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Original Articles

PD-L1 promotes colorectal cancer stem cell expansion by activating HMGA1-dependent signaling pathways

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ABSTRACT

PD-L1 is critical for tumor cell escape from immune surveillance by inhibiting T cell function via the PD-1 receptor. Accumulating evidence demonstrates that anti-PD-L1 monoclonal antibodies might potently enhance antitumor effects in various tumors, but the effect of PD-L1 on colorectal cancer stem cells (CSCs) remains unclear. We observed high PD-L1 expression in $CD133^+CD44^+$ colorectal CSCs and CSC-enriched tumorspheres. Altering PD-L1 expression promoted colorectal CSC self-renewal by increasing the expression of stemness genes, the $CD133^+CD44^+$ cell population sizes and the ability to form tumorspheres. Additionally, PD-L1 expression was markedly increased in chemoresistant colorectal cancer (CRC) cells in vitro and in vivo. More importantly, PD-L1 enhanced CRC cell tumorigenicity in nude mice; the inoculation of 1×10^4 cells resulted in high tumor formation efficiency. Mechanistically, PD-L1 directly interacted with HMGA1, and HMGA1 upregulation by PD-L1 activated HMGA1-dependent pathways, including the PI3K/Akt and MEK/ERK pathways, and promoted CSC expansion. HMGA1 downregulation rescued the PD-L1-induced phenotypes, highlighting the role of HMGA1 in PD-L1-mediated colorectal CSC self-renewal. Moreover, PD-L1 expression was correlated with the expression of CSC markers and HMGA1 in clinical CRC specimens. Thus, PD-L1 could crucially contribute to the maintenance of CSC self-renewal by activating HMGA1-dependent signaling pathways.

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide [1]. Although surgical resection is an efficient method for CRC therapy, cancer recurrence and distant metastases remain major problems in CRC treatment [2,3]. Due to their acquisition of normal stem cell traits, such as self-renewal, multipotency and a limitless proliferation potential [4,5], cancer stem cells (CSCs) are currently considered responsible for the occurrence, progression and recurrence of all types of tumors [6–8]. Thus, the identification of specific molecules that target CSCs may be an effective approach for eradicating CRC.

The effect of the immune system on eliminating tumor cells was recently recognized after the great success of blocking PD-L1, a T cell inhibitor expressed on T cells, B cells, and natural killer T cells that is considered one of the most significant members of the B7 family [9,10]. The binding of PD-L1 with PD-1 delivers the effective inhibitory signal that negatively regulates T cell or other immune cell functions and is critical for maintaining the homeostasis of the immune system. PD-L1 was recently found to be upregulated in a variety of cancer cells, which results in the transmission of inhibitory signals and thereby in immunosuppression via a PD-1 − PD-L1-mediated interaction between tumor cells and T cells [11,12]. Nevertheless, aside from its known effect on the immunological response, PD-L1 expression plays an inherent role in tumor cells themselves, where it works as a “molecular shield” to protect cancer cells from cytolysis [13]. Nevertheless, the mechanism through which PD-L1 exerts this effect on colorectal CSCs remains largely unknown.

In this study, we investigated the role of PD-L1 in regulating CSC properties and further clarified that PD-L1 maintains colorectal CSCs by activating HMGA1-dependent signaling pathways.

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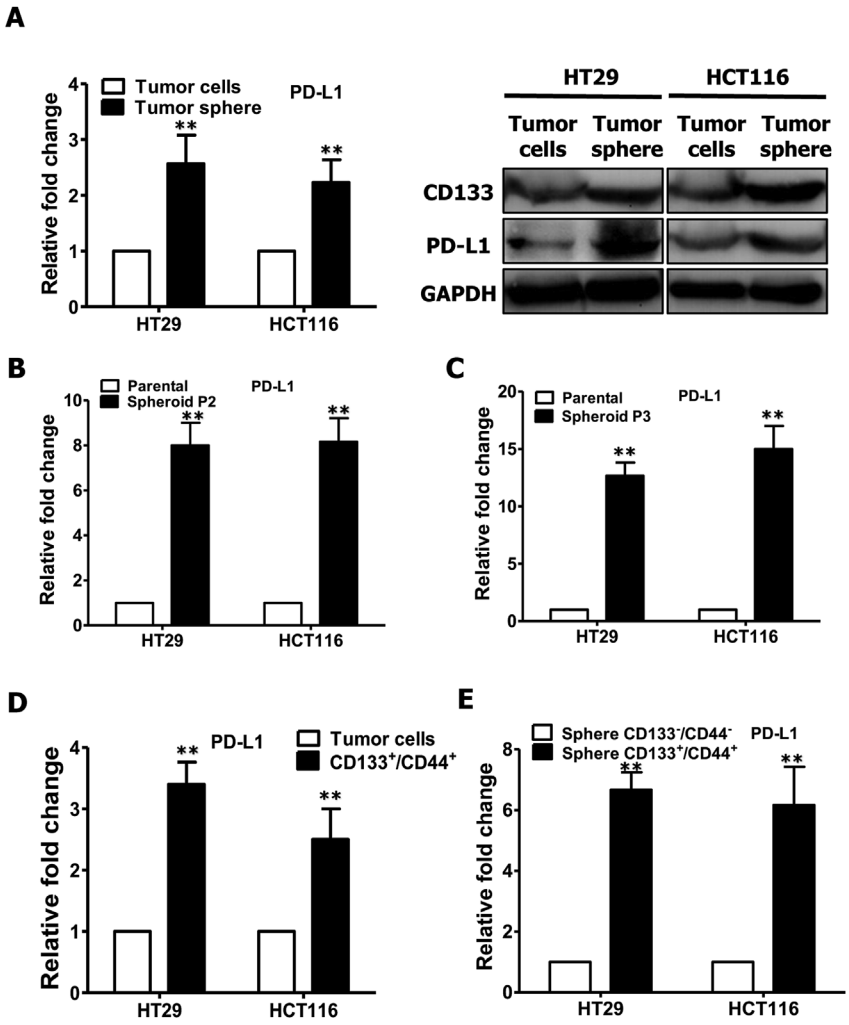

Fig. 1. PD-L1 levels are elevated in colorectal CSCs.
(A) The PD-L1 expression levels in tumorspheres and tumor-attached CRC cells were compared by qRT-PCR and Western blotting.
(B) HT29 and HCT116 cell-derived spheroids were trypsinized and cultured under ultralow-attachment conditions for the second passage to enrich colorectal CSCs. The PD-L1 expression levels in the primary spheroids and the passage 2 spheres were compared by qRT-PCR.
(C) HT29 and HCT116 cell-derived tumor spheres were trypsinized and cultured under ultralow-attachment environments for the third passage to enrich for colorectal CSCs. The PD-L1 expression levels in the primary spheroids and the passage 3 spheroids were compared by real-time PCR.
(D) The PD-L1 expression levels in $CD133^+CD44^+$ CRC cells and tumor-attached CRC cells were compared by qRT-PCR.
(E) The PD-L1 expression level in $CD133^+CD44^+$CRC cells sorted from trypsinized spheroids was compared with that in $CD133^-CD44^-$ cells.

2. Materials and methods

2.1. Colorectal cell lines

HT29 and HCT116 CRC cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and were maintained at 37 °C with 5% CO_2.

2.2. Establishment of drug-resistant HCT116 cells

To obtain drug-resistant cells, HCT116 cells were exposed to increasing concentrations of 5-fluorouracil (5-FU) ranging from 10 to 80 μM or cisplatin ranging from 10 to 80 μg/ml in complete medium. Briefly, HCT116 cells were cultured in 60-mm culture plates for 24 h, and after 10 μM 5-FU or 10 μg/ml cisplatin was added to the medium, and the cells were further incubated for 48 h. The medium was then replaced with drug-free fresh medium, and the cells were incubated until they reached 90% confluence. Subsequently, the cells were re-plated and re-exposed to a double dose of the drug. This process was repeated until the cells exhibited resistance to 80 μM 5-FU or 80 μg/ml cisplatin. The living cells after exposure to increasing concentrations of 5-FU and cisplatin for about 2 months were collected, termed drug-resistant cells and used for subsequent experiments.

2.3. Clinical samples

Primary CRC tissues and adjacent normal tissues were collected from Guangzhou First People's Hospital, Guangdong, China. Written informed consent from all the subjects and approval from the Ethics Committee of Guangzhou First People's Hospital were obtained for the use of human biological materials for research purposes. In all, 20 CRC and 20 adjacent normal specimens were used for qRT-PCR analysis, and a cohort of 100 CRC specimens was collected for analysis of the association of PD-L1 expression with the expression of stem cell markers and HMGA1.

2.4. Tumor sphere formation assay

CRC cells were harvested and counted, and 5000 cells in serum-free DMEM-F12 (Gibco) containing EGF (20 ng/ml, PeproTech), b-FGF (10 ng/ml, PeproTech), and B27 (1:50 dilution, BD Biosciences) were plated in each well of six-well ultralow attachment plates (Corning, NY, USA). After one week, the formed tumor spheres were counted under a phase-contrast microscope.

