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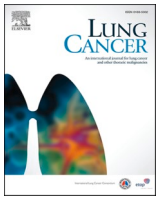

Chemotherapy-induced PDL-1 expression in cancer-associated fibroblasts promotes chemoresistance in NSCLC

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ABSTRACT

Objectives: A cure for cancer is out of reach for most patients due to chemoresistance. Cancer-associated fibroblasts (CAFs) play a vital role in cancer chemoresistance, but detailed understanding of the process particularly in chemoresistant lung cancer is lacking. In this study, we investigated programmed death-ligand 1 (PDL-1) as a potential biomarker for CAF-induced chemoresistance and evaluated its role and the underlying mechanisms of chemoresistance in non-small cell lung cancer (NSCLC).

Materials and Methods: A systemic search of gene expression profiles of multiple tissues in NSCLC was carried out to determine the expression intensities of traditional fibroblast biomarkers and CAF-secreted protumorigenic cytokines. PDL-1 expression in CAFs was analyzed by ELISA, Western blotting, and flow cytometry. Human cytokine array was used to identify specific cytokines secreted from CAFs. Role of PDL-1 in NSCLC chemoresistance was assessed using CRISPR/Cas9 knockdown and various functional assays including MTT, cell invasion, sphere formation, and cell apoptosis. *In vivo* experiments were conducted using a co-implantation xenograft mouse model with live cell imaging and immunohistochemistry.

Results: We demonstrated that chemotherapy-stimulated CAFs promoted tumorigenic and stem cell-like properties of NSCLC cells, which contribute to their chemoresistance. Subsequently, we revealed that PDL-1 expression is upregulated in chemotherapy-treated CAFs and is associated with poor prognosis. Silencing PDL-1 expression suppressed CAFs' ability to promote stem cell-like properties and invasiveness of lung cancer cells, favoring chemoresistance. Mechanistically, an upregulation of PDL-1 in chemotherapy-treated CAFs led to an increase in hepatocyte growth factor (HGF) secretion, which stimulates cancer progression, cell invasion, and stemness of lung cancer cells, while inhibiting apoptosis.

Conclusion: Our results show that PDL-1-positive CAFs modulate stem cell-like properties of NSCLC cells by secreting elevated HGF, thereby promoting chemoresistance. Our finding supports PDL-1 in CAFs as a chemotherapy response biomarker and as a drug delivery and therapeutic target for chemoresistant NSCLC.

1. Introduction

Lung cancer is rising in prominence as a leading cause of death, accounting for 26.5% of all cancer-related deaths [1,2]. Despite the recent advances in clinical research, the prognosis for non-small cell lung cancer (NSCLC) remains poor, and the five-year survival rate for lung cancer is approximately 15% [3]. The key factor that limits the therapeutic success of lung cancer treatment is chemoresistance [4,5]. While tumor cells' autonomous mechanisms have been extensively studied as a primary cause for chemoresistance, tumor microenvironment (TME) has emerged as a key player in chemoresistance and recurrence of cancer [6,7].

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As a key component of the TME, CAFs have been implicated in promoting and enabling chemoresistance in multiple cancer types, including breast cancer [8], squamous cell carcinoma [9], ovarian cancer,[10] colorectal cancer [11], and lung cancer [12]. The pro-tumorigenic functions of CAFs are primarily driven by CAF-secreted growth factors and cytokines, such as insulin-like growth factor (IGF), hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β), interleukin (IL)-6,-10, C-X-C motif chemokine ligand 12 (CXCL12) [13,14]. Evidence suggests that CAF-secreted factors and specific ECM remodeling features linked with it could contribute to chemoresistance by promoting dissemination and invasion of cancer cells via ECM remodeling and degradation [15,16]. Therefore, targeting CAFs is emerging as a new avenue to enhance the efficacy of current cancer therapies.

Though CAFs implicate a strong tumor-modulating effect, a precise molecular definition of CAFs is still debatable due to the lack of CAF-specific markers [14]. A number of traditional CAF biomarkers, including α-smooth muscle actin (α-SMA), podoplanin (PDPN), or fibroblast activation protein (FAP) have been widely used to isolate the CAF population [17]. However, there is a concern about the ability of these markers to distinguish CAFs from other tissue types due to their relatively low specificity [18]. As a result of the advancement in single-cell sequencing, novel biomarkers are being discovered to define CAF heterogeneity. For example, programmed death ligand 1 (PDL-1), also known as CD274, has been suggested as a new CAF biomarker in NSCLC [19] and breast cancer [20], and studies have confirmed its role as a "prognostic biomarker".

PDL-1 has also gained significant interest as a biomarker for chemotherapy and targeted therapy due to improved clinical outcomes of PD-1/PDL-1 immunotherapy [21]. Although the expression of PDL-1 on some stromal cell types such as macrophages and tumor-infiltrating lymphocytes has been determined in the context of tumor progression [22], PDL-1 expression in CAFs and its role in chemoresistance and recurrence of NSCLC has never been studied. We, therefore, evaluated the PDL-1 expression in CAFs from human NSCLC and deciphered its functional role in CAF-mediated chemoresistance. We found that chemotherapy potently upregulates PDL-1 expression in CAFs. We also revealed that PDL-1-positive CAFs regulate stem cell-like properties of lung cancer cells that contribute to chemoresistance of NSCLC through a mechanism that involves HGF secretion. The results of this study suggest that PDL-1 on CAFs can serve as a potential therapeutic target for chemoresistant NSCLC in addition to its role as a clinical biomarker.

2. Materials and Methods

2.1. Cell culture

Human NSCLC cell lines (H460 and PC9) and HEK-293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Lung CAFs and normal fibroblasts (NFs) utilized in this study were derived from primary culture and were generously gifted to us by the Weizmann Institute of Science (Rehovot, Israel)[23]. The cells were isolated from newly diagnosed lung cancer patients with the method of enzymatic digestion[23]. NSCLC cells were cultured in RPMI-1640 (Corning, Corning city, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, New York, NY) and 1% penicillin and streptomycin (P/S) (Gibco, New York, NY). DMEM supplemented with 10% FBS was used to culture HEK-293 T cells.

2.2. Collection of conditioned media (CM)

For the generation of CMs, CAFs and NFs were seeded into six-well plates at 0.8×10^5 cells per well density. After adhesion of the cells to the plate overnight, cells were treated with 1 μM cisplatin (Sigma-Aldrich, St Louis, MO) or its vehicle (0.9% NaCl) as well as 0.5 μM gefitinib (MedChemExpress, Monmouth Junction, NJ) or its vehicle (10 mM DMSO in culture medium). CMs from CAFs and NFs treated with chemotherapy, or their corresponding vehicles were collected after 48 hrs. and filtered through a 0.2 μm filter.

2.3. Pre-incubation of NSCLC cells with fibroblast-derived CMs

Before being utilized in functional studies, including ABCG2 protein analysis, NSCLC cells were incubated with fibroblast-derived CMs (CAFs or NFs). As a first step, H460 and PC9 cells were seeded into 6-well plates at a density of 0.15×10^5 cells per well. The following day, culture medium was replaced with fresh medium or combination with fibroblast-derived CM at a 3:1 ratio (2 ml/well) and incubated for 48 hrs. in a humidified incubator containing 5% CO_2 at 37 ℃. Exogenous HGF (10 ng/ml: PeproTech, Cranbury, NJ Cat: 100-39H-25UG) was added to CM obtained from chemotherapy-treated CAFs with or without PDL-1 knockdown prior to being utilized in rescue experiments. Before being used in tumorsphere formation and cell invasion assays, pre-incubated H460 and PC9 cells were treated with cisplatin or gefitinib. NSCLC cells treated with their corresponding vehicles were used as control untreated cells.

2.4. Indirect co-culture assay using conditioned medium and transwell system.

NSCLC cells, which had been pre-incubated with fibroblast-derived CM, were seeded in 96-well plates at a density of 3,000 cells/well. A combination of RPMI 1640 and fibroblast-derived CM at a 3:1 ratio was utilized as the culture medium. For the Transwell assay, NSCLC cells (lower chamber) were co-cultured with fibroblasts treated with chemotherapy or vehicle (upper chamber) in a transwell system featuring inserts with a 0.4 μm pore size polycarbonate membrane (Corning, Corning city, NY). The cell viability was detected by MTT assay (Tokyo Chemical Industry Co. Ltd, Portland, OR) following the manufacturer's instructions after 48 hrs. of chemotherapy. IC_{50} values were calculated using GraphPad Prism 5.0 software (La Jolla, CA, USA).

2.5. Tumorsphere formation assay

Chemotherapy-treated NSCLC cells were seeded in ultra-low 24-well plates (Corning, Corning city, NY) at a density of 200 cells/well. The cells were maintained in a semi-solid medium (MethoCult H4100; STEMCELL Technologies, Canada) containing serum-free DMEM/F-12 medium (HyClone Laboratories, Inc. Logan, UT) supplemented with 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich, St Louis, MO), 20 ng/ml basic fibroblast growth factor (bFGF) (Gibco, New York, NY), and 4 μg/ml insulin (Gibco, New York, NY). After two weeks of incubation at 37 °C in 5% CO_2, tumorspheres were photographed and enumerated.

2.6. Invasion assay

Transwell chambers with 8 μm pore polyester membrane inserts (Corning, Corning city, NY) were used for cell invasion assay. The top chambers were coated with Matrigel (Corning, Corning city, NY). The upper compartment of the transwell chamber was seeded with 3×10^4 cells, while the lower compartment was filled with RPMI 1640, either alone or mixed with fibroblast-derived CM in a 3:1 ratio. After 24 hrs, filters were fixed with ice-cold methanol: acetone (1:1) for 5 mins and stained with crystal violet (Sigma Aldrich, St. Louis, MO) for 30 mins. Cells that had not invaded were wiped away with a cotton swab. Invading cells were imaged under a microscope.

2.7. Cell apoptosis assay

Cell apoptosis was evaluated using an annexin V-FITC apoptosis detection kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, pre-incubated NSCLC cells were treated

with chemotherapy for 48 hrs. Cells treated with their respective vehicles are referred as untreated cells (control). Afterward, the cells were harvested, washed with PBS (HyClone Laboratories, Inc. Logan, UT), and resuspended in annexin-V binding buffer. Annexin V-FITC and propidium iodide (PI) were added to the cells and incubated for 15 mins at room temperature in the dark. Apoptosis was quantified by flow cytometry.

2.8. Western blot analysis

After specific treatments, cells were lysed with lysis buffer (PhosphoSolutions, Aurora, CO) containing 100 mM phenylmethylsulfonyl fluoride (PMSF; Roche Diagnostics, Germany) and protease inhibitor (Roche Diagnostics, Germany). Protein lysate (30–50 μg) was then separated by running SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Inc Hercules, CA). After blocking with 5% nonfat dry milk (Bio-Rad Laboratories, Inc Hercules, CA), immunodetection was performed using primary and HRP-conjugated secondary antibodies (Sigma-Aldrich, St Louis, MO), followed by detection with an enhanced chemiluminescence detection system. Protein levels were normalized to β-actin levels. The primary antibodies used in Western blotting were as follows: ABCG2 (Sigma Aldrich, St. Louis, MO), PDL-1 (Millipore Sigma, Temecula, CA), and β-actin (Santa Cruz Biotechnology, Inc. Dallas, TX).

2.9. Elisa

Human PDL-1 ELISA kit (Proteintech Group, Inc Rosemont, IL) and human HGF ELISA kit (R&D systems, Minneapolis, