenance of stemness.

Table 1. Realtime PCR primers as follow.

Realtime-PCR	Primers 5′→3′	Genes
PD-L1 F	CTACTGGCATTTGCTGAACG	qPCR for human PD-L1
PD-L1 R	TGCAGCCAGGTCTAATTGTTT	
PD-L2 F	AAAGAGGGAAGTGAACAGTGCT	qPCR for human PD-L2
PD-L2 R	GCTTCTTTAGATGTCATATCAGGTCA	
ALDH1 F	GCTCCATCATCTATCACCCGT	qPCR for human ALDH1
ALDH1 R	ATCTCCGTGAATGAGGGTCCA	
CD133 F	CAGAAGGCATATGAATCCAAAA	qPCR for human CD133
CD133 R	ATAAACAGCAGCCCCAGGAC	
SOX2 F	GACAGTTACGCGCACATGAA	qPCR for human SOX2
SOX2 R	TAGGTCTGCGAGCTGGTCAT	
OCT4 F	GGTATTCAGCCAAACGACCA	qPCR for human OCT4
OCT4 R	CACACTCGGACCACATCCTT	
NANOG F	TTTGTGGGCCTGAAGAAAACT	qPCR for human NANOG
NANOG R	AGGGCTGTCCTGAATAAGCAG	

In this study, we found that PD-L1 expression increased with the ECSC separation process from day 1 to day 8, and the elevated PD-L1 can promote the growth and proliferation of ECSCs, while PD-L1 inhibition prevented this effect. Further analysis of the HIF signaling pathway in the hypoxic microenvironment showed that it regulated PD-L1 by interfering with its promoter region. Thus, we speculate that detecting the expression and mechanism of action of PD-L1 in ECSCs may be critical for the future application of immunotherapy, and the molecular mechanism for the self-renewal in ECSC, therefore, represent a new reference point and basis for further research and clinical application.

2. Materials and methods

2.1 Cell culture

The endometrial cancer cell line Ishikawa (ISK) cells were cultured in the medium of DMEM/F12 (11320082, Gibco, Carlsbad, CA, USA), supplemented with 1 percent penicillin/ streptomycin (15140122, Gibco, Carlsbad, CA, USA), and 10 percent FBS (SV30087, HyClone). ECSC cells were cultured in DMEM/F12 media supplemented with 10 ng/mL Human Fibroblast Growth Factor Protein (FGF) (AA 10-155, Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL Human Epidermal Growth Factor Protein (EGF) (HY-P7109, MedChemExpress, Monmouth Junction, NJ, USA), 0.4% Bovine Serum Albumin (BSA) (HZB0148, Sigma-Aldrich, St. Louis, MO, USA) 20 ng/mL Insulin (HY-P73243, MedChemExpress, Monmouth Junction, NJ, USA) and 1% penicillin/ streptomycin. For hypoxia exposure, cells were placed in a modular incubator chamber (Billups-Rothenberg, San Diego, CA, USA), which was reported before [15]. In testing conditions, ECSC cells were cultured in defined media before supplemented chemicals as indicated as $CoCl_2$, the HIFs activator (7646-79-9, Sigma-Aldrich, St. Louis, MO, USA), and KC7F2, the

HIFs inhibitor (HY-18777, MedChemExpress, Monmouth Junction, NJ, USA) as used in the experiments.

2.2 Flow cytometry analysis

Analysis of CD133 and ALDH1 population of the ECSCs isolated from Ishikawa cells by flow cytometry. The ECSCs spheres were harvested after 3 days after formed and dissociated into single cells, and ALDH1, CD133 antibody was added, then analysed by flow cytometry. We used cell lysis (C3702, Beyotime, Shanghai, China) buffer of Red blood cell to remove red blood cells in tumor sample. Cells were suspended with 2 percent FBS in PBS solution, blocked with 5 percent BSA in PBS before CD133 and ALDH1 antibody labelling. Cells were stained with antibodies on ice for 20 min before washing. The CD133 (1:100, CST, Mouse mAb #60577), with the second antibody anti-mouse IgG (H+L) (Alexa Fluor® 555 Conjugate), and the ALDH1A1 (1:100, CST, Rabbit mAb #36671), with the second antibody anti-rabbit IgG (H+L) (Alexa Fluor® 488 Conjugate) (1:100, CST, #4412). Cells were analysed on the FACS Calibur cell analyser (BD Biosciences, San Jose, CA, USA), or sorted on the FACS cell sorter with Aria^{TM} II (BD Biosciences, San Jose, CA, USA), respectively.

2.3 RT-qPCR

We used TRIzol (15596026, LIFE TECHNOLOGIES, Carlsbad, CA, USA) to extracted the total RNA according to the manufacturer's instructions. cDNA synthesis was performed using the High Capacity RNA-to-cDNA Kit (4388950, LIFE TECHNOLOGIES, Carlsbad, CA, USA) and SYBR green master mix (Q111-03, Vazyme, Piscataway, NJ, USA). Primers used in this study are shown in Table 1. All results were performed in triplicate, and three independent experiments.

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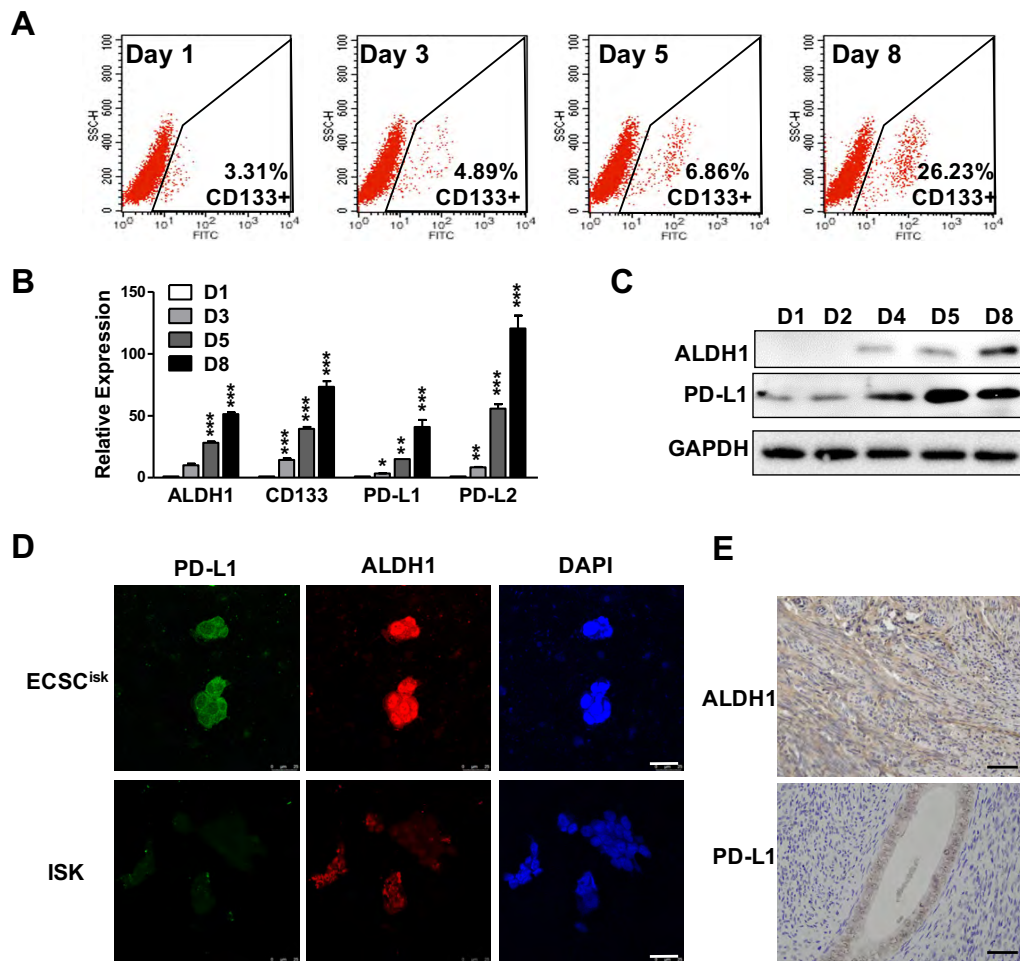
Fig. 1. PD-L1 is crucial for pluripotency in ECSCs. (A) FACS showing the percentage of CD133 positive cells in ECSC^{isk} on days 1, 3, 5 and 8. (B) The genes of ALDH1, PD-L1 and PD-L2 were analysised by qRT-PCR in ECSC^{isk}. (C) The protein of ALDH1 and PD-L1 were analysised by immunoblotting in ECSC^{isk} on different days of day 1, day 2, day 4, day 5 and day 8. (D) The ECSC^{isk} Morphology and the ALDH1 and PD-L1 immunofluorescent staining in ECSC^{isk}. (E) Immunohistochemical staining for PD-L1 in tumors. Means ± SEM (N = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

2.4 Western-blot assay

The modified RIPA lysis buffer (50 mM Tris·HCl, pH 7.5, 1 mM β-mercaptoethanol, 150 mM NaCl, 1 mM Na3VO4, 1 mM NaF, 1 mM EDTA, 0.25% sodium deoxycholate and 1% Igepal CA-630) was prepared for the cell lysates. Blots were probed with PD-L1 (1:1000, Abcam, Rabbit mAb #205921), OCT4 (1:1000, Abcam, Rabbit mAb #19857), ALDH1 (1:1000, CST, Rabbit mAb #36671), and GAPDH (ab9485, 1:5000, Abcam) antibodies. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were used and the chemiluminescent signal was detected by using ECL Plus (C510045-0100, Sangon, Shanghai, China).

2.5 SiRNA and transduction

SiRNA targeting PD-L1 were purchased from Ribo Bio. SiRNA sequences were designed as follows: *PD-L1*, Sense: 5′- CCA GCA CAC UGA GAA UCA ATT-

3′ and antisense: 5′- UUG AUU CUC AGU GUG CUG GTT-3′. *HIF1α*, Sense: 5′-GCC ACU UCG AAG UAG UGC UTT-3′ and antisense: 5′-AGC ACU ACU UCG AAG UGG CTT-3′. *HIF2α*, Sense: 5′-GCG ACA GCU GGA GUA UGA ATT-3′ and antisense: 5′-UUC AUA CUC CAG CUG UCG CTT-3′. All siRNAs were transfected into ECSC cells for interfering the expression of *PD-L1, HIF1α, HIF2α*. For lentivirus mediated *PD-L1* knockdown, lentiviral vector FG12 (Addgene, Watertown, MA, USA) were used, and the packaging plasmids were pRSV/REV, pMDLG/pRRE and pHCMVG. The RNA and protein were collected after 48 h and detected by Real-time Quantitative PCR Detecting System (QPCR) or Western Blot (WB), and the cell functions were identified by Immunofluorescence staining for marker genes and clonal counting. For PD-L1 promoter (sequence –2685 to +86) reporter plasmid was constructed in PGL3 basic vector at PD-L1 promoter sites.
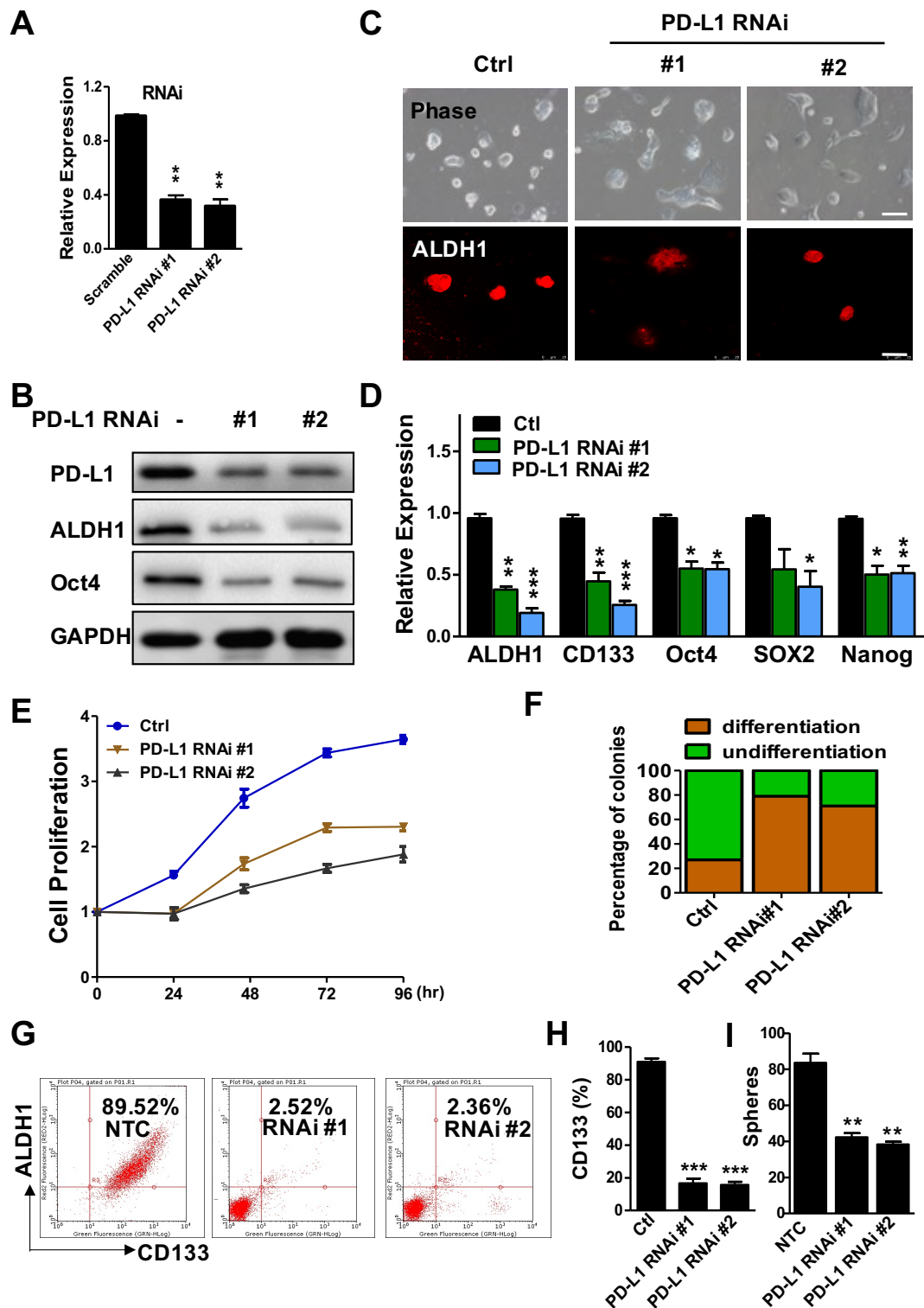
Fig. 2. The effect of PD-L1 on ECSC stemness. Validation of the siRNAs targeted to PD-L1 by qRT-PCR (A) and western blot (B). ALDH1 and OCT-4 protein levels detected in ECSC^{isk} transfected with control and PD-L1 siRNAs. (C) Images showing the morphology of ECSC^{isk} and immunofluorescent staining for ALDH1 when using PD-L1 targeted siRNAs. (D) Stemness genes (*ALDH1, CD133, OCT4, SOX2* and *NANOG*) using PD-L1 targeted siRNAs were analysis by qRT-PCR. (E) CCK-8 analysis of the cell proliferation of ECSC^{isk} cells transfected with control and PD-L1 siRNAs. (F) The percent of undifferentiated and differentiated colonies were calculated according to their morphology after PD-L1 was targeted by siRNAs. (G) FACS analysis showing the percentages of positive ALDH1 and CD133 ECSC^{isk} cells in transfected with control and PD-L1 siRNAs. Percentage of CD133-positive cells (H) and spheres (I) in ECSC^{isk} transfected with control and PD-L1 siRNAs. Mean $\pm$ SEM (N = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.
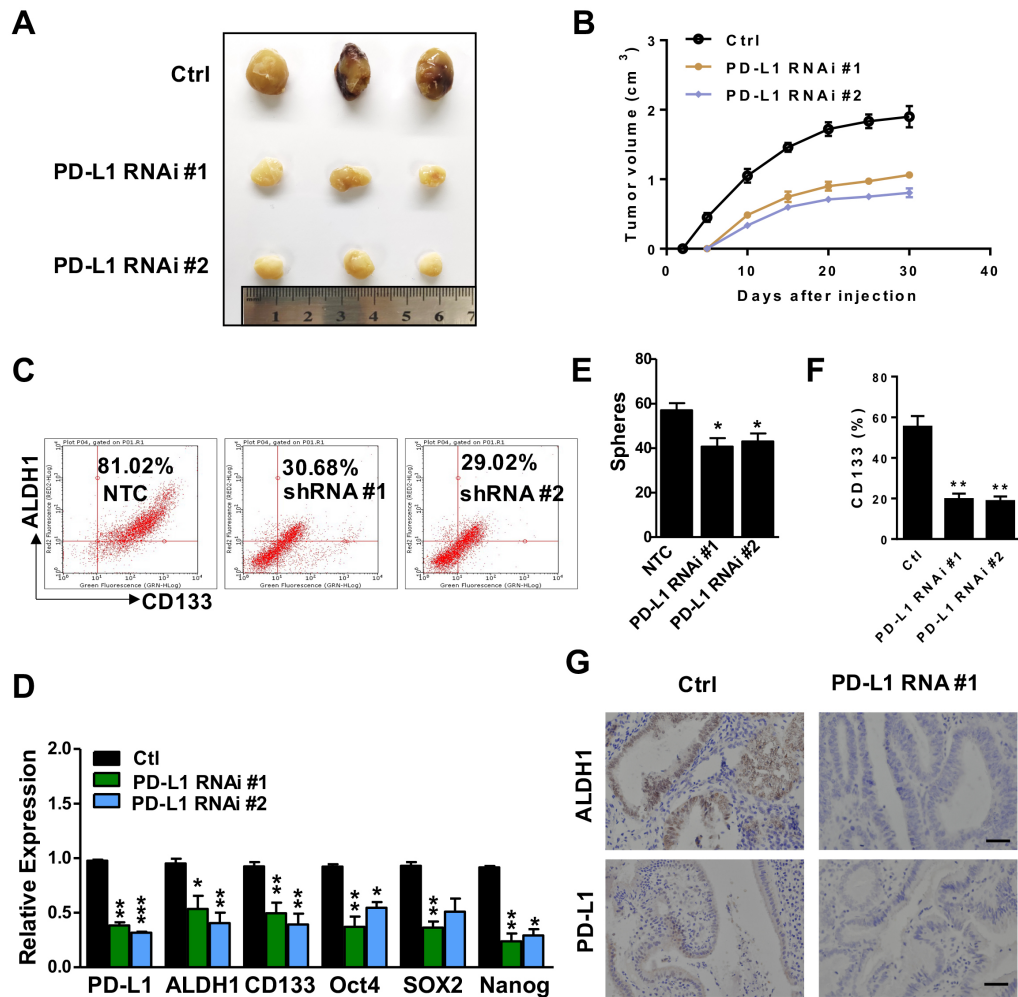
Fig. 3. PD-L1 induces an endometrial cancer stem-like state. Tumorigenicity of $ECSC^{isk}$ cells and PD-L1 knockdown $ECSC^{isk}$ cells with PD-L1 RNAi-Lentivirus for stable PD-L1 knockdown cell lines detected by morphology (A) xenografts volumes (B). (C) FACS analysis the percentage of ALDH1 and CD133 positive $ECSC^{isk}$ cells transfected with control and PD-L1 knockdown $ECSC^{isk}$ cells. (D) qRT-PCR was used to analysis the stemness genes (*CD133, OCT4, ALDH1, SOX2* and *NANOG*) in the tumors from the two groups analysised by. Percentage of spheres (E) and CD133-positive cells (F) in $ECSC^{isk}$ transfected with control and PD-L1 knockdown $ECSC^{isk}$ cells. (G) Immunostaining for ALDH1 and PD-L1 in tumors using Immunohistochemistry. Mean $\pm$ SEM (N = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

2.6 PD-L1 promoter reporter assay

For luciferase assay, HEK 293T cells were plated into a 24-well plate and transfected with reporter plasmid plus a Renilla luciferase plasmid as internal reference using Lipofectamine 2000 reagent in 21% O_2 or 2% O_2 condition. 24 h later, CoCl2, KC7F2 was added into the cells for additional 24 h separately and the cells were lysed and the luciferase activities were assessed measured using a dual Luciferase Reporter Assay System (N1610, Promega, Madison, WI, USA).

2.7 Sphere formation assay

Cells were trypsinizedinto single-cell suspensions, then seeded in six-well plates (3736, Corning) with 5000 cells per well. The cells were photographed 3 days later.

2.8 Orthotopic transplantation

Followed with the Tongji University Caring and Laboratory Animals using Guide, approved by the Ethics Review Committee and Animal Care. We randomly assigned each group blind. A tatal of 18 female BALB/c mice used each time, there were 3 groups for both NTC and ECSC cells, and six mice were randomly assigned to each group with three times repeat. A total of 1×10^4 NTC ECSCs or 1×10^4 PD-L1 knocking down ECSCs subclone cells were injected into the mammary fat pad of female nonobese diabetic/SCID/IL2Rγ-null (NSG) immunodeficient mice in PBS solution separately. At three weeks after injection, mice were examined for the presence of tumors, which were

harvested for analysis.

2.9 Statistical analysis

The mean $\pm$ SEM was reported for all values. Student's *t*-test was used to calculate *p* values. GraphPad Prism 6 software (Version 6, Shanghai, China) was used to analysis all the graphs.

3. Results

3.1 PD-L1 is crucial for pluripotency in ECSCs

We studied the expression of PD-L1/L2 in ECSCs from ECs by firstly obtaining ECSCs from an EC cell line and tumor samples using our previously described method [16]. We also enriched ECSCs from endometial cancer cell lines, the commonly reported stemness marker, CD133, was used to characterize the endometrial cancer stem cell (CSC). In this study, ECSCs isolated from ISK were investigated from day 1 to day 8, and we found that the expression of CD133 increased by FACS data (Fig. 1A). To further confirm the high expression of the PD-L1 in ECSC^{isk}, we used qPCR (Fig. 1B) and western blotting (Fig. 1C) to study the stemness marker ALDH1 in mRNA and protein level. As expected, morphological changes in ECSC^{isk} were observed and recorded (Fig. 1D), we found that ECSC^{isk} gradually developed a uniform shape when the expression of PD-L1 and ALDH1 was high. However, the EC cells appeared dispersed and flattened when the expression of PD-L1 and ALDH1 was low (Fig. 1D). In addition, immunohistochemical findings showed that ALDH1 and PD-L1 were increased in ECSC^{isk} (Fig. 1E) and ECSC^{isk} showed the highest expression of PD-L1 exhibited the most stem cell-like state.

3.2 The effect of PD-L1 on ECSC stemness

PD-L1 has been reported to have a regulatory effect on tumor stem cells, and therefore, this study investigated whether inhibition of PD-L1 signaling could influence ECSC phenotypes. We focused on PD-L1 signaling and its effects on ECSCs. PD-L1 RNA interference was used to elucidate the effect of PD-L1 on the state of the ECSCs and to further study the molecular mechanism of PD-L1 and its effect on the stem-like state of ECSCs. siRNAs were designed to specifically target PD-L1, and quantitative RT-PCR and western blots were used to confirm the effectiveness of the siRNAs (Fig. 2A and 2B). We found that PD-L1 was significantly knocked down after 3 days in ECSC, and these cells also lost their compact colonial morphology. However, the expression of a scrambled siRNA maintained ECSC-like morphology (Fig. 2C), whereas when PD-L1 was knocked down the ECSC cells lost their colonial morphology as well as a reduced expression of pluripotency-related genes (CD133, ALDH1, OCT4, SOX2 and NANOG) (Fig. 2D). Furthermore, cell proliferation and the presence of undifferentiation colonies were consistently decreased by knocking down PD-L1

(Fig. 2E & 2F). FACS analysis revealed that the scrambled siRNA maintained high levels of pluripotency-related markers, including ALDH1 and CD133, while the PD-L1 knockdown in ECSCs did not prevent the loss of these markers (Fig. 2G). The CD133 positivity was significantly reduced, and the percentage of CD133-positive ECSCs was highly correlated with results from PD-L1 knockdown in ECSC cells (Fig. 2H), along with a decrease in the number of spherical cells (Fig. 2I). Therefore, RNA interference of PD-L1 demonstrated that this protein is required for the stem-like state of ECSCs.

3.3 PD-L1 induces an ECSC stem-like state

Here, we found that PD-L1 promoted a stem-like state in ECSCs and to further reveal a role for PD-L1 in the modification of ECSCs, we identified the tumorigenicity of the stem cells using a mouse xenograft assay (Fig. 3A). By injecting mice with cells (1×10^4) derived from NTC (Negative Control) ECSCs and ECSCs with knocked down PD-L1 with PD-L1 RNAi-Lentivirus for stable PD-L1 knockdown cell lines, they developed palpable tumors, and in particularly the NTC ECSC subclones could easily form tumors but this was not so for the ECSCs with knocked down PD-L1. The tumors were visible in the mice injected with NTC ECSCs after 7 days, while the mice receiving ECSCs with knocked down PD-L1 had detectable tumors after 14 days (Fig. 3B). Notably, FACS assays showed a much stronger CD133 and ALDH1 expression in tumor tissues from NTC ECSCs when compared to tissues from ECSCs with knocked down PD-L1 (Fig. 3C). Furthermore, tumor tissues from ECSCs with knocked down PD-L1 also showed significantly decreased expression of several pluripotency markers (Fig. 3D). To determine the properties associated with stemness in ECSC injected tumor tissues, we isolated cells from NTC and PD-L1 knocked down ECSCs and the injected tumor tissues were cultured and subjected to sphere formation assays. The number of spheres from PD-L1 knocked down ECSC injected tumors significantly decreased when compared to NTC ECSCs (Fig. 3E). Furthermore, the percentage of CD133-positive ECSCs showed similar results and PD-L1 knocked down ECSCs significantly reduced the enrichment of CD133 positive cells (Fig. 3F) and decreased the percentage with weaker ALDH1 and PD-L1 immunostaining (Fig. 3G) when compared to NTC ECSCs. Therefore, tumorigenicity of cells in NOD/SCID mice characterized showed decreased stemness properties in PD-L1 knocked down ECSCs.

3.4 The expression of PD-L1 is regulated by hypoxia

We have found that PD-L1 promoted the stem-like state of ECSCs and to further explore the underlying molecular mechanism responsible for the regulation of PD-L1 expression during modification of ECSCs, we analyzed the PD-L1 promoter sequence. We constructed a truncated PD-
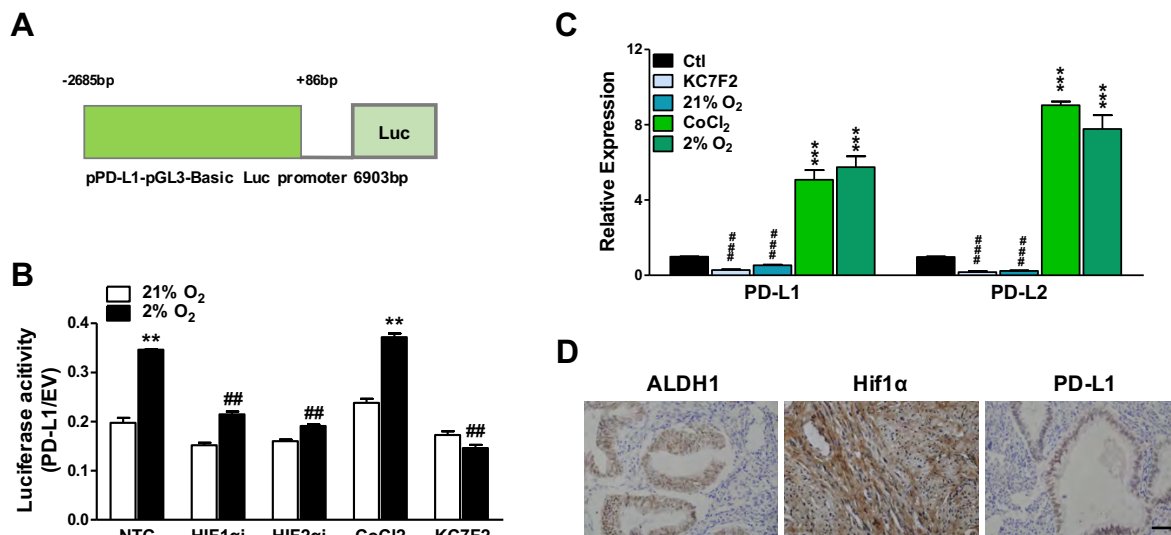
Fig. 4. The expression of PD-L1 is regulated by hypoxia. (A) A full-length plasmid with truncated PD-L1 and luciferase reporter containing the sites –2685/+86. (B) ECSC^{isk} were transfected with the PD-L1 promoter reporter plasmid for 24 h, and renilla plasmid was used as control. Cells were treated with a HIF signal inducer, CoCl$_2$, and the inhibitor KC7F2 under normoxic or hypoxic conditions for a further 24 h and then luciferase activities were measured. (C) qRT-PCR analysis of PD-L1 and PD-L2 genes in ECSC^{isk} cells treated with the HIF signal inducer, CoCl$_2$, and the inhibitor KC7F2 under normoxic or hypoxic conditions for a further 24 h and then luciferase activities were measured. (D) Immunostaining for ALDH1, HIF1α and PD-L1 in tumors by immunohistochemistry (IHC).

L1 promoter luciferase reporter, which contained the sites –2685/+86 which are related to hypoxia (Fig. 4A). Hypoxia has been reported to have a regulatory effect on ECSCs [16] and our luciferase analysis showed that the HIF signal inducer, CoCl$_2$, was able to significantly increase the transcription of full-length plasmids under hypoxic conditions, while addition of the inhibitor KC7F2 prevented the transcription of the full-length plasmid (Fig. 4B). In addition, the increased expression of PD-L1 was observed under both hypoxic conditions and by the addition of the HIF signaling inducer, COCl$_2$, but was decreased after HIF-1α or HIF-2α inhibition, or under normoxia (Fig. 4C). These effects of high expression of ALDH1, HIF-1α and PD-L1 were seen by immunostaining (Fig. 4D) and the results suggest that hypoxia increases PD-L1 levels which then bind to the PD-L1 promoter and increase the expression of PD-L1 further thus producing the ECSC phenotype.

Therefore, with the isolated ECSCs, we found that PD-L1 promoted a sustained stem-like state. Mechanically, HIF-1α and HIF-2α activated the PD-L1 promoter, thereby increasing PD-L1 expression. Additionally, PD-L1 knockdown inhibited ALDH1 expression and ECSC stemness, supporting the theory that HIF can activate the PD-L1 promoter, thus enhancing the expression of PD-L1 and the ECSC phenotype (Fig. 5). These data indicate that increased PD-L1 expression in ECSCs, can promote the ECSCs stem-like state and is regulated by hypoxia, thus representing a promising target for future PD1/PD-L1 based tumor immunotherapy.

4. Discussion

PD-1/PD-L1 is a critical member of the immunoglobulin superfamily of costimulatory molecules and participates in many immunomodulatory processes. Under normal conditions, PD1 is an inhibitory receptor expressed on activated T cells. When bound to its ligand, PD-L1, it plays an important immune regulatory role, by inhibiting the activation and proliferation of T cells, regulate the expression and secretion of cytokines, and can participate in the immune escape mechanism. Currently, many studies have shown that PD-L1 is highly expressed in tumors, and is closely related to clinical pathology and prognosis and has become a biological indicator for tumor detection and future prognoses. When PD-L1 is expressed, tumor cells bind to the PD1 receptor on T cells which can lead to the formation of an immunosuppressive tumor microenvironment, transduce negative regulatory signals, and lead to the induction of apoptosis and immunosuppression of tumor antigen-specific T cells. These effects enable tumor cells to escape immune monitoring and destruction. However, PD-1/PD-L1 inhibitors can reverse this tumor immunosuppression microenvironment.

PD-1/PD-L1 is regulated by a multi-level network, and the regulation of the expression of PD-L1 can be divided into five levels: genome level, transcriptional level, miRNA level, post-transcriptional level and protein level [17]. In many tumors, soluble factors, oncogenic signals, miRNAs, genetic variations and post-transcriptional modifications produced by immune cells are involved in regulating the expression of PD-L1 such as INF-γ [18], IFN-α and
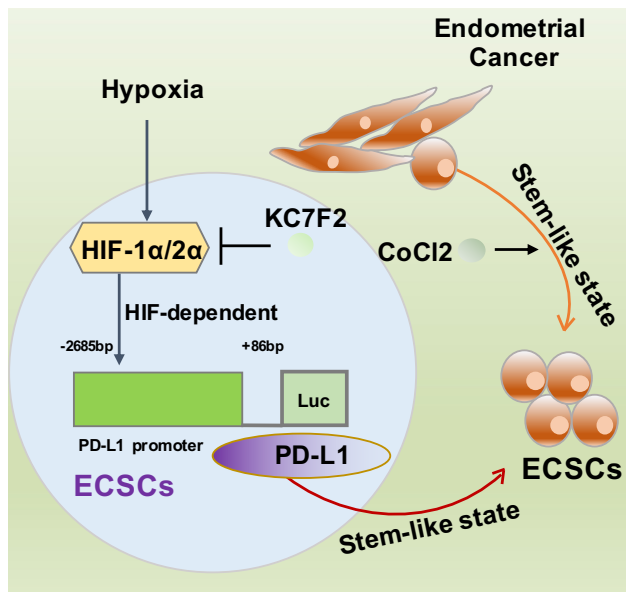
Fig. 5. Mechanism of HIF-dependent expression of PD-L1 in ECSCs. In ECSCs, the expression of PD-L1 is activated by HIF-1α and HIF-2α under hypoxic conditions, which facilitates the ECSC phenotype. Knockdown of PD-L1 prevents the ECSCs stem-like state and inhibits the tumorigenicity, which is reversed by a HIF activator, CoCl$_2$ or exposure to hypoxia. Moreover, knockdown of HIF-1α or HIF-2α reduces PD-L1 promoter activity, which can be reversed with CoCl$_2$ addition. Collectively, hypoxia promotes the expression of PD-L1 by inducing PD-L1 promoter activity, thus enhancing the expression of ALDH1 and the stemness of ECSCs.

IFN-β [19], TLR4s [20], TGF-β [21] and other soluble factors such as MYC [22], HIF1α [23], HIF2α [24], STAT3 [25], MUC1-C [26], CDK5 [27], PI3K-Akt-MTOR [28], and EGFR [29]. Hypoxia is an important marker for cancer, and principally HIF1α and HIF2α, are induced within the hypoxic microenvironment and are closely related to the degree of tumor malignancy. Hypoxia also plays a vitalt role in the activation and self-renewal of CSCs [30]. During tumor formation, HIFs are activated and consequently activate downstream signaling cascades and stimulate vascular endothelial growth factor (VEGF) generation and angiogenesis, thus causing epithelial-mesenchymal transition (EMT) and tumor metastasis. They also increase glycolytic pathways and improve survival and drug resistance under oxygen deficiency, nutrient deficiency, or metabolic abnormalities. It also promotes the activation of transcription factors and self-renewal of tumor stem cells. It has been reported that HIF1α and HIF2α act on the PD-L1 promoter region through the hypoxia response element (HRE) in various tumor cells to promote PD-L1 expression [24,31,32], leading to tumor immune escape.

The above preliminary data suggest that the increased expression of PD-L1 in ECSCs effectively maintains their self-renewal, and HIFs bind to the PD-L1 promoter region to participate in the regulation of PD-L1 expression, thus promoting self-renewal of cancer stem cells. Therefore, our research mainly focused on to explore the mode of action, sites of interaction and specific regulatory mechanisms of HIF1α in promoting PD-L1 in ECSCs, and then to determine the regulatory mechanism of HIF1α in enhancing the expression of PD-L1 and promoting self-renewal of ECSCs. Thus, ECSCs are promoted to self-renew and participate in the immune escape of tumor cells, making cancer stem cells more able to survive and exert drug resistance when compared to ordinary tumor cells. This research also provides sufficient evidence for the use of immunotherapy rather than chemotherapy or radiotherapy to better target the effective destruction of refractory tumor stem cells.

5. Conclusions

In conclusion, we found that the increased PD-L1 expression in the ECSCs was regulated in the hypoxic microenvironment as HIFs bound to the PD-L1 promoter region, thus resulted the self-renewal of ECSCs. This work represent a new target of PD-L1 in cancer stem cells and may benefit for further cancer research and clinical therapy.

Abbreviations

ECSCs: Endometrial cancer stem-like cells; HIFs: Hypoxia inducible factors; PD-L1/L2: Programmed cell death ligand 1/2; ALDH1: Aldehyde dehydrogenase 1.

Author contributions

GC, SY designed the study and write the draft. SY performed most of the experiments, with assistance from YG and XW conducted the scientific editing of language. HZ assisted with the data analyses. GC wrote the manuscript.

Ethics approval and consent to participate

This study was approved by the Investigation Ethical Committee of Shanghai First Maternity and Infant Hospital, Tongji university school of Medicine, in accordance with the ethical standards or comparable ethical standards. The ethics approval code is KS21289. Written informed consent was obtained from each participant after detailed explanations regarding the study objectives and procedures were provided.

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Conflict of interest

The authors declare no conflict of interest.

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