2.5. Determination of $CD133^+CD44^+$ cell percentages by flow cytometry

The $CD133^+CD44^+$ cell ratio was analyzed by fluorescence-activated cell sorting (FACS) according to the manufacturer's recommended protocol (MACS Miltenyi Biotec). The cells were washed and re-suspended in phosphate-buffered saline (PBS). Subsequently, 10 μl of anti-CD133/1 (AC133)-phycoerythrin (PE) antibody and 10 μl of anti-CD44-fluorescein isothiocyanate (FITC) antibody were added to 100 μl of cell suspension, and the mixture was mixed well and incubated for

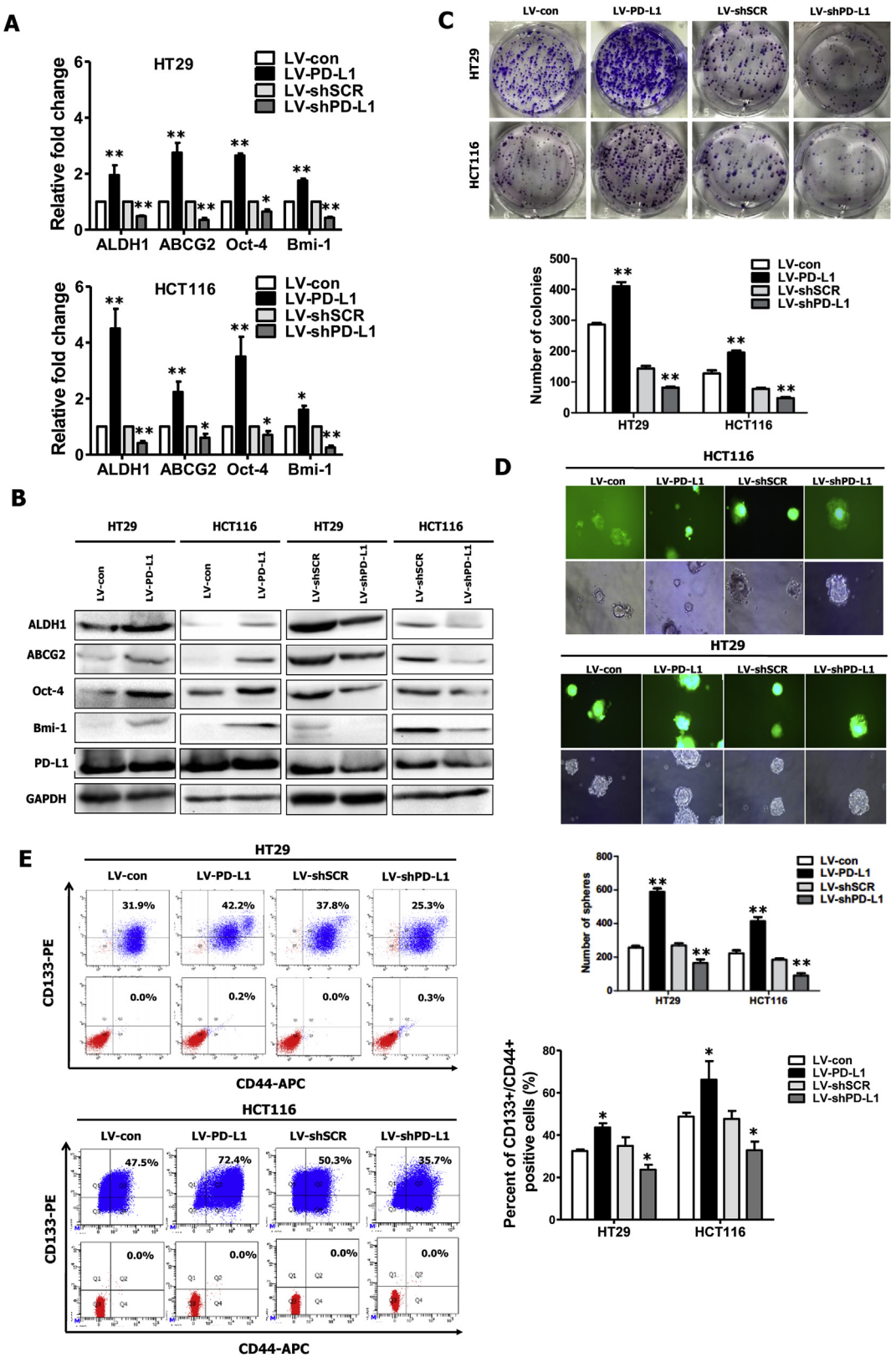

Fig. 2. PD-L1 overexpression increases colorectal CRC self-renewal.

(A) The mRNA levels of ALDH1, ABCG2, Bmi-1, and Oct4 in PD-L1-expressing and shPD-L1-expressing CRC cells were determined by qRT-PCR.

(B) The protein levels of ALDH1, ABCG2, Bmi-1 and Oct4 in PD-L1-expressing and shPD-L1-expressing CRC cells were determined via Western blot analysis.

(C) PD-L1 promotes CRC cell proliferation. The colony sizes are shown in the left panels, and the colony numbers are shown in the right panels.

(D) PD-L1 promotes CRC cell tumor sphere formation. The tumor sphere sizes are shown in the left panels, and the tumor sphere numbers are shown in the right panels.

(E) PD-L1 increases the percentages of $CD133^{+}CD44^{+}$ cells in the PD-L1- and shPD-L1-expressing CRC cell populations.

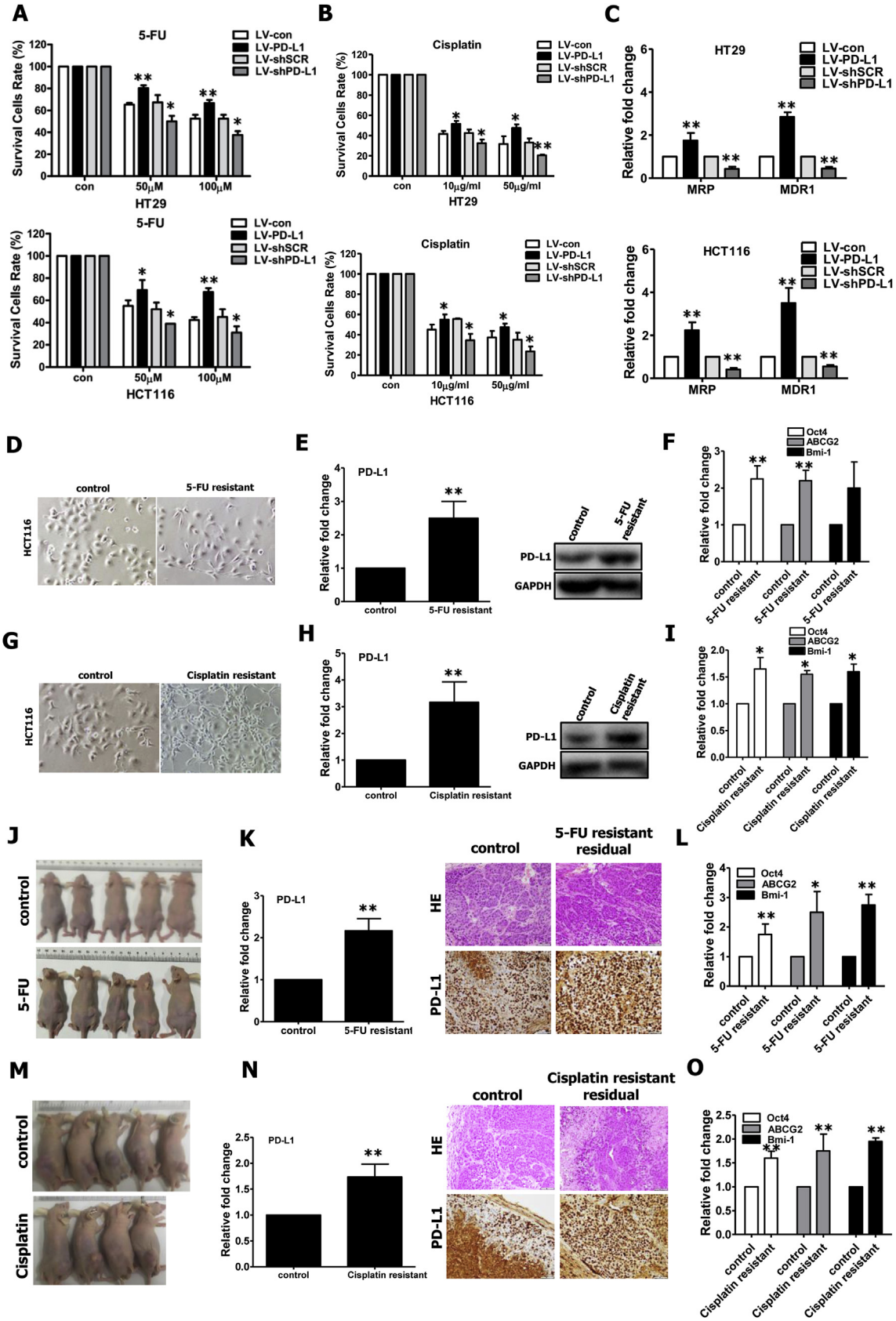

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Fig. 3. PD-L1 expression is associated with the chemoresistance of CRC.
(A) PD-L1- and shPD-L1-expressing CRC cells were treated with 50 or 100 μM 5-FU for 48 h, and the cell survival percentages are shown.
(B) PD-L1- and shPD-L1-expressing CRC cells were treated with 10 or 50 μg/ml cisplatin for 48 h, and the cell survival percentages are demonstrated.
(C) The mRNA levels of drug resistance-related genes were detected by RT-PCR.
(D) Morphology of 5-FU-resistant HCT116 cells established in vitro through continuous exposure to 5-FU (200 ×).
(E) The expression of PD-L1 in the 5-FU-resistant HCT116 cells was assessed via qRT-PCR and Western blotting.
(F) The expression levels of stemness genes (Oct4, ABCG2 and Bmi-1) in the 5-FU-resistant HCT116 cells were assessed via qRT-PCR.
(G) Morphology of cisplatin-resistant HCT116 cells established in vitro through continuous exposure to 5-FU (200 ×).
(H) The expression of PD-L1 in the cisplatin-resistant HCT116 cells was assessed via qRT-PCR and Western blotting.
(I) The expression levels of stemness genes (Oct4, ABCG2 and Bmi-1) in the cisplatin-resistant HCT116 cells were assessed via qRT-PCR.
(J) A total of 1×10^6 HCT116 cells were subcutaneously inoculated into nude mice. HCT116 cell-derived xenograft tumors were cut into pieces with a volume of 0.1 cm^3 and subcutaneously inoculated into 10 nude mice. The mice were administered five intratumoral injections of 2 mg/kg 5-FU or PBS every other day. The residual tumor tissues enriched in 5-FU-resistant cells were resected for subsequent experiments.
(K) The expression of PD-L1 in the 5-FU-resistant xenograft tumors was assessed via qRT-PCR and IHC.
(L) The expression levels of stemness genes (Oct4, ABCG2 and Bmi-1) in the 5-FU-resistant xenograft tumors were assessed via qRT-PCR.
(M) HCT116 cell-derived xenograft tumors were inoculated subcutaneously into 10 nude mice. The mice were administered five intratumoral injections of 2 mg/kg cisplatin or PBS every other day. The residual tumor tissues enriched in cisplatin-resistant cells were resected for subsequent experiments.
(N) The expression of PD-L1 in the cisplatin-resistant xenograft tumors was assessed via qRT-PCR and IHC·
(O) The expression levels of stemness genes (Oct4, ABCG2 and Bmi-1) in the cisplatin-resistant xenograft tumors were assessed via qRT-PCR.

15 min in the dark at 4 °C. After two washes with PBS, the cell pellet was resuspended in 400–500 μl of PBS and maintained on ice until flow cytometric analysis.

2.6. Sorting of $CD133^+CD44^+$ CRC cells by flow cytometry

CRC cells (HCT116 and HT29) were labeled with the CD44-FITC and CD133-PE antibodies as described above. The $CD133^+CD44^+$ cells and non-$CD133^+CD44^+$ cells were sorted by flow cytometry for tumor sphere formation assays.

2.7. qRT-PCR

The total RNA from CRC cells was extracted with TRIzol reagent (TaKaRa), and cDNA was synthesized using a PrimeScript RT reagent kit (TaKaRa) following the manufacturer's recommended protocol. mRNA expression levels were analyzed by qRT-PCR using SYBR Green Premix ExTaq (TaKaRa). The primers used in the qRT-PCR assay are listed in Supplementary Table S3. GAPDH was utilized for the normalization of mRNA expression. The fold changes were estimated using the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot analysis and antibodies

Cells were collected and lysed in RIPA containing protease and phosphatase inhibitors. Protein lysates were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). The membranes were first probed with the indicated primary antibodies listed in Supplementary Table S4 and then with an HRP-labeled secondary antibody. The hybridization signal bands were visualized using enhanced chemiluminescence (CWBIO Technology). GAPDH was used as the protein loading control.

2.9. Mass spectrometry analysis

As described previously, the tryptic peptides were measured and analyzed using a nanoUPLC-ESILTQ Orbitrap mass spectrometer [14]. The mass spectrometer was operated following the manufacturer's recommended protocol. Only he proteins identified by unique peptides were considered, and the proteins identified by the same set of peptides were grouped.

2.10. Immunoprecipitation (IP) analysis

Immunoprecipitates were obtained by co-immunoprecipitation (Co-IP) according to the manufacturer's instructions. Using this approach allows the isolation of protein complexes from cell lysates using purified antibodies on an agarose support. After washing and elution, the immunoprecipitates were subjected to Western blot assays.

2.11. GST-pull down assay

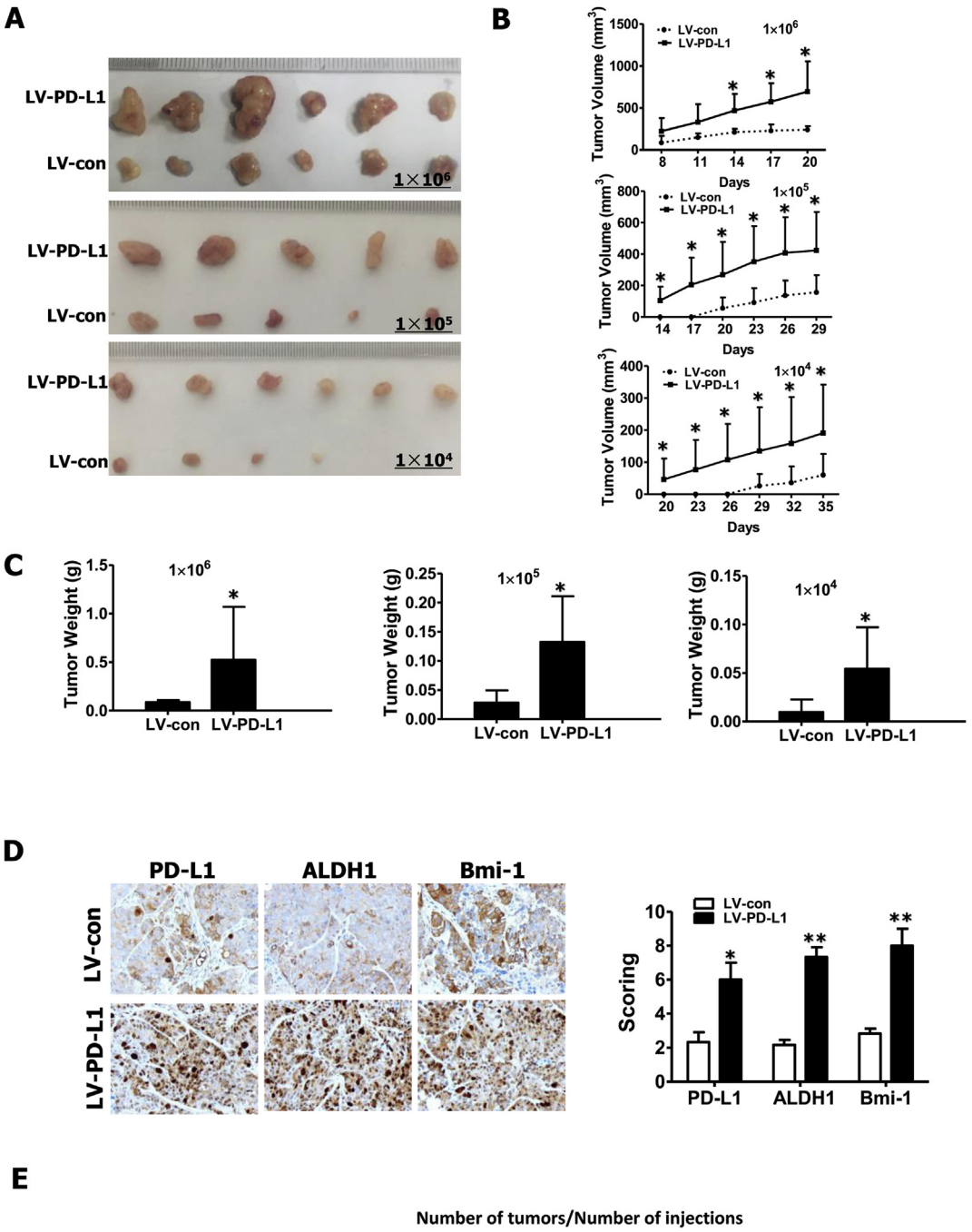
For pull-down assay, 300 μg HIS-HMGA1 protein,200 μl immobilized GST-tag purification resin and 100 μg GST-PD-L1 protein were added to 1000 μl of pull down buffer (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 1% protease inhibitor cocktail [pH 8.0]), then incubated at 4 °C for 16 h. As negative control, 300 μg HIS-HMGA1 protein was incubated with 200 μl immobilized GST-tag purification resin and 100 μg GST protein. Resin were washed three times with the pull-down buffer. Retained proteins were released by adding 2 × loading buffer and boiled for 5 min at 95 °C, then resolved by SDS-PAGE and subsequently subjected to Western blot analysis.

2.12. Lentiviral plasmids

The pLV-PD-L1-puro-EGFP, pLV-shPD-L1-puro-EGFP, pLV-HMGA1-puro-EGFP and pLV-shHMGA1-puro-EGFP plasmids were purchased from GeneCopoeia, and the lentiviral packaging plasmids pMD2.G and psPAX2 were provided by Prof. Dong Xiao (Cancer Research Institute, Southern Medical University, China). The scramble sequence was 5′-GCTTCGCGCCGTAGTCTTA-3'. The shRNAs targeting PD-L1 sequences were as follows: 5′-CCCATTAATACTCTGGTTGAC-3' (shRNA-1); 5′-CCTGTTGTGATAACCACTATT-3' (shRNA-2); and 5′-GCCTTTGC CATATAATCTAAT-3' (shRNA-3). The shRNAs targeting HMGA1 sequences were as follows: 5′-CAACTCCAGGAAGGAAACCAA-3′ (shRNA-1); and 5′-CCTTGGCCTCCAAGCAGGAAA-3′ (shRNA-2). For gene knockdown, the shRNA oligonucleotides were ligated into the lentivirus vector (pU6–EGFP-IRES-Puro), and lentiviral packaging and infection were performed. For gene overexpression, the lentivirus plasmid vector pCMV-ORF-EGFP-IRES-Puro was used for infection.

2.13. Lentivirus production and transduction

To establish stable CRC cell lines, recombinant lentiviruses (pLV-PD-L1-puro-EGFP, pLV-shPD-L1-puro-EGFP, pLV-HMGA1-puro-EGFP and pLV-shHMGA1-puro-EGFP) were established as previously described [15] and were transduced into HCT116 and HT29 cells for 72 h. Virus-infected CRC cells were then selected with puromycin at a concentration of 4 μg/ml and subjected to a green fluorescent protein assay with an inverted fluorescence microscope (Nikon, Japan).



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		Number of tumors/Number of injections									
		Days after injection									
Cell No.	Name	8	11	14	17	20	23	26	29	32	35
1×10⁶	LV-con	4/6	6/6	6/6	6/6	6/6	-	-	-	-	-
	LV-PD-L1	6/6	6/6	6/6	6/6	6/6	-	-	-	-	-
1×10⁵	LV-con					3/6	4/6	5/6	5/6	-	-
	LV-PD-L1			4/6	5/6	5/6	5/6	5/6	5/6	-	-
1×10⁴	LV-con								3/8	3/8	4/8
	LV-PD-L1					3/8	4/8	5/8	5/8	5/8	6/8

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Fig. 4. PD-L1 promotes the tumorigenesis of HCT116 cells in vivo.
(A) For the in vivo limiting dilution assay, PD-L1- and vector-expressing control cells were injected subcutaneously into nude mice. A representative image is shown.
(B) Tumor growth curves of tumors in nude mice injected with HCT116 cells expressing PD-L1 (LV-PD-L1) or vector control (LV-con). Once the tumors became palpable, the PD-L1-expressing tumors grew at a higher rate than the vector-expressing control tumors in all mice. Significant differences were obtained among the injections of 10^6, 10^5 and 10^4 cells.
(C) Weights of tumors from nude mice injected with HCT116 cells expressing PD-L1 (LV-PD-L1) or vector control (LV-con).
(D) The expression levels of PD-L1, ALDH1 and Bmi-1 in PD-L1-expressing or control tumors were determined via IHC.
(E) Tumors of PD-L1-expressing cells formed at an earlier time point and developed at a faster rate than those of control cells. The formation of tumors was checked for 20–35 days after the injection.

2.14. Tumor xenograft experiments in nude mice

The animal experiments were approved by the Ethics Committee for Animal Experiments of South China University of Technology. All nude mice were purchased from the Medical Laboratory Animal Center of Guangdong Province. To evaluate tumor growth, PD-L1- or shPD-L1-expressing HCT116 cells were resuspended at different concentrations in a mixture of PBS and Matrigel (1:1) and were subcutaneously injected into the left (control group) and right (experimental group) dorsal thighs of each mouse. The tumor growth was detected regularly, and the volume was calculated using formula V = $(L \times l^2)/2$, where L and l are the long and short diameters of the tumor, respectively. All the mice were sacrificed when they reached the ethical endpoint, and the tumor tissues were further processed for histological analysis.

2.15. IHC staining and antibodies

To further evaluate the association of PD-L1 with CSC-related markers and HMGA1, we performed IHC analysis using the routine protocols. The primary antibodies used for IHC are listed in Supplementary Table S5. The staining intensity was scored as negative (0), weak (1), moderate (2) or strong (3). The staining extent was scored as 1 (< =10%), 2 (11–50%), 3 (51–75%) or 4 (> 75%). A total expression score was calculated by multiplying the score of the staining intensity with that of the staining extent.

2.16. Statistical analysis

The statistical analyses were performed using the SPSS 17.0 software package and GraphPad 5.0. A two-tailed Student's *t*-test was used for comparisons between two independent groups. The mixed model analysis of variance (two-way ANOVA) was employed for the evaluation of in vivo tumorigenesis. The data are presented as the means ± SDs. $*P < 0.05$ and $**P < 0.01$ were defined as indicating statistical significance.

3. Results

3.1. PD-L1 is highly expressed in colorectal CSCs

In view of the close relationship between colorectal CSCs and chemoresistance, we examined the expression of PD-L1 in colorectal CSCs. As shown in Fig. 1A, PD-L1 expression was significantly higher in CSC-enriched tumorspheres than in monolayer-cultured cells, and PD-L1 expression was further elevated in passaged spheroids (Fig. 1B and C). Compared with non-$CD133^+CD44^+$ cells, CRC cells that were either $CD133^+$ or $CD44^+$, which are considered colorectal CSCs, exhibited enhanced PD-L1 expression (Fig. 1D). Consistently, $CD133^+CD44^+$ CSCs sorted from trypsinized spheroids of CRC cells displayed even higher PD-L1 levels than did $CD133^-CD44^-$ CSCs (Fig. 1E). Thus, we concluded that PD-L1 is highly expressed in colorectal CSCs.

3.2. PD-L1 promotes the expansion of colorectal CSCs

To explore the role of PD-L1 in the regulation of colorectal CSCs, PD-L1- and shPD-L1-expressing stably transfected CRC cells and their corresponding control CRC cells were utilized. CSC-associated genes (ALDH1, Bmi-1, ABCG2 and Oct4) have frequently been utilized to recognize CSCs in clinical tissues and many cancer cell lines. To assess the role of altering PD-L1 expression in the expression of stemness-related genes in CRC cells, cancer stem cell markers were detected by qRT-PCR and Western blotting. As indicated in Fig. 2A and B, compared with control cells, PD-L1-overexpressing and PD-L1-depleted cells showed substantially increased and reduced expression of stemness-associated genes (i.e., Oct4, ABCG2, ALDH1 and Bmi-1), respectively. These findings indicate that PD-L1 induces alterations in the levels of stem cell markers.

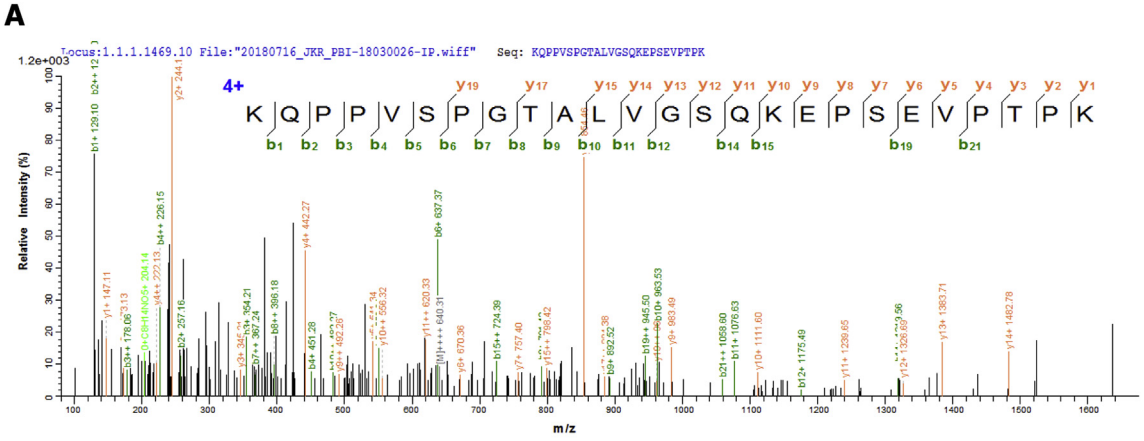
The colony formation assay revealed significantly higher and lower numbers of colonies in PD-L1-expressing and shPD-L1-expressing CRC cells, respectively, compared with the numbers in empty vector-expressing cells (Fig. 2C). Previous studies reported that CSCs can form tumor spheroids in a serum-free, non-adhesive environment and can even be enriched under these conditions [16]. Therefore, we examined the capacity of PD-L1- and shPD-L1-expressing stably transfected CRC cells to form tumor spheroids through an in vitro tumorsphere formation assay. As shown in Fig. 2D, PD-L1-overexpressing cells formed more spheres than did the vector control cells, as expected, and the opposite result was observed for shPD-L1-overexpressing cells. $CD133^+CD44^+$ cells have been reported to exhibit CSC characteristics. As shown in Fig. 2E, PD-L1 and shPD-L1 overexpression increased and reduced the percentages of CRC $CD133^+CD44^+$cells, respectively. Therefore, we concluded that PD-L1 can promote the self-renewal capacity of colorectal CSCs and can thereby enhance the size of these populations.

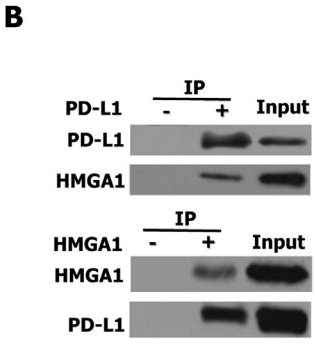
3.3. PD-L1 expression is correlated with chemoresistance

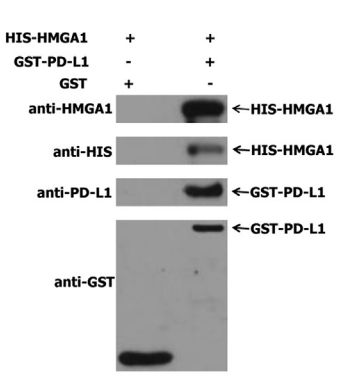
To explore the relationship between PD-L1 expression and chemoresistance, we first tested whether PD-L1- and shPD-L1-expressing CRC cells have different sensitivities to 5-FU and cisplatin. The data demonstrated that PD-L1-expressing and shPD-L1-expressing cells were more resistant and more sensitive to chemical agents than were vector-expressing cells, respectively (Fig. 3A and B). Moreover, multiple drug-resistance genes, such as those encoding multidrug resistance protein 1 (MDR1) and multidrug protein (MRP), were preferentially expressed in PD-L1-expressing cells compared with vector cells; in contrast, the expression levels of these genes were lower in shPD-L1-expressing cells than in vector cells (Fig. 3C).

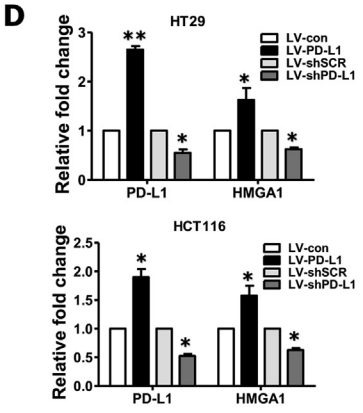
To further determine whether PD-L1 expression is upregulated in drug-resistant cell lines, we developed 5-FU- or cisplatin-resistant cells derived from HCT116 cells in vitro. As shown in Fig. 3D and G, 5-FU- or cisplatin-resistant cells exhibited an elongated spindle shape, whereas the control cells showed a rounded appearance. Interestingly, PD-L1 expression was found to be upregulated in 5-FU- or cisplatin-resistant cells by qRT-PCR and Western blotting (Fig. 3E, H). In addition, the mRNA expression levels of stemness-related genes (Bmi-1, ABCG2 and Oct4) were also higher in 5-FU- or cisplatin-resistant cells than in the control cells (Fig. 3F, I). These results might indicate that PD-L1 is upregulated in 5-FU- or cisplatin-resistant cells endowed with CSC characteristics.

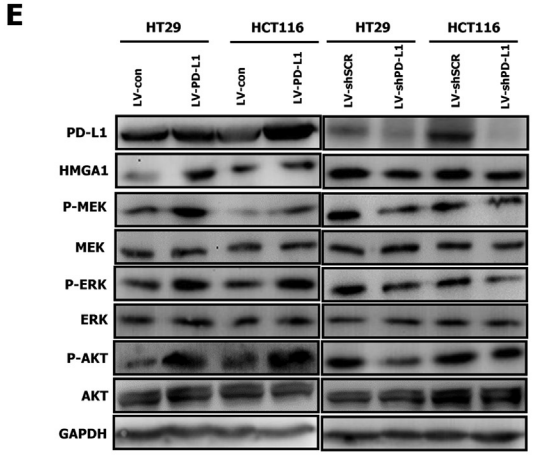
For the in vivo experiments, CRC xenografts resistant to 5-FU were established as shown in Fig. 3J. PD-L1 expression was significantly

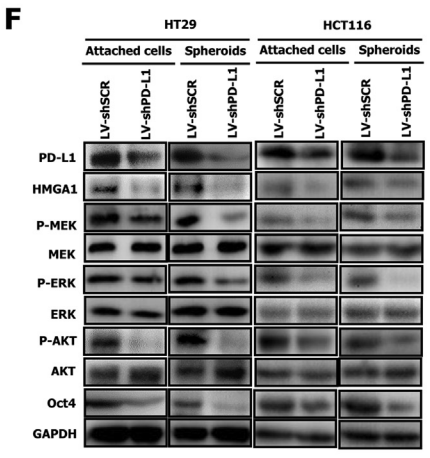












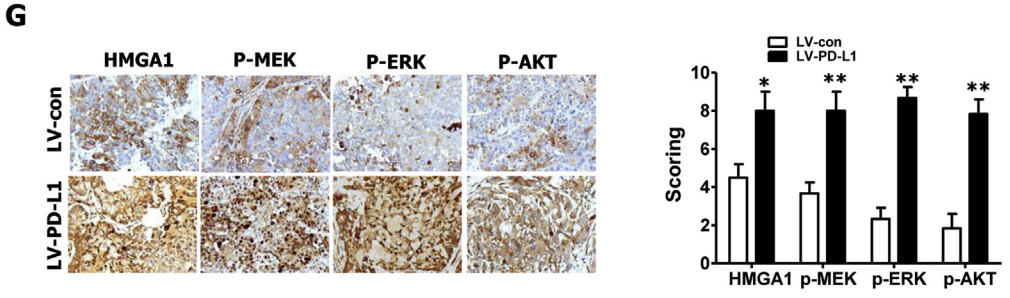


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Fig. 5. PD-L1 interacts with HMGA1 and activates HMGA1-dependent signaling pathways.
(A) Representative MS/MS spectra of the peptides from HMGA1.
(B) The immunoprecipitates of PD-L1 were purified using anti-PD-L1 antibody and were separated by SDS-PAGE. The presence of HMGA1 was analyzed by Western blotting. The immunoprecipitates of HMGA1 were purified using anti-HMGA1 antibody and were separated by SDS-PAGE. The presence of PD-L1 was analyzed by Western blotting. Normal IgG was used as the negative control.
(C) Recombinant GST or GST-PD-L1 fusion proteins purified by glutathione-Sepharose were incubated with HIS-HMGA1-transfected cell lysates, and the bound proteins were analyzed by Western blotting with anti-HMGA1, anti-HIS, anti-PD-L1 and anti-GST antibodies.
(D) The expression levels of PD-L1 and HMGA1 in CRC cells transfected with different lentivirus plasmids were determined by qRT-PCR.
(E) The protein levels of HMGA1 and related signaling pathway proteins in CRC cells transfected with different lentivirus plasmids were determined by Western blotting.
(F) The protein levels of HMGA1, Oct4 and related signaling pathway proteins in attached and spheroid CRC cells (LV-shPD-L1 versus LV-shSCR) were determined by Western blotting.
(G) The upregulation of PD-L1 increased the expression levels of HMGA1, p-AKT, p-MEK and p-ERK1/2 in tumor sections, as revealed by immunohistochemical staining (HCT116-PD-L1 versus HCT116-con).

higher in the 5-FU-resistant CRC residual cells than in the control cells, as demonstrated by qRT-PCR and IHC (Fig. 3K), and the stemness-related gene (Oct4, ABCG2 and Bmi-1) expression levels were also increased in the 5-FU-resistant CRC residual cells (Fig. 3L). Similarly, CRC xenografts resistant to cisplatin were established using the same protocol (Fig. 3M). Interestingly, enhanced PD-L1 and stemness-related gene (Oct4, ABCG2 and Bmi-1) expression levels were detected in the cisplatin-resistant CRC residual cells (Figure 3N, O), indicating that PD-L1 expression was related to chemoresistance. Collectively, these data revealed a correlation between PD-L1 expression and chemoresistance.

3.4. PD-L1 increases the number of tumor-initiating cells in vivo

As indicated above, PD-L1 promoted the stemness of CRC cells in vitro. We then evaluated the effects of PD-L1 on the tumorigenicity of CRC cells in nude mice using a limiting dilution assay. The data presented in Fig. 4A and E shows that the injection of 1×10^6, 1×10^5 and 1×10^4 PD-L1-expressing HCT116 cells into nude mice resulted in tumor development in 100% (6/6), 83.3% (5/6) and 75% (6/8) of the mice, respectively, whereas the injection of the same numbers of control cells led to tumor development in 100% (6/6), 83.3% (5/6) and 50% (4/8) of the mice, respectively. As shown in Fig. 4B and E, the growth rates of the PD-L1-overexpressing tumors were substantially higher than those of the control tumors. The injection of 1×10^6 PD-L1-expressing or control cells led to the formation of tumors that appeared at the same time; however, as the number of injected cells was reduced to 1×10^5 or 1×10^4, the first palpable tumor in the PD-L1-overexpressing group appeared 6 or 9 days earlier, respectively, than the first palpable tumor in the control group. After 20–35 days, the mice were sacrificed, and the tumors were weighed (Fig. 4C). The data demonstrated that the tumors formed with PD-L1-expressing CRC cells were heavier than those formed with the vector control cells, and the expression levels of PD-L1, ALDH1 and Bmi-1 in the PD-L1-expressing tumors were higher than those in the control tumors (Fig. 4D). Therefore, PD-L1 promotes the formation of tumor-initiating cells in CRC.

Furthermore, we assessed the effects of PD-L1 on the tumorigenicity of CRC cells in nude mice using HCT116 cells in which endogenous PD-L1 was silenced (Fig. S1). Our results showed that shPD-L1 delays the formation of tumor-initiating cells in CRC (Fig. S1).

3.5. PD-L1 interacts with HMGA1 and activates HMGA1-dependent signaling pathways

To gain insight into the mechanism through which PD-L1 maintains the stemness of colorectal CSCs, we used mass spectrometry to identify a possible protein with which PD-L1 directly interacts. A total of 19 differentially expressed proteins were identified between the IP sample and the IgG sample (Fig. S2 and Table S1). Following a literature search, HMGA1, a protein associated with CSC self-renewal, was selected as a candidate PD-L1-interacting protein. HMGA1 was detected from the PD-L1 immunoprecipitate with unique peptides, and the representative mass spectra are shown in Fig. 5A. As shown in Fig. 5B and Fig. S3, the coimmunoprecipitation experiment revealed that HMGA1 was pulled down by PD-L1. Moreover, PD-L1 was also detected by the immunoprecipitation of HMGA1. We also performed a GST pull-down assay, and the capacity of the GST-PD-L1 affinity resin to specifically retain in vitro-translated HIS-labeled HMGA1 was analyzed. As shown in Fig. 5C and Fig. S4, HMGA1 was retained by GST-PD-L1 but not GST alone, validating a reciprocal direct interaction between PD-L1 and HMGA1. To further validate whether PD-L1 can regulate HMGA1 and its downstream signaling pathways, we compared HMGA1 expression among PD-L1- and shPD-L1-expressing cells and the corresponding control cells. qRT-PCR and Western blotting assays revealed that PD-L1 overexpression led to the upregulation of HMGA1, p-AKT, p-MEK and p-ERK expression, whereas PD-L1 silencing resulted in significant downregulation of HMGA1, p-AKT, p-MEK and p-ERK expression (Fig. 5D and E). As shown in Fig. 5F, PD-L1 depletion notably suppressed HMGA1, p-AKT, p-MEK and p-ERK expression in CRC CSCs compared with the expression levels in attached CRC cells, indicating that PD-L1 regulates HMGA1 and its downstream signaling pathways in CRC CSCs. The immunohistochemical staining of tumor sections from xenografts demonstrated that the upregulation of PD-L1 significantly increased the expression of HMGA1 and the levels of p-AKT, p-MEK, and p-ERK in PD-L1-overexpressing tumors (Fig. 5G). Collectively, these results indicate that PD-L1 interacts with HMGA1 and activates HMGA1-dependent signaling pathways in CRC.

3.6. PD-L1 promotes the self-renewal of colorectal CSCs by increasing HMGA1 expression

To analyze the relationship between HMGA1 and PD-L1, we further investigated whether the stable shRNA-mediated knockdown of HMGA1 in PD-L1-overexpressing CRC cells could downregulate HMGA1-dependent signaling pathways and whether stable HMGA1 overexpression in PD-L1-silenced CRC cells could reactivate HMGA1-dependent signaling pathways. As shown in Fig. 6A, HMGA1 overexpression markedly increased the levels of p-AKT, p-MEK and p-ERK in CRC cells, similar to the effect of PD-L1 overexpression. In addition, the silencing of endogenous HMGA1 in PD-L1-overexpressing CRC cells decreased the levels of p-AKT, p-MEK and p-ERK. As expected, HMGA1 silencing decreased p-AKT, p-MEK and p-ERK, as observed in PD-L1-silenced CRC cells. Furthermore, the overexpression of HMGA1 in PD-L1-silenced CRC cells restored the levels of p-AKT, p-MEK and p-ERK.

To understand whether the PD-L1-induced promotion of CRC stemness maintenance is mediated by HMGA1, we performed gain- and loss-of-function experiments. First, we examined the role of HMGA1 in the stemness maintenance of CRC cells. As shown in Fig. 6, the gain of HMGA1 function increased the expression of stemness-related markers (Oct4 and Bmi-1) (Fig. 6A) and promoted tumorsphere and colony formation by CRC cells, similar to the effects of PD-L1 overexpression (Fig. 6B and C). As expected, the opposite results were found in the HMGA1 loss-of-function experiments (Fig. 6B and C). Subsequently, we

