Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2025**; 30(11): 46743
https://doi.org/10.31083/FBL46743

Original Research

HMGA1/SMAD3 Promoter Complex Mediates PD-L1-Dependent Transcriptional Regulation of GAS6, EGR1 and PD-L1

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Academic Editor: Eun Sook Hwang

Submitted: 19 September 2025 Revised: 20 October 2025 Accepted: 31 October 2025 Published: 20 November 2025

Abstract

Background: Programmed death-ligand 1 (PD-L1) partners with specificity Protein 1 (SP1) or signal transducer and activator of transcription 3 (STAT3) to modulate the transcription of growth arrest-specific 6 (GAS6) and early growth response protein 1 (EGR1), necessitating mediators to avoid feedback. Based on binding and stemness data, high mobility group A1 (HMGA1) and Small Mother Against Decapentaplegic3 (SMAD3) were identified as potential mediators in this context. While the SMAD3–P300–STAT3 complex facilitates SMAD3–STAT3 crosstalk, it remains unclear whether the PD-L1–HMGA1–SP1 or PD-L1–SMAD3–SP1 complexes bind to GAS6 and EGR1 promoters to regulate their transcription. **Methods**: MG63 osteosarcoma cells and SW620 colon cancer cells with unidentified nuclear PD-L1 function were chosen for our study. Chromatin immunoprecipitation and co-immunoprecipitation assays were performed to evaluate SP1, HMGA1, SMAD3, STAT3, P300 and PD-L1 (also denoted CD274) enrichment at the GAS6 and EGR1 promoters; the existence of the PD-L1–(HMGA1 or SMAD3)–SP1 complexes; whether P300 binds to STAT3; and whether HMGA1 and SMAD3 bind to P300. The alterations in GAS6, EGR1 and PD-L1 mRNA levels after their combined over-expression and/or knockdown were assessed via qPCR. Two representative target genes identified via PD-L1 chromatin immunoprecipitation (ChIP)-seq were examined to determine whether HMGA1 and SMAD3 were enriched at their promoters. **Results**: PD-L1, HMGA1, SMAD3, SP1, P300 and STAT3 were enriched at GAS6 and EGR1 promoters in two cell lines. HMGA1 or SMAD3 antibody pulled down PD-L1 and SP1; PD-L1 antibody pulled down HMGA1, SMAD3 and SP1; P300 antibody pulled down STAT3; and, surprisingly, HMGA1 and SMAD3 antibodies pulled down P300. Combined over-expression or knockdown significantly altered GAS6, EGR1 and PD-L1 mRNA levels. PD-L1 ChIP-seq indicated 114 target genes, among which PD-L1 and beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC) were chosen to verify the promoter enrichment of HMGA1 and SMAD3. **Conclusion**: Our study provides initial evidence that PD-L1 might form HMGA1- and SMAD3-dependent complexes to bind the GAS6, EGR1 and CD274 promoters, thus modulating the transcription of GAS6, EGR1 and PD-L1 mRNA in cancer and sarcoma cells.

Keywords: PD-L1; HMGA1 protein; SMAD3 protein; growth arrest-specific protein 6; early growth response protein 1; neoplasms; sarcoma

1. Introduction

The interactions between programmed death-ligand 1 (PD-L1) on cancer and sarcoma cell membranes with programmed death protein 1 (PD-1) on T cells inhibit T cells from attacking tumor cells, thus promoting immune checkpoint escape [1,2]. Membrane PD-L1 also has pro-survival and invasive-enhancing functions in cancer and sarcoma cells, but the definite mechanisms underpinning signal transduction remain poorly understood [3,4]. PD-L1/PD-1 blockade therapies have been clinically used to restore the anti-tumor immune response; however, checkpoint blockade drugs are prone to low response rates, serious adverse effects and therapeutic resistance [5,6]. Recent research has shown that PD-L1 expressed on the cell surface has a secondary mechanism of regulating immune responses that is mediated via its endocytic and nucleocytoplasmic translocation into the nucleus [7]. P300-mediated acetylation of PD-L1 blocks its translocation, whereas histone deacetylase 2 (HDAC2)-driven deacetylation promotes this process [7]. In previous research, TGF-β has been shown to stimulate PD-L1 nuclear translocation and the fibroblast to myofibroblast transition in the lung [8]. Nuclear PD-L1 has been identified as a critical regulator of 1144 genes involved in immune responses, angiogenesis and signal transduction, determining anti-PD-1 treatment outcomes in cancer patients to a great extent [9]. Moreover, although the possibility of PD-L1 directly interacting with DNA to regulate gene expression has been proposed, it is also possible that PD-L1 indirectly interacts with DNA via transcription factors, with the latter idea being increasingly supported in the literature.

Regarding PD-L1-modulated transcription within the nucleus, PD-L1 forms a complex with the transcription factor specificity protein 1 (SP1) and enhances the expression of Growth Arrest-Specific 6 (*GAS6*) by binding to its promoter. subsequent activation of MerTK signaling by up-

regulated GAS6 has been shown to drive tumor proliferation in non-small cell lung cancer [10]. Furthermore, nuclear PD-L1 drives the progression of uveal melanoma by co-localizing with phosphorylated signal transducer and activator of transcription 3 (STAT3) at promoter regions, leading to transcriptional up-regulation of Early Growth Response protein 1 (*EGR1*) [9]. In addition, p-STAT3 physically interacts with PD-L1 to enter the nucleus and binds to the *gasdermin C* promoter to enhance its transcription under hypoxic conditions, leading to the activation of a non-canonical pyroptosis pathway that fuels tumor progression [11]. Better comprehension of the mechanisms of PD-L1-activated signal pathways and PD-L1-regulated genes is critically needed to "harness" PD-L1 for more effective therapeutic management of sarcoma and cancer.

Emerging studies have identified novel transcription factors governing the expression of PD-L1. A polymorphism in the *PD-L1* promoter enhances its affinity for SP1, resulting in up-regulated PD-L1 expression and increased risk of gastric cancer [12]. In Kaposi's sarcoma-associated herpesvirus infection, the viral replication and transcription activator (K-RTA) cooperates with host SP1 near the transcription start site to transactivate the *CD274/PD-L1* promoter. This mechanism facilitates immune evasion by elevating PD-L1 expression [13]. Furthermore, SP1 and STAT3 not only serve as established transcriptional activators of *PD-L1*, but are also reciprocally up-regulated by PD-L1 over-expression via Myc- or HIF-1α-mediated pathways [14,15]. These findings strongly suggest that the interaction of PD-L1 with SP1 or STAT3 may form a positive/negative feedback loop regulating PD-L1 expression, which would logically result in either continuously increasing up-regulation or stable suppression of PD-L1; however, this apparently contradicts the bidirectional dynamic fluctuation in PD-L1 levels observed during oncogenesis [16]. Even if mediators exist, a similar problem still arises; however, this issue may be resolved by considering the (direct or indirect) combination of mediators, as elucidated in this paper. Nonetheless, we should still proceed by initially determining the involved mediators, leading to further analyses regarding their combinations. Therefore, it can be hypothesized that there are potential combinations of mediators of PD-L1, SP1 and/or STAT3 that result in the dynamic and differentiated expression of PD-L1.

Transcriptional complexes formed by High Mobility Group A1 (HMGA1) or SMAD3 with SP1 orchestrate the expression of oncogenic targets such as insulin-like growth factor 1 receptor (*IGF-1R*) and Tenascin-C (*TNC*), thereby promoting tumor progression through activation of growth and matricellular signaling pathways [17,18]. Interestingly, both HMGA1 and SMAD3 have been demonstrated to directly bind PD-L1 [8,19]; therefore, both can be considered as candidate mediators, and their combination as mediators should also be validated. Whether HMGA1 and SMAD3 are enriched at GAS6 and EGR1 promoters has not yet been studied; nevertheless, they might mediate the binding of PD-L1 and SP1 to those promoters to modulate the expression of GAS6 and/or EGR1. Although this concept may seem strange and complicated at first, we present the results of systematic tests performed to determine its fundamental viability in this study. Direct P300–STAT3 and SMAD3–STAT3 interactions have been reported in previous studies, but whether these complexes are enriched at the *GAS6* and *EGR1* promoters has not been studied [20]. In the present study, we over-expressed PD-L1/CD274 in colon cancer and osteocarcinoma cells, as the role of their nuclear PD-L1 in regulating *GAS6* and *EGR1* expression has not yet been explored. We treated the cells with Transforming growth factor-β (TGF-β, 5 ng/mL for 6 h) to increase nuclear PD-L1, HMGA1 and SMAD3 expression. The TGF-β was then either removed as blank control,or the TGF-β-treated cells were subjected to 50 ng/mL IL-6 as multiple treatments to investigate their effects on the binding of PD-L1 to the *GAS6* and *EGR1* promoters [21,22]. PD-L1, HMGA1, SMAD3, STAT3, P300 and SP1 enrichment at the GAS6 and EGR1 promoters was evaluated via chromatin immunoprecipitation (ChIP) assay. Subsequently, via co-immunoprecipitation (CoIP) assay, we assessed whether HMGA1 or SMAD3 antibodies co-immunoprecipitate with PD-L1 and SP1; PD-L1 antibody co-immunoprecipitates HMGA1, SMAD3 and SP1; P300 antibody co-immunoprecipitates with p-STAT3; and HMGA1 and SMAD3 antibodies co-immunoprecipitate with P300. Next, we over-expressed or knocked-down typical combinations of the abovementioned molecules to examine the associated effects on *GAS6*, *EGR1* and *PD-L1* transcription. We employed ChIP-seq profiling to identify PD-L1-regulated target genes and found *CD274* (encoding PD-L1) and beta-transducin repeat containing E3 ubiquitin protein ligase (*BTRC*)—a key ubiquitin E3 ligase for β-catenin—as prominent transcriptional targets. We then assessed whether HMGA1 and SMAD3 are enriched at *PD-L1* and *BTRC* promoters in gastric cancer AGS cells and lung cancer SK-MES-1 cells, as predicted by Chip-sequence as PD-L1-regulated genes. Finally, we validated whether HMGA1 and SMAD3 were also enriched at the *PD-L1* and *BTRC* promoters for the first time.

Taken together, the results of these experiments validate the critical role of PD-L1 in modulating the transcription of *GAS6*, *EGR1* and *PD-L1* itself through the formation of HMGA1- and SMAD3-dependent complexes to bind relevant promoters. Given the complexity of the proposed concept, further validation using other sources and more in-depth exploration are warranted.

2. Materials and Methods

2.1 Cell Lines and Reagents

SW620 and MG63 cells were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were validated via STR profiling and

Table 1. Primer sequences used for ChIP-qPCR analysis.

Gene	Primer set	Forward primer (5′→3′)	Reverse primer (5′→3′)
GAS6	F1/R1	CCAAGACAAGAGCCAGAA	CGAGTGAAATGCGACG
GAS6	F2/R2	GGAACCCTTAGGAAATAATAGC	TTCTGCCCCGAAACCAC
GAS6	F3/R3	CAATCCTATTCCATTCTTCC	AATCCCCACACCCCA
EGR1	F1/R1	CTGGACTGGATAAAGGGGGG	TGTCGCTGGGAAATTGAGGAT
EGR1	F2/R2	GGGACAGCCACAGAGGGATT	AAGGAGGGTGCTTGACGACA
EGR1	F3/R3	CAGCGACACCCGGAAAGACA	TCAGGGACCGTGAGAGCGAA
CD274	F1/R1	AAAGAAAAGGGAGCACA	CAAGATGACAGACGATGG
CD274	F2/R2	ACCTGTAAACTGTATTGCC	CAGCTCAGATGTTCCTTC
CD274	F3/R3	TAAAAGATGTAGCTCGGG	TGTGTGTGTGTATGGGTGT
BTRC	F1/R1	AGTCCTCTGAAAACCTCC	CTTTGTCCTAGCCCCTA
BTRC	F2/R2	CAAGGCTGTCACAATCTAA	AAACCCCCGTCTCTACTA
BTRC	F3/R3	GCTGTGGGGGAGAAAAG	GGCAAGAATCACGGGTC

ChIP-qPCR, ChIP-quantitative polymerase chain reaction.

tested negative for mycoplasma. According to previous research focused on PD-L1 regulation, TGF-β stimulation might induce PD-L1 up-regulation and nuclear translocation, while the PD-L1–HMGA1 interaction in colon cancer and the PD-L1–SMAD3 interaction in osteocarcinoma cells might also facilitate HMGA1 and SMAD3 nuclear translocation, respectively, subsequently increasing the possibility of observing their interactions and gene regulation. IL-6 stimulation can activate STAT3 and induce its nuclear translocation; according to some reports, it activates P300 to facilitate STAT3 binding to DNA. As such, TGF-β and IL-6 co-simulation has been used to simulate cancerous settings characterized by multiple factors. Cells were transfected with a PD-L1-expressing plasmid and cultured using established protocols [23]. To activate nuclear translocation, the cells were treated with 5 ng/mL TGF-β (ab50036, Abcam, Cambridge, UK) for 6 h before harvest. To further investigate the factors influencing the binding of PD-L1 to the GAS6 and EGR1 promoters, the 5 ng/mL TGF-β was removed, and 50 ng/mL IL-6 (Acrobiosystems, Beijing, China) was added to the cells. Normoxic conditions are defined as 5% CO_2 and 95% air, while hypoxic conditions are characterized by 3% O_2, 5% CO_2 and N_2 to balance. The following antibodies were obtained from Abcam (Cambridge, UK): anti-HMGA1 (ab252930), anti-SMAD3 (ab208182), anti-phospho-STAT3 (Y705; ab267373), anti-P300 (ab275378) and anti-SP1 (ab231778). For PD-L1 detection, the E1L3N monoclonal antibody (CST #13684S) from Cell Signaling Technology (Danvers, MA, USA) was applied in ChIP experiments, while an alternative PD-L1 antibody (ab213480; Abcam) was utilized for IP. A commercial co-immunoprecipitation and Western blotting kit (GeneCreate, Wuhan, Hubei, China) was employed, following the manufacturer's protocol.

2.2 ChIP-Quantitative Polymerase Chain Reaction (ChIP-qPCR)

ChIP was conducted using a commercial Millipore kit (GeneCreate, Wuhan, Hubei, China), following established methods [6]. After crosslinking, cells were lysed, and chromatin was fragmented via sonication on ice. Immunoprecipitation was performed using Protein G agarose beads with antibodies against SP1, HMGA1, SMAD3, STAT3 or PD-L1, with normal IgG as a control. Following reversal of crosslinking and proteinase K treatment, purified DNA was analyzed via qPCR using primers specific to the *GAS6* and *EGR1* promoter regions, whose 5′ to 3′ sequences are listed as follows [6,7] (Table 1).

2.3 ChIP-Seq Analysis

The generation and sequencing of short DNA reads from immunoprecipitated samples were performed at GeneCreate (Wuhan, Hubei, China) using an Illumina NovaSeq6000 (San Diego, CA, USA). Reads were mapped against the human reference genome hg38 with Bowtie 2 version 2.5.0 (https://bowtie-bio.sourceforge.net/) [24]. Peak calling was carried out using MACS3 (version 3.0.0, Buffalo, New York, NY, USA), peak gene annotation was accomplished using ChIPseeker (version 1.36, Guangzhou, Guangdong, China), and Homer (version 4.11, San Diego, CA, USA) analysis was performed to identify enriched motifs.

2.4 Co-Immunoprecipitation–Western Blotting (CoIP-WB)

Cell lysates were prepared in RIPA buffer supplemented with protease inhibitors and cleared by centrifugation. Target protein complexes were immunoprecipitated using antibody-conjugated agarose beads. Following extensive washing and elution, immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

Table 2. Primer sequences used for quantitative real-time PCR (qPCR).

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
SMAD3	GGGGGTTGGACTTTCCTTCC	GACTCCAAGTGGCAGCAGAA
SP1	CCCTTGAGCTTGTCCCTCAG	TGAAAAGGCACCACCACCAT
P300	GCAGTGTGCCAAACCAGATG	GGGTTTGCCGGGGTACAATA
GAS6	ACGACCCCGAGACGGATTAT	GGCGAAGCCTGAGTTTTTGG
STAT3	TCTGTGTGACACCAACGACC	TCCTCACATGGGGGAGGTAG
EGR1	CCCACCATGGACAACTACCC	AAAGACTCTGCGGTCAGGTG
HMGA1	CATCCGCATTTGCTACCAGC	TCTCAGTGCCGTCCTTTTCC
CD274	ACTGGCATTTGCTGAACG	TCCTCCATTTCCCAATAGAC
ACTB	GCGTGACATTAAGGAGAAGC	CCACGTCACACTTCATGATGG
GAPDH	CTGACTTCAACAGCGACACC	GTGGTCCAGGGGTCTTACTC

Table 3. Sequences of siRNAs used for gene knockdown experiments.

siRNA Name	Sense Strand (5′-3′)	Antisense Strand (5′-3′)
si-h-CD274_007	GCUGUCUUUAUAUUCAUGATT	UCAUGAAUAUAAAGACAGCTT
HMGA1-Homo-256	GAGUCGAGCUCGAAGUCCATT	UGGACUUCGAGCUCGACUCTT
HMGA1-Homo-1378	GCUCCCCUAACCCUACUUUTT	AAAGUAGGGUUAGGGGAGCTT
HMGA1-Homo-402	GCCAACACCUAAGAGACCUTT	AGGUCUCUUAGGUGUUGGCTT
SMAD3-homo-660	GCGUGAAUCCCUACCACUATT	UAGUGGUAGGGAUUCACGCTT
SMAD3-homo-1307	GGAUGCAACCUGAAGAUCUTT	AGAUCUUCAGGUUGCAUCCTT
SMAD3-homo-1414	CCGCAUGAGCUUCGUCAAATT	UUUGACGAAGCUCAUGCGGTT
SP1-homo-2414	GCAGAUCUGCAGUCCAUUATT	UAAUGGACUGCAGAUCUGCTT
SP1-homo-379	CCUCACAGCCACACAACUUTT	AAGUUGUGUGGCUGUGAGGTT
SP1-homo-1689	CCAUUAACCUCAGUGCAUUTT	AAUGCACUGAGGUUAAUGGTT
Negative Control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

2.5 Quantitative Polymerase Chain Reaction (qPCR)

MG63 and SW620 cells were divided into five groups to be successively transfected with control, *PD-L1OE*, *PD-L1OE+HMGA1OE+SP1OE*, *PD-L1OE+SMAD3OE+SP1OE* or *P300OE+STAT3OE+SP1OE* plasmids (Honorgene, Changsha, China) for 48 h under normoxic conditions, following which PCR was performed to confirm their successful over-expression and measure *GAS6* and *EGR1* mRNA. As p-STAT3 physically interacts with PD-L1 in the nucleus to regulate *gasdermin C* transcription under hypoxic conditions, we assessed their expression levels under hypoxia and their effects with or without SP1, PD-L1, HMGA1 and SMAD3 knockdown [11]. SW620 cells with adequate PD-L1 expression were divided into five groups to be successively transfected with control small interfering ribonucleic acids (siRNAs) and siRNAs targeting *SP1*, *PD-L1+SP1*, *HMGA1* and *HMGA1+SMAD3* (Honorgene, Changsha, Hunan, China) for 48 h under normoxic conditions or two cycles of 20 h under normoxic and 4 h under hypoxic conditions. qPCR was then performed to confirm their successful knockdown and to measure *GAS6* and *EGR1* mRNA. Total RNA was isolated using a commercial RNA purification kit (Honorgene, Changsha, Hunan, China), and cDNA was synthesized with the PrimeScript RT reagent kit (Honorgene, Changsha, Hunan, China). Quantitative real-time PCR was performed using SYBR Green master mix (Honorgene, Changsha, Hunan, China) with *ACTB* or *GAPDH* as the endogenous control for over-expression or knockdown experiments, respectively. To evaluate the knockdown efficiency of siRNA in preliminary experiments, the specific si-SP1, si-SP1+PD-L1, si-HMGA1 and si-HMGA1+SMAD3 combinations were selected based on their knockdown performance both independently and in combinations. Each sample was analyzed in triplicate, and the cycle threshold (Ct) value was calculated. The ΔCt value was determined by subtracting the mean Ct value of ACTB or GAPDH from that of the target gene. Gene expression fold changes were determined using the $2^{-\Delta\Delta Ct}$ method.

The primer sequences used are listed below (Tables 2,3).

The siRNA sense and antisense sequences used are also listed below (sense: 5′-...-3′; antisense: 5′-...-3′).

2.6 Statistical Analyses

Statistical analyses were conducted using SPSS 20.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). ChIP-qPCR, Co-IP and Western blotting experiments were performed with three independent replicates, while qRT-PCR was performed with six independent replicates. Data are presented as the mean $\pm$ SD. For ChIP-qPCR results,

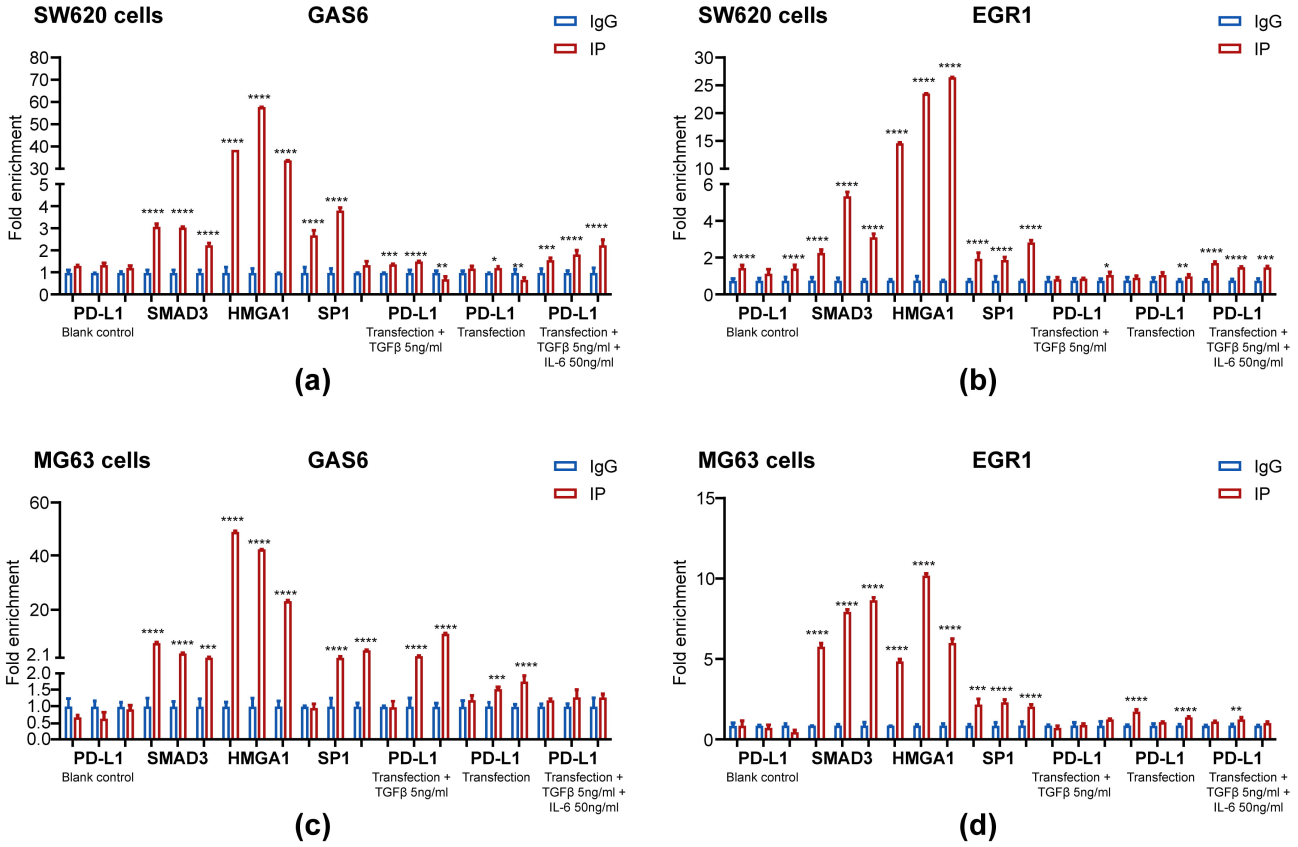

Fig. 1. Enrichment of HMGA1, SMAD3, SP1 and PD-L1 at the GAS6 and EGR1 promoters. SW620 (a,c) and MG63 cells (b,d) were transfected with PD-L1 plasmid for 42 h. Then, the cells were treated with 5 ng/mL TGF-β for 6 h and HMGA1, SMAD3, SP1 and PD-L1 ChIP assays were performed using appropriate antibodies, followed by qPCR analysis. The detected enrichment results of primer pairs 1, 2 and 3 for GAS6 or EGR1 promoter for each ChIP antibody are displayed from left to right. SW620 and MG63 cells transfected with PD-L1 plasmid for 48 h or 45 h with 5 ng/mL TGF-β and 50 ng/mL IL-6 for an additional 3 h before harvest were also subjected to PD-L1 ChIP-qPCR analysis. HMGA1, High Mobility Group A1; PD-L1, programmed death-ligand 1; GAS6, Growth Arrest-Specific 6; EGR1, Early Growth Response protein 1; SMAD3, SmallMother Against Decapentaplegic 3; SP1, Specificity Protein 1. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

two-way ANOVA with Sidak's multiple comparisons test was applied to compare experimental groups against the IgG control. For qPCR data, two-tailed Student's *t*-test was used to assess differences between control and experimental conditions. Significance levels are denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

3.1 PD-L1, SP1, HMGA1 and SMAD3 are Enriched at the GAS6 Promoter

We performed ChIP assays to evaluate the mechanisms underlying the binding of PD-L1 to the promoter of *GAS6*. In contrast with blank control and control IgG, PD-L1, SMAD3, HMGA1 and SP1 were enriched at the *GAS6* promoter after the 6 h treatment with 5 ng/mL TGF-β in both SW620 and MG63 cells. Without the 5 ng/mL TGF-β treatment for 6 h, PD-L1 was not enriched at the *GAS6* promoter in SW620 cells, but was still enriched in MG63 cells (Fig. 1a,c). Notably, when using 5 ng/mL TGF-β and IL-6 50 ng/mL treatment for 6 h, PD-L1 was not enriched at the GAS6 promoter in MG63 cells, but was still enriched in SW620 cells (Fig. 1a,c). Given that there are no direct binding sites for HMGA1 and SMAD3 in the *GAS6* promoter, and as HMGA1 and SMAD3 are known to bind PD-L1 and SP1, these data support the possibility that PD-L1, HMGA1, SMAD3 and SP1 form complex(es) at the *GAS6* promoter that are differently affected by IL-6 and TGF-β stimulation, depending on the cellular context.

3.2 PD-L1, SP1, SMAD3 and HMGA1 are Enriched at the EGR1 Promoter

We also performed ChIP assays at the *EGR1* promoter to gain additional insights into the potential roles of protein complexes containing HMGA1, SMAD3, SP1 and PD-L1. Consistent with the results for the *GAS6* promoter, SMAD3, HMGA1 and SP1 were enriched at the *EGR1* promoter in MG63 and SW620 cells after treatment with 5 ng/mL TGF-β for 6 h; however, the pattern of PD-L1 en-

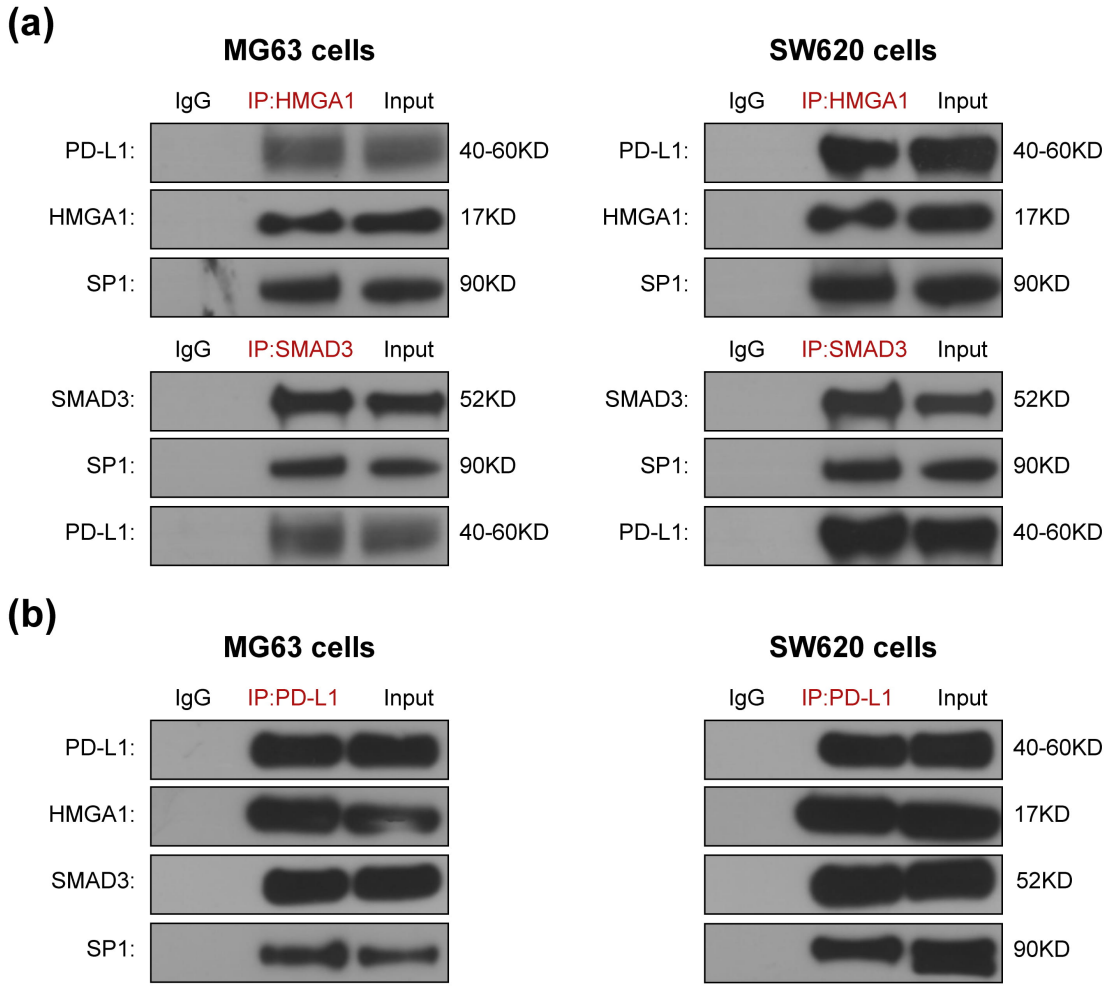

Fig. 2. The comfirmation of HMGA1 and SMAD3 as mediators for PD-L1 and SP1 combination. HMGA1 and SMAD3 antibodies pull down PD-L1 and SP1 (a). PD-L1 antibody pulls down HMGA1, SMAD3 and SP1 (b). SW620 and MG63 cells were transfected with PD-L1 plasmids. After 42 h, they were treated for 6 h with 5 ng/mL TGF-β before harvest. Immunoprecipitation was performed, followed by Western blotting with specific antibodies. IgG and input were used as negative and positive controls.

richment differed from that for the *GAS6* promoter. The levels of promoter-bound PD-L1 were marginal in the presence of 5 ng/mL TGF-β but were significantly enhanced when removing TGF-β treatment or adding 50 ng/mL IL-6 to SW620 and MG63 cells (Fig. 1b,d). Given that there are no direct binding sites for HMGA1 and SMAD3 in the *EGR1* promoter, and that HMGA1 and SMAD3 are known to bind PD-L1 and SP1, these data further support the idea that PD-L1, HMGA1, SMAD3 and SP1 form complexes at *EGR1* promoters, and that the binding of these complexes are differently affected by IL-6 and TGF-β depending on the cellular context.

3.3 PD-L1 and SP1 Binding to the GAS6 and EGR1 Promoters is HMGA1- and SMAD3-Dependent

SMAD3 has been reported to directly interact with PD-L1 [8], STAT3 [21,22] and SP1 [18]; while HMGA1 has been reported to bind to PD-L1 and SP1 [17,19]. To determine whether SMAD3 and HMGA1 interact with PD-L1 and SP1 within PD-L1–SMAD3–SP1 and PD-L1–HMGA1–SP1 ternary complexes or HMGA1 complexes with SMAD3, we performed CoIP assays. In SW620 and MG63 cells, PD-L1 and SP1 were pulled down by both anti-SMAD3 and anti-HMGA1 antibodies (Fig. 2a). Furthermore, HMGA1, SMAD3 and SP1 were pulled down by PD-L1 antibody in both cells (Fig. 2b). These results are consistent with the existence of PD-L1–SMAD3–SP1 and PD-L1–HMGA1–SP1 ternary complex or a connection between HMGA1 and SMAD3, PD-L1 and SP1 were reduced to at least one pair in SW620 and MG63 cells.

3.4 P300–STAT3 Binding to the GAS6 and EGR1 Promoters is Also HMGA1- and SMAD3-Dependent

To further assess the role of STAT3, we performed Western blot assays in PD-L1-transfected SW620 and MG63 cells using an antibody that binds its activated form, p-STAT3. The results demonstrated that, compared to the blank control, 5 ng/mL TGF-β maintained the activation

of p-STAT3, which diminished after removal of 5 ng/mL TGF-β and nearly disappeared after adding 50 ng/mL IL-6. We did not detect STAT3 Lys-685 acetylation and p-AKT after PD-L1 over-expression with and without TGF-β or IL-6 stimulation in SW620 and MG63 cells [25] (Fig. 3a). The reduction in free p-STAT3 might be correlated with increased DNA-bound p-STAT3. Consistently, in TGF-β-treated SW620 and MG63 cells, the CoIP assay revealed adequate P300-bound p-STAT3 (Fig. 3b). Surprisingly, the CoIP assay results confirmed that both HMGA1 and SMAD3 bound P300 in TGF-β-treated SW620 and MG63 cells (Fig. 3b). To the best of our knowledge, this is the first time that it has been definitively reported that HMGA1 and SMAD3 both bind to P300. These results are in accordance with previous reports, stating that five lysine residues of HMGA1a could be acetylated by P300 [26] and that increased P300–SMAD3–SP1 interactions were detected in systemic sclerosis fibroblasts [27]. These data support a possible model in which P300 concomitantly binds HMGA1, SMAD3 and p-STAT3 in such a way that bridges PD-L1–HMGA1–SP1 and PD-L1–SMAD3–SP1 while facilitating the binding of p-STAT3 to gene promoters. If HMGA1 directly binds SMAD3, the combined PD-L1 and SP1 reduce to at least one pair; this simpler structure also conforms to the CoIP results, although this concept needs future validation.

The ChIP results confirmed that P-STAT3 was enriched at the GAS6 and EGR1 promoters, both in the absence and presence of 5 ng/mL TGF-β in PD-L1 over-expressed MG63 cells (Fig. 3c); furthermore, P300 was also enriched at the GAS6 and EGR1 promoters, both in PD-L1 over-expressed MG63 and SW620 cells (Fig. 3d). Taken together, these findings support that P300–p-STAT3 connects to PD-L1–HMGA1–SP1 and/or PD-L1–SMAD3–SP1 to form a complex and bind to the GAS6 and EGR1 promoters; or that HMGA1 binds directly to SMAD3 then, as a whole, laterally associates with the P300–STAT3 complex while vertically engaging with PD-L1 and SP1.

3.5 PD-L1 Complex Combined Over-Expression or Knockdown Significantly Elevated or Decreased GAS6 and EGR1 mRNA

We performed a series of ChIP and CoIP assays to determine whether PD-L1, SP1 and P300–STAT3 form an expedient complex at the GAS6 and EGR1 promoters in a HMGA1- and SMAD3-dependent manner; however, whether this actually facilitates the transcription of target genes GAS6 and EGR1 has not been examined. Over-expression of PD-L1, PD-L1–HMGA1–SP1, PD-L1–SMAD3–SP1 and P300–STAT3–SP1 in MG63 cells significantly elevated GAS6 mRNA by 31-, 17-, 19- and 15-fold ($p < 0.01$) in contrast to negative control, respectively (Fig. 4a). Over-expression of PD-L1, PD-L1–HMGA1–SP1, PD-L1–SMAD3–SP1 and P300–STAT3–SP1 in SW620 cells elevated GAS6 mRNA by 1.14-, 1.12-, 1.29- and 0.84-fold (all groups $p > 0.05$; except PD-L1–SMAD3–SP1, $p < 0.01$) in contrast to negative control, respectively (Fig. 4a). PD-L1 over-expression was the dominant GAS6 mRNA up-regulator in MG63 cells, while PD-L1–SMAD3–SP1 was the only statistically significant up-regulator in SW620 cells.

Over-expression of PD-L1, PD-L1–HMGA1–SP1, PD-L1–SMAD3–SP1 and P300–STAT3–SP1 in MG63 cells elevated EGR1 mRNA expression by 5-, 77-, 204- and 51-fold (all groups $p < 0.01$) in contrast to negative control, respectively; as such, PD-L1–SMAD3–SP1 over-expression was the dominant EGR1 transcription up-regulator in MG63 cells (Fig. 4a). In quite dramatic contrast, over-expression of the above-mentioned molecules in SW620 cells significantly decreased EGR1 mRNA expression by 0.44-, 0.40-, 0.42- and 0.96-fold (all groups $p < 0.01$; except P300–STAT3–SP1, $p > 0.05$) in contrast to negative control, respectively. All treatments significantly down-regulated EGR1 transcription in SW620 cells except for P300–STAT3, which did not have a significant effect (Fig. 4a).

Our PD-L1 ChIP-seq results revealed that PD-L1 was enriched in the CD274/PD-L1 promoter (<1 kb from the transcript start site). As such, it is likely that PD-L1 forms a complex at this promoter, which was validated via HMGA1 and SMAD3 co-enrichment at the PD-L1 promoter: PD-L1 mRNA increased in each group in MG63 cells, by 384-, 4647-, 1628- and 0.64-fold (all groups $p < 0.01$; except P300–STAT3–SP1, $p > 0.05$) in contrast to negative control, respectively (Fig. 4a); and in SW620 cells by 15573-, 11907-, 2316- and 0.86-fold (all groups $p < 0.01$; except P300–STAT3–SP1, $p > 0.05$) in contrast to negative control, respectively. All treatments strikingly increased PD-L1 mRNA in MG63 and SW620 cells except for P300–STAT3–SP1, which insignificantly decreased this level (Fig. 4a).

As PD-L1 expression in MG63 cells was low in our preliminary study, SW620 with adequate endogenous PD-L1 expression was chosen for knockdown experiments. Successive knockdown of SP1, PD-L1–SP1 and HMGA1–SMAD3 altered PD-L1 mRNA by 0.641-, 0.578- and 0.589-fold, while knockdown of HMGA1 increased it by 1.140-fold (all $p < 0.05$) under normoxic conditions; the associated values under hypoxic conditions were 1.139-, 0.943- (both $p > 0.05$), 2.080- and 1.718-fold (both $p > 0.05$), respectively (Fig. 4b). Successive knockdown of SP1, PD-L1–SP1, HMGA1 and HMGA1–SMAD3 altered EGR1 mRNA by 0.510-, 0.827- (both $p < 0.05$), 1.359- and 1.235-fold (both $p < 0.05$) under normoxic condition; and by 0.314-, 1.047-, 1.716- and 2.071-fold (all $p < 0.05$ except PD-L1–SP1 knockdown) under hypoxic conditions, respectively (Fig. 4b). Knockdown of SP1, PD-L1–SP1, HMGA1 and HMGA1–SMAD3 altered GAS6 mRNA by 1.019- ($p > 0.05$), 1.472-, 1.639- and 1.484-fold (all $p < 0.05$) under normoxic conditions; and by 1.313-, 1.764-

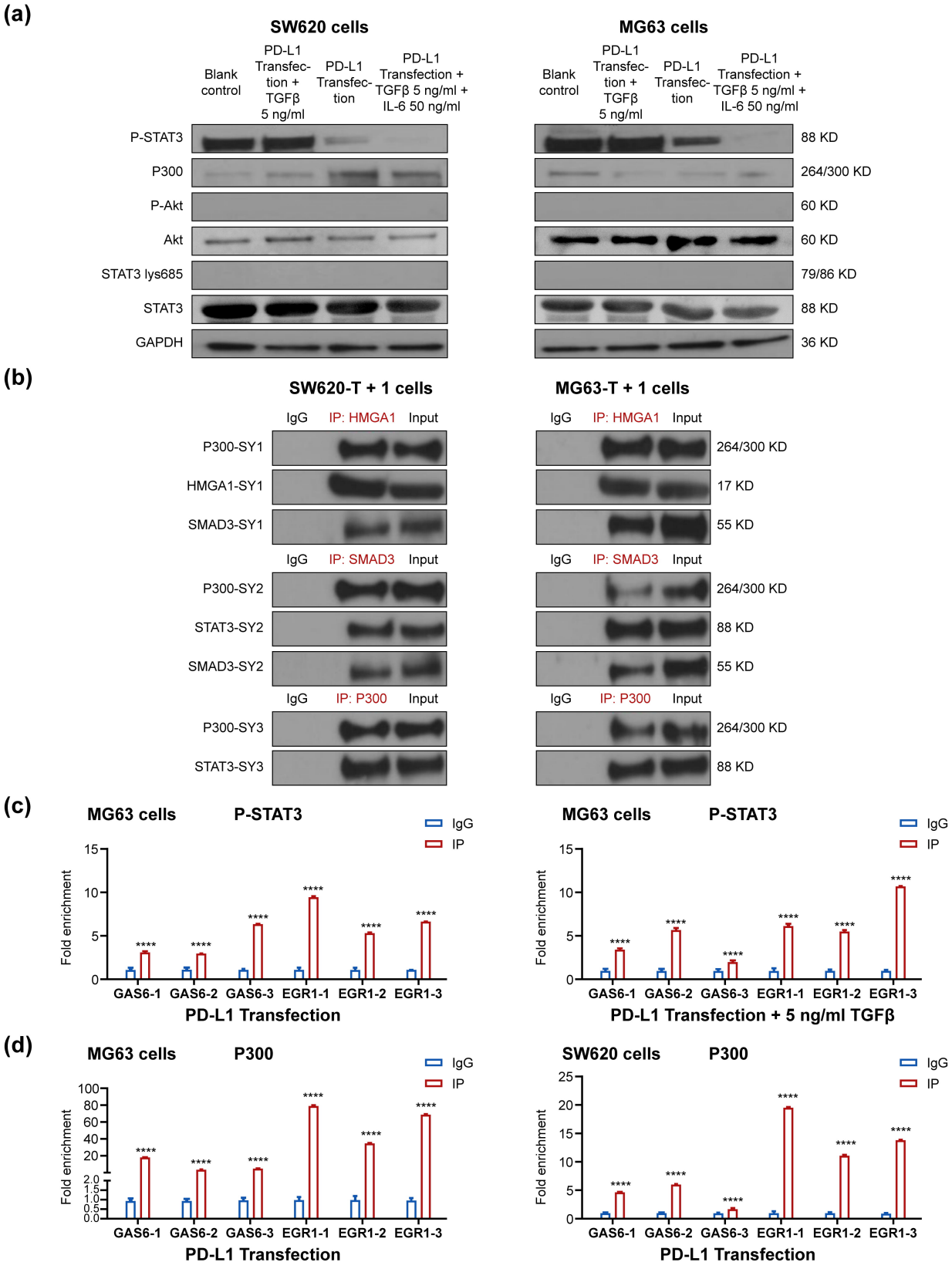

Fig. 3. SW620 and MG63 cells were transfected with PD-L1 plasmids for 48 h, 42 h followed by 5 ng/mL TGF-β for 6 h, or 45 h followed by 5 ng/mL TGF-β and 50 ng/mL for 3 h. SW620 and MG63 cells were then harvested and evaluated via Western blotting (a); CoIP assays between HMGA1-P300, SMAD3-P300 and P300-pSTAT3 (b); and ChIP-qPCR analyses using specific P-STAT3 ChIP-grade antibody (c) or P300 ChIP-grade antibody (d). IgG and input were used as negative and positive controls, respectively. ****$p <$ 0.0001.

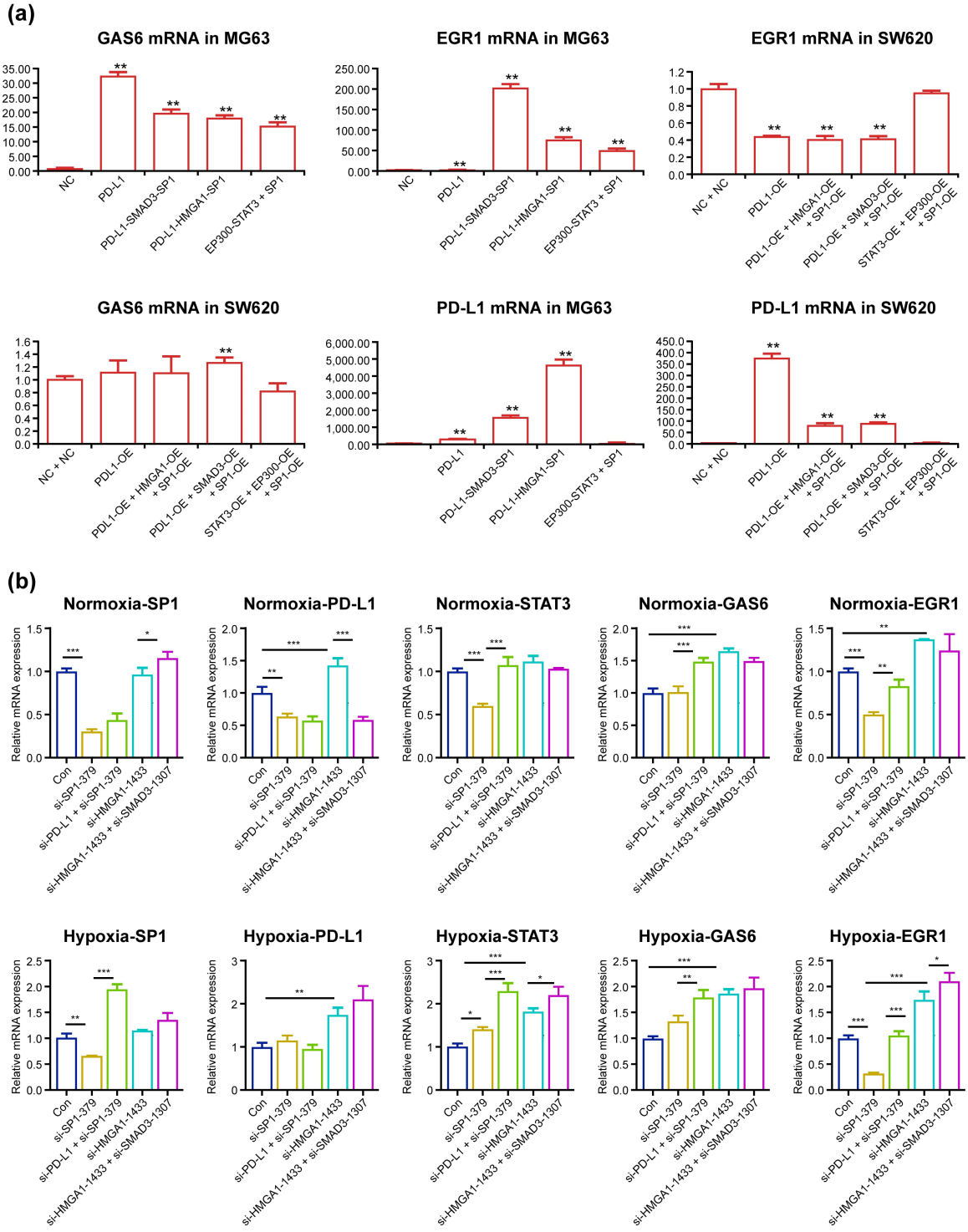

Fig. 4. GAS6 mRNA and EGR1 mRNA significantly increased after PD-L1, PD-L1–HMGA1–SP1, PD-L1–SMAD3–SP1 and P300–STAT3–SP1 over-expression and decreased after SP1, PD-L1–SP1, HMGA1 and HMGA1–SMAD3 knockdown in SW620 cells. Control plasmid or PD-L1 and HMGA1, SP1 or SMAD3 plasmids were transfected for 48 h under normoxic conditions or two cycles of 20 h under normoxic and 4 h under hypoxic conditions. SW620 and MG63 cells were then harvested and subjected to qPCR (a). Control plasmid or PD-L1 and other specific siRNAs against HMGA1, SMAD3 and SP1 were co-transfected for 48 h under normoxic conditions or two cycles of 20 h under normoxic and 4 h under hypoxic conditions. Then, SW620 cells were harvested and subjected to qPCR (b). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Details of confirmations of knockdown experiments could be found in **Supplementary Fig. 1.**

, 1.836- and 1.940-fold under hypoxic conditions, respectively (Fig. 4b).

3.6 PD-L1 ChIP-seq Newly Identified CD274 and BTRC as Target Genes, While ChIP-QPCR Validated HMGA1 and SMAD3 Enrichment at CD274 and BTRC Promoters

PD-L1 ChIP-seq using PD-L1 over-expressed SW620 cells enabled the identification of 114 target genes, in contrast to the 1144 target genes previously reported via Cut&Tag in MUM2B cells [7] (Fig. 5a–d). Among these 114 target genes, we found two interesting newly delineated genes: CD274/PD-L1 and beta-transducin repeat-containing E3 ubiquitin protein ligase (BTRC), which targets many important proteins for ubiquitination and degradation (Fig. 5e,f).

PD-L1 complexes were found to boost CD274/PD-L1 transcription, generally correlating with HMGA1, SMAD3, SP1, STAT3 and P300 up-regulation. TGF-β treatment promoted their nuclear translocation, facilitating complex assembly and transcript modulations. To further assess the binding of PD-L1 complexes with promoters, we performed ChIP-QPCR to validate that HMGA1 and SMAD3 were also enriched at the CD274/PD-L1 and BTRC promoters in a gastric cancer cell line (AGS) and a lung cancer cell line (SK-MES-1) (Fig. 5g,h). These findings partially validated our hypothesis that PD-L1 complexes modulate the expression of target genes.

4. Discussion

Nuclear expression of PD-L1 in lung, breast, colon, renal cell and hepatic cell cancers might be clinically associated with shorter survival duration or higher resistance to anti-PD1/PD-L1 therapies [9,10]. Nuclear PD-L1 has been reported to regulate caspase-8-mediated pyroptosis [11], immune-related gene expression [7], genomic stability and sister chromatid cohesion [28] and GAS6 expression through coupling with SP1 to activate MerTK signaling in lung cancer [10]. To the best of our knowledge, it has not been determined whether nuclear PD-L1 binds to DNA directly or through its interaction with transcription factors. There are 1144 PD-L1 binding sites genome-wide, according to the latest report [9]. These are mainly distributed near the transcription start sites, supporting a transcription regulatory function; however, the number of PD-L1 binding sites is much lower than that for SP1, with over 12,000 known sites [29]. Therefore, it is quite likely that there exist mediators that facilitate the PD-L1- and SP1-associated regulation of target gene transcription.

Hydrogen peroxide, serum deprivation and temozolomide have been shown to up-regulate cancer stem cell (CSC)-related genes in glioblastoma cells via SP1 [30]. Furthermore, PD-L1 expression is increased in chemo-resistant colorectal cells, promoting colorectal CSC self-renewal by up-regulating stemness genes via direct interaction with HMGA1 [19]. On the other hand, TGF-β1-induced deficiency of linc00261 negatively modulated SMAD3 phosphorylation to promote stemness of hepatocellular carcinoma [31], and TGF-β has also been shown to induce PD-L1 expression and its interaction with SMAD3, which mediates the fibroblast to myofibroblast transition in the lung [8]. Taken together, these reports suggest that PD-L1 might interact through HMGA1 and SMAD3 with SP1 to increase cancer stemness. Consistently, in our study, HMGA1 or SMAD3 antibodies pulled down PD-L1 and SP1, while PD-L1 antibody pulled down HMGA1, SMAD3 and SP1, thus supporting the idea that PD-L1 forms complexes with HMGA1–SP1 and SMAD3–SP1. We also proved that HMGA1 connects to SMAD3 for the first time, as both pulled down P300, while P300 pulled down STAT3. These data support the idea that P300 concomitantly binds HMGA1, SMAD3 and STAT3 to bridge the PD-L1–HMGA1–SP1 and PD-L1–SMAD3–SP1 complexes, while also facilitating the binding of p-STAT3 to DNA. Another possibility is that SMAD3 directly connects to HMGA1 and then, as a whole, connects to P300–STAT3; in this way, PD-L1 and SP1 binding to this complex could be reduced to one pair. This simpler structure conforms to the CoIP data, but further validation is required. Consistently, PD-L1, HMGA1, SMAD3, SP1, p-STAT3 and P300 were enriched at the GAS6 promoter in MG63 and SW620 cells. According to our analysis of mediators between PD-L1 and SP1, in order to avoid contradictory positive or negative feedback, these factors inevitably form integrated complexes. Conforming with previous reports that the over-expression of PD-L1 increased GAS6 mRNA through SP1 promoter binding [10], successive over-expression of PD-L1, PD-L1–HMGA1–SP1, PD-L1–SMAD3–SP1 and P300–STAT3–SP1 in MG63 cells significantly elevated GAS6 mRNA expression by 31-, 17-, 19- and 15-fold, respectively; while, in SW620 cells, they did not significantly elevate GAS6 mRNA expression except for PD-L1–SMAD3–SP1 (by 1.29-fold); the reason for this is discussed below (see Fig. 4).

The EGR1 promoter comprises five serum response elements and two SP1 sites [32], similarly to the Tenascin-C promoter, in which the SMAD3 complex translocates to the nucleus and binds Ets1 and SP1 to regulate gene expression in cooperation with P300 [11]. In our study, SMAD3 and HMGA1 were confirmed to concomitantly bind PD-L1 and SP1, while P300 was shown to concomitantly bind p-STAT3, HMGA1 and SMAD3. These results support the idea that P300–STAT3 joins up with the PD-L1–SMAD3–SP1 and PD-L1–HMGA1–SP1 complexes to form a whole to bind the EGR1 promoter. In the case that HMGA1 directly connects to SMAD3 as a whole to bind P300–STAT3, the combined PD-L1 and SP1 could be reduced to only one pair. PD-L1, HMGA1, SMAD3, SP1, STAT3 and P300 were consistently enriched at the EGR1 promoter in MG63 and SW620 cells. PD-L1 over-expression has been shown to activate the PI3K/AKT pathway in head and

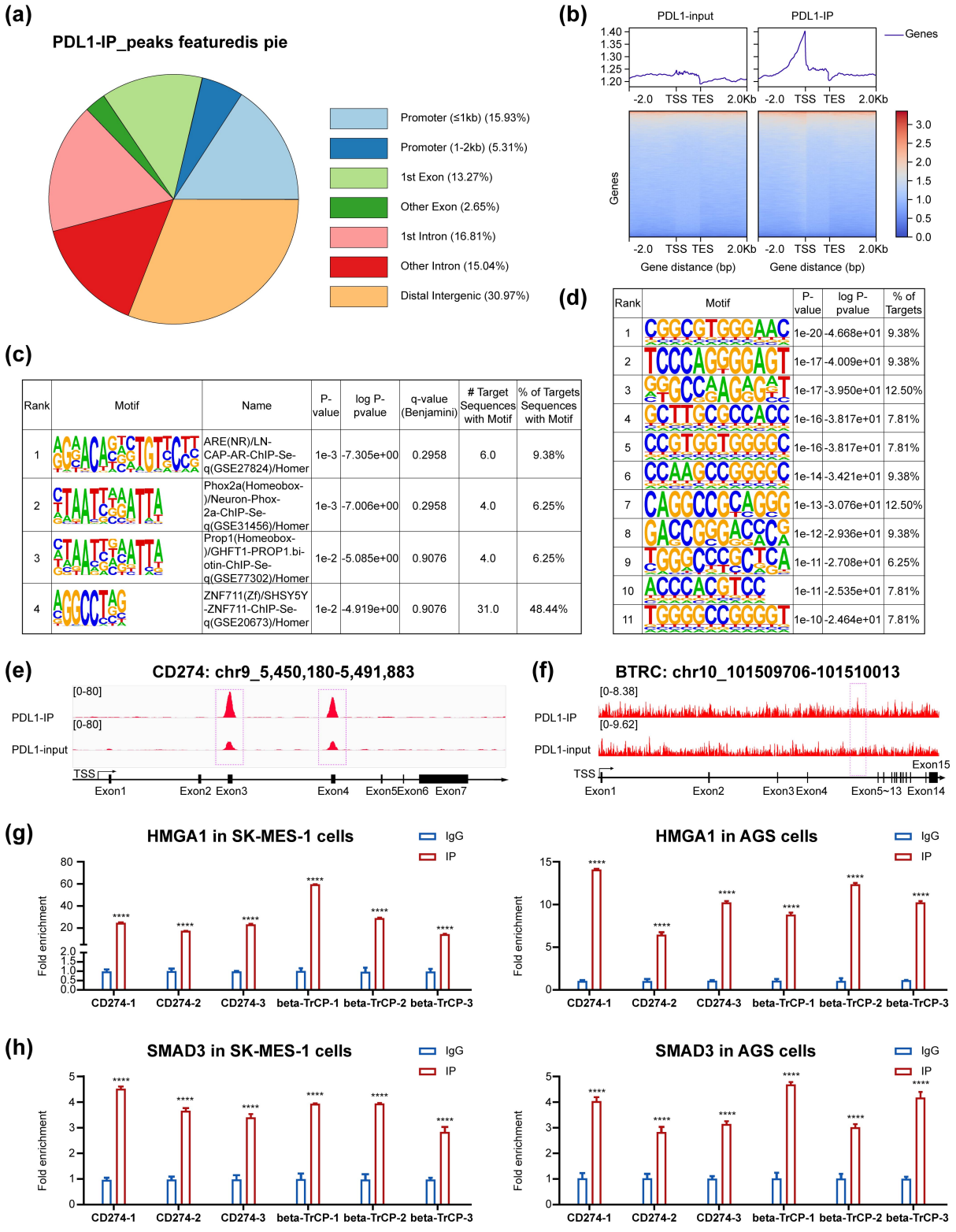

Fig. 5. The PD-L1 ChIP-seq results, *PD-L1* and *BTRC* identified as PD-L1 target genes,HMGA1 and SMAD3 enrichment in newly identified genes' promoters in two new cell-lines. PD-L1 ChIP-seq revealed 114 target genes, mostly near the transcript start site (a,b). Previously reported binding motifs were confirmed in our study, with no new special binding motifs identified (c,d). PD-L1 was newly found to be enriched at the CD274 and BTRC promoters (e,f), and concomitant enrichment of HMGA1 and SMAD3 was also validated at these promoters (g,h). BTRC, beta-transducin repeat containing E3 ubiquitin protein ligase. ****$p < 0.0001$.

neck squamous cell carcinoma. This pathway is also triggered by IL-6, leukemia inhibitory factor (LIF) and oncostatin M, leading to P300-mediated acetylation of STAT3 at Lys685, which enhances its DNA binding affinity and transcriptional activity [25]. In contrast, we did not detect STAT3 Lys-685 acetylation and p-AKT after PD-L1 over-expression with/without TGF-β or IL-6 stimulation in SW620 and MG63 cells. Therefore, these effects might be context- or cell-type-dependent, and were not supported by our experimental results. Comparably, HMGA1 has been reported to bind the minor groove of DNA, recruit transcription factors to the major groove and interact to stabilize the enhanceosome [33]. In addition, interactions between the P300 bromodomain and STAT3 NH2-terminal domain might help to stabilize the enhanceosome, which further recruits RNA polymerase II holoenzyme to accomplish the initiation of transcription [34]. In line with the former report that PD-L1 over-expression increased EGR1 expression with the help of p-STAT3, successive over-expression of PD-L1, PD-L1–HMGA1–SP1, PD-L1–SMAD3–SP1 and P300–STAT3–SP1 in MG63 cells significantly elevated EGR1 mRNA expression by 5-, 77-, 204- and 51-fold, respectively. In dramatic contrast, over-expression of former three abovementioned molecules in SW620 cells significantly decreased EGR1 mRNA expression by 0.44-, 0.40- and 0.42-fold, respectively, while P300–STAT3–SP1 over-expression resulted in no significant change; the reason for this is discussed below.

The PD-L1 complexes dramatically elevated GAS6 and EGR1 mRNA in MG63 cells, but only one group slightly increased GAS6 mRNA, and all (except one) did not decrease EGR1 mRNA in SW620 cells in a significant manner. Although the underlying reasons for these discrepancies are currently unknown and warrant further investigation, we offer some explanations here. The existence of RNA polymerase II holoenzyme assembly, initiation repressing [35] or transcription silencing [36] mechanisms might underlie the transcript differences in MG63 and SW620 cells. Furthermore, the PD-L1 complex formation process might incorporate or upregulate some transcription factors, leading to down- or up-regulation of target gene transcription. When the transcription enhancement mechanism is at work, the mRNA expression levels increased by more than 5-fold; meanwhile, when the enhancement mechanism was impeded, with regard to whether the transcript factors were incorporated or released, the mRNA expression levels fluctuated between 0.40- and 2-fold. Our PD-L1 ChIP-seq results revealed for the first time that PD-L1 was enriched at the CD274/PD-L1 gene promoter, and HMGA1 and SMAD3 were also newly found to be enriched at the same promoter, thus confirming the existence of our proposed complex. Accordingly, PD-L1 mRNA considerably increased in PD-L1, PD-L1–HMGA1–SP1 and PD-L1–SMAD3–SP1 groups in MG63 and SW620 cells. The aforementioned treatments dramatically elevated PD-L1 transcription in MG63 cells by 384-, 4647- and 1628-fold ($p < 0.01$), and in SW620 cells by 15573-, 11907- and 2316-fold ($p < 0.01$), while P300–STAT3–SP1 slightly decreased it ($p > 0.05$).

Knockdown of SP1 and PD-L1–SP1 significantly reduced SP1 and PD-L1 expression under normoxic conditions, while knockdown of both HMGA1 and HMGA1–SMAD3 elevated GAS6 and EGR1 mRNA under normoxic conditions; this is likely due to knockdown of HMGA1 elevating PD-L1 mRNA expression through some unknown compensation mechanism. Under hypoxic conditions, PD-L1 mRNA expression was elevated with the knockdown of HMGA1 and HMGA1–SMAD3, likely through Hypoxia-inducible Factor-1 binding with the PD-L1 gene promoter, which accordingly increased GAS6 and EGR1 mRNA levels. Intriguingly, under both conditions, knockdown of SP1 in SW620 cells significantly reduced EGR1 mRNA ($p < 0.05$) but did not alter GAS6 mRNA ($p > 0.05$).

Taken together, despite the complexity of the proposed concept and the crudeness of our methods, our findings support a novel model in which PD-L1, SMAD3, SP1 and HMGA1 complexes and P300–STAT3 form an expedient complex to bind the GAS6 and EGR1 promoters in SW620 and MG63 cells in order to modulate mRNA transcription. Consistent with this model, PD-L1, HMGA1 and SMAD3 were confirmed for the first time to be enriched at the newly identified CD274/PD-L1 and BTRC gene promoters. These novel findings validate our proposed complex, which dramatically boosted PD-L1 mRNA expression in the PD-L1, PD-L1–HMGA1–SP1 and PD-L1–SMAD3–SP1 groups and slightly decreased it in the P300–STAT3 group in MG63 and SW620 cells. The associated effects on BTRC expression and functions warrant further investigation. Our identification of PD-L1 enrichment at the CD274 and BTRC promoters not only evidences an unprecedented self-reinforcing transcriptional circuitry driving PD-L1 expression, but also opens new avenues for targeting this regulatory axis to overcome immune evasion in cancer through the modulation of PD-L1 protein homeostasis, inducing its targeted degradation or inhibiting its nuclear transport [37]. The discovery of PD-L1-mediated regulation of BTRC—a key modulator of protein stability and signaling cascades—provides a novel mechanistic link between immune checkpoint control and cellular degradation machinery, suggesting potential dual-therapeutic strategies for aggressive cancers [38]. By elucidating the cooperative binding of PD-L1-containing complexes to the GAS6 and EGR1 promoters, our work establishes a foundational framework for exploiting transcriptional co-regulators in high-PD-L1 tumors, thereby paving the way for next-generation epigenetic and immune combination therapies [39,40]. The accurate structure of the expediently modeled PD-L1 complex and its precise mode of action in conjunction with RNA polymerase II to modulate gene transcription are still in the initial stage of exploration. However, these insights

are expected to pave the way for further research on PD-L1-regulated immune and non-immune genes, which might lead to innovation in PD-L1-targeted therapies for cancer and sarcoma.

5. Conclusions

Our findings support a model wherein a complex comprising HMGA1, SMAD3, SP1 and PD-L1 binds to the promoters of 114 target genes—including GAS6, EGR1 and CD274 (PD-L1 itself)—to regulate their expression. Although this proposed mechanism provides a foundational framework for understanding this regulatory axis, further validation is required to fully elucidate the composition and functional dynamics of the proposed complex.

Availability of Data and Materials

The relevant data are presented in the five figures and the **Supplemnentary Material**. All data are available from the corresponding authors by reasonable request.

Author Contributions

WL: conception of the work. WL and HG: composition of the figures. WL, PT: acquisition, analysis, and interpretation of the literature. WL: drafting and revising the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

We thank all the reviewers for contributing to this article. We would like to express our gratitude to all those who helped us during the experiments and the writing of this manuscript.

Funding

Funding was obtained from Wei Liu, Hunan Provincial Health Commission Foundation of China (No.20257169).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL46743.

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