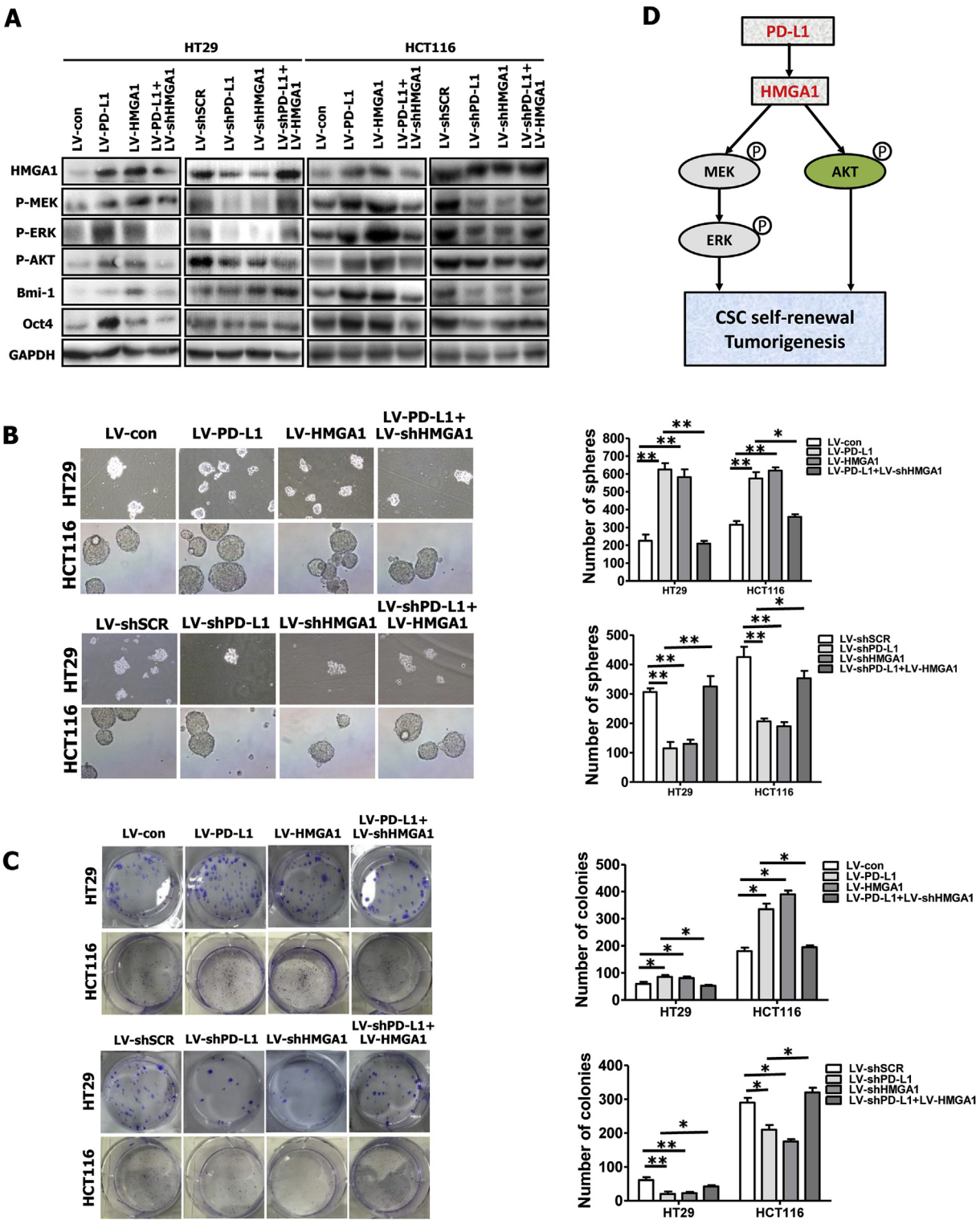

Fig. 6. PD-L1 promotes the self-renewal of colorectal CSCs by increasing HMGA1 expression.
(A) The differences in protein levels among CRC cells transfected with different plasmids were analyzed by Western blotting.
(B) PD-L1 promoted the formation of tumorspheres of CRC cells by activating HMGA1-dependent signaling pathways. The sphere sizes and densities are shown. Original magnification: 200 × .
(C) PD-L1 promoted the formation of colonies of CRC cells by activating HMGA1-dependent signaling pathways. The colony densities are shown.
(D) Schematic diagram showing the effect of PD-L1 on the stemness of colorectal cancer cells via HMGA1-dependent MEK/ERK and AKT signaling pathways.

investigated whether the ectopic expression of shHMGA1/HMGA1 reverses the PD-L1-induced alteration of stemness gene expression and tumor sphere and colony formation by CRC cells. We found that shHMGA1 reversed the upregulation of stemness-related marker expression (Fig. 6A) and abrogated the promotion of tumor sphere and colony formation induced by PD-L1 in CRC cells (Fig. 6B and C). Conversely, HMGA1 overexpression in shPD-L1-expressing cells abrogated the downregulation of stemness gene expression (Fig. 6A) and

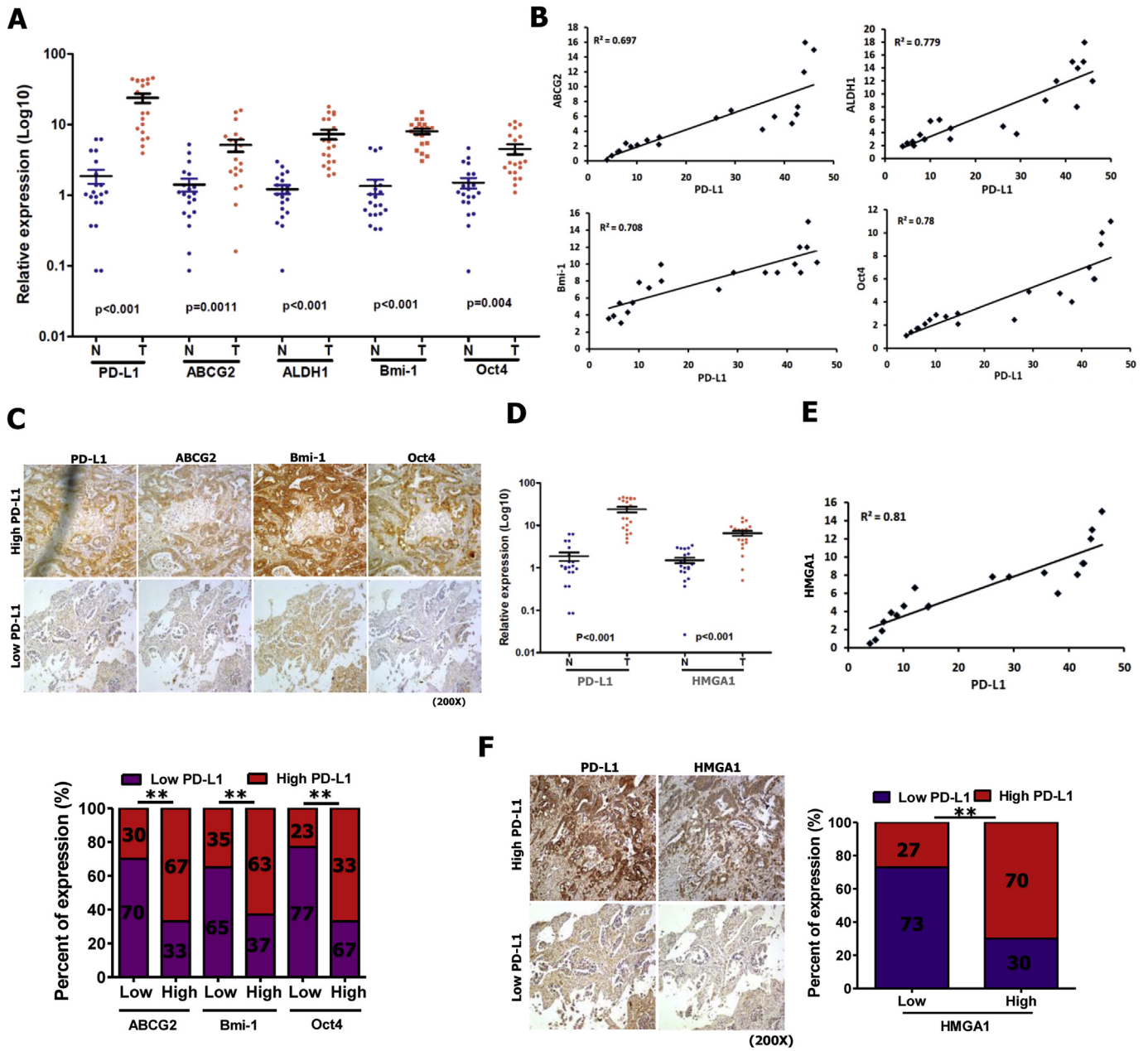

Fig. 7. Association between PD-L1 expression and HMGA1 and stem cell-related marker expression.

(A) The expression levels of PD-L1, ALDH1, ABCG2, Bmi-1 and Oct4 in CRC samples and control normal samples were detected by qRT-PCR. N = normal, T = tumor.

(B) PD-L1 expression was positively correlated with the ABCG2, ALDH1, Bmi-1 and Oct4 expression levels in CRC tissue samples.

(C) PD-L1 expression was positively correlated with the ABCG2, Bmi-1 and Oct4 expression levels in CRC tissue samples, as determined by IHC.

(D) The mRNA expression levels of PD-L1 and HMGA1 in CRC biopsy samples and control normal biopsy samples were measured by qRT-PCR. N = normal, T = tumor.

(E) PD-L1 expression was positively correlated with HMGA1 expression in CRC tissue samples.

(F) PD-L1 expression was positively correlated with HMGA1 expression in CRC tissue samples, as determined by IHC.

tumor sphere and colony formation induced by shPD-L1 in CRC cells (Fig. 6B and C). Taken together, these results suggest that HMGA1 is involved in the PD-L1-induced maintenance of the stemness of CRC cells.

Based on the above-presented data, we propose that PD-L1 regulates CRC CSC self-renewal by modulating HMGA1-dependent signaling pathways, including the MEK/ERK and AKT pathways (Fig. 6D).

3.7. Association of PD-L1 expression with HMGA1 and stem cell-related marker expression in CRC patients

To determine the potential correlations between PD-L1 expression and stem cell marker expression in CRC tissues, we obtained total RNA from 20 adjacent normal tissue samples and 20 CRC tissue samples and analyzed the mRNA levels of ALDH1, ABCG2, Bmi-1 and Oct4 by qRT-PCR. Our research indicated that PD-L1 and the CSC-related markers were clearly upregulated in the CRC samples in comparison with the adjacent normal samples (Fig. 7A). In addition, we further showed that PD-L1 expression was positively related to ALDH1, ABCG2, Bmi-1 and

Oct4 expression (Fig. 7B). Furthermore, high PD-L1 expression was associated with high ABCG2, Bmi-1 and Oct4 expression in the CRC biopsies, as determined by IHC (Fig. 7C and Table S2). Statistical analyses revealed significant positive correlations between the expression levels of ABCG2, Bmi-1 and Oct4 and that of PD-L1 in the 100 CRC biopsies.

In addition, we performed qRT-PCR and IHC analyses to identify the associations between HMGA1 and PD-L1 expression in the CRC biopsies. HMGA1 was upregulated in the CRC tissue compared with the normal tissue (Fig. 7D), and PD-L1 expression was positively correlated with HMGA1 expression (Fig. 7E and F and Table S2). Statistical analyses demonstrated a significant positive association between HMGA1 and PD-L1 expression in the 100 CRC biopsies.

4. Discussion

Accumulating evidence indicates that human tumors are stem cell diseases and that only a minor population of tumor cells with CSC characteristics might be responsible for tumor occurrence, development and chemoresistance [17]. Tumor recurrence after surgical resection might also be due in part to CSCs [18]. As reported by the CSC hypothesis, CRC can be deemed a disease in which mutations transform normal stem cells into abnormal stem cells responsible for tumor occurrence and development [19]. Therefore, it is important to clarify the molecular mechanism underlying colorectal CSC regulation to develop novel therapeutic strategies targeting CSCs. Here, we report that PD-L1 plays a critical role in colorectal CSC expansion that is independent of its immune checkpoint role. In addition, we provide intriguing evidence showing that PD-L1 directly interacts with HMGA1 and activates HMGA1-dependent signaling pathways, leading to the acceleration of CRC development.

CSCs are believed to be enriched by chemotherapy due to their unique survival mechanisms [20]. In our study, PD-L1-expressing CRC cells were resistant to therapeutic agents (e.g., 5-FU and cisplatin), and PD-L1 induced the increased expression of drug resistance genes. In addition, colorectal CSCs were enriched through the establishment of chemoresistant cells and chemoresistant CRC xenograft tumors, and the expression of PD-L1 in these chemoresistant cells and xenografts was significantly upregulated. Given the significance of CSCs in cancer recurrence and chemoresistance, we researched the role of PD-L1 in colorectal CSCs. The culture of tumor spheres comprised of tumor cells is a regular approach for the enrichment of CSCs. We found high PD-L1 expression in CRC tumorspheres and even higher PD-L1 expression in serially passaged tumorspheres. Currently, CD44 and CD133 are accepted as the predominant biomarkers of colorectal CSCs. We observed high PD-L1 expression in $CD133^+CD44^+$ CRC cells and even higher PD-L1 expression in $CD133^+CD44^+$ tumorspheres. Thus, our data showed that PD-L1 expression was increased in colorectal CSCs.

PD-L1 was previously reported to play a prosurvival role in cholangiocarcinoma stem cells, and the deletion of PD-L1 increased tumorigenesis and CSC phenotypes [21]. In contrast, PD-L1 overexpression enhances the self-renewal capacity of breast CSCs and leukemia-initiating cells (LICs) [13,22]. High PD-L1 expression has also been reported in colorectal CSCs [23].However, the role of PD-L1 in CSCs remains largely unclear. Our research indicates that PD-L1 induces a stem cell-like state, as confirmed by an improved self-renewal capacity and increases the number of cancer stem cells in vivo. This discovery was verified by the formation of tumor spheres, an increased population of $CD133^+CD44^+$ cells and increased expression of CSC-related markers, such as ALDH1, ABCG2, Bmi-1 and Oct4. In addition, our studies revealed that these CSC markers are upregulated in CRC tissue in comparison with tumor-adjacent normal tissue. Previous reports have proven that ALDH1, ABCG2, Oct4 and Bmi-1 play important functions in the tumorigenesis and development of CRC [24]. These findings indicate that PD-L1 is critical for colorectal CSC expansion.

To date, few studies have focused on clarifying the mechanism of PD-L1 in colorectal CSCs. Interestingly, Fang et al. found that PD-L1 plays an essential role in the proliferation of LICs. The PD-L1/JNK/Cyclin D2 signaling pathway promotes cell cycle entry in lymphoma stem cells [22]. Almozyan et al. showed that CD274 promotes Oct4 and Nanog expression in breast CSCs by activating the PI3K/AKT signaling pathway [13]. However, the mechanism of PD-L1 in CSCs remains largely unclear. Here, we found that PD-L1 significantly increased the levels of p-AKT, p-MEK and p-ERK in CRC cell lines. Importantly, such alterations in expression also occurred in vivo, as revealed by the immunohistochemical staining of xenograft tumor sections. However, the mechanism through which PD-L1 regulates the expression of the MEK/ERK and PI3K/AKT signaling pathways awaits further investigation.

The findings of a previous study indicated that doxorubicin can downregulate the expression of PD-L1 on the surface membrane and can promote its nuclear translocation in breast cancer cells [25]. It has also been reported that nuclear PD-L1 expression in colorectal cancer patients is significantly associated with short survival durations [26]. In this study, we found that an increase in the expression of PD-L1 in the nucleus (Fig. 4D) activates nuclear MEK/ERK and PI3K/AKT signaling pathways. Furthermore, the effect of PD-L1 on the nuclear fraction of ERK and particularly that of AKT, which plays a significant role in CSC self-renewal and tumorigenesis, supports an exclusive role in nuclear interactive pathways.

To further demonstrate the molecular mechanisms involved in PD-L1-mediated self-renewal activity, we performed a mass spectrometry analysis of the proteins from PD-L1- and vector-expressing CRC cells. Our data showed that HMGA1 was one of most differentially expressed proteins in PD-L1- and vector-expressing CRC cells. HMGA1 is a structural transcription factor that is located in the nucleus and has transcription-regulatory functions. The HMGA1 protein has been reported to be overexpressed in various human cancers, including pancreatic adenocarcinoma and breast and colon cancers [27–29]. In addition, Belton and colleagues have demonstrated that HMGA1 promotes cell proliferation and polyp formation in the intestines of HMGA1-transgenic mice and results in metastatic development and stem cell-like characteristics in colon cancer cells [27]. Moreover, HMGA1 can regulate MAPK-ERK1/2 signaling through the phosphorylation of Shc (leading to the activation of Ras, Raf and ultimately ERK1/2). Furthermore, HMGA1 is able to increase PI3K-AKT signaling by promoting PIP3-dependent processes and AKT and PKC activation [28,29]. In CRC, the knockdown of PD-L1 decreased and the overexpression of PD-L1 increased HMGA1 expression, and HMGA1 was found to be involved in the maintenance of CRC cell stemness. In addition, we found that PD-L1 directly interacted with HMGA1 and significantly increased the levels of p-MEK, p-ERK1/2 and p-Akt in CRC cells. Importantly, such alterations in expression also occurred in vivo, as revealed by the immunohistochemical staining of xenograft tumor sections. Therefore, we concluded that PD-L1 promotes CSC expansion by targeting HMGA1 and consequently activating the PI3K-Akt and MEK-ERK pathways in CRC.

Regarding the interaction between PD-L1 and HMGA1, a reciprocal interaction between PD-L1 and HMGA1 was observed by mass spectrometry and coimmunoprecipitation. We further performed a GST pull-down assay to validate the direct interaction between PD-L1 and HMGA1, and the results showed that HMGA1 was retained by GST-PD-L1 but not GST alone. Future studies will verify the specific domain in PD-L1 that is critical for its interaction with HMGA1 and will delineate which region of HMGA1 is necessary for association with PD-L1. This study provides the first demonstration that PD-L1 directly interplays with HMGA1 to further activate the PI3K-Akt and MEK-ERK pathways in CRC. Our current findings help elucidate the effects of PD-L1 on colorectal CSC expansion and provide a detailed molecular mechanism explaining the efficacy of PD-L1 in modulating CSCs. As a result, PD-L1 might be an optimal target in colorectal CSC-targeted therapy.

To date, a phase II clinical trial revealed that the administration of pembrolizumab (an anti-PD-L1 Mab) yielded partial objective response

rates of 40% and 0% in dMMR and pMMR CRC patients, respectively [30]. The phase II trial by Overman et al. [31] is the largest immunotherapy trial in CRC and highlights a partial response to nivolumab alone or in combination with ipilimumab in 31% of microsatellite instability (MSI) patients versus 10% of microsatellite stability (MSS) patients. Despite these encouraging results, it is clear that most MSI CRCs do not respond to immunotherapy; this finding represents the rationale for testing other markers that are able to predict cancer populations who will be eligible for personalized immune approaches [32].

A retrospective study of a CRC cohort with and without MSI clearly demonstrated the types and densities of inflammatory cells populating the tumor microenvironment and analyzed their PD-L1 expression, and the results identified three different tumor groups: group A (non-PD-L1-expressing neoplastic cells (NCs) and tumor-infiltrating immune cells (IICs)), group B (PD-L1-expressing IICs), and group C (PD-L1-expressing NCs and IICs). Group A is characterized by tumors with poor immunogenic competence and a poor immune response but massive granulocyte infiltrates, justifying the absence of PD-L1 as an immunoinhibiting receptor. Group B is likely characterized by more immunogenic CRCs, justifying the strong IICs-mediated immune response, and PD-L1 expression is upregulated only on IICs. Group C is likely characterized by CRCs with large amounts of tumor neo-antigens, resulting in a marked infiltration of lymphocytes and PD-L1-upregulating NCs. This study prompted us to assess PD-L1 expression in both NCs and IICs to better stratify CRCs with different immunological patterns [32].

Thus, the utility of PD-L1 in personalized medicine for CRC patients is worthy of further investigation.

Conflicts of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.022.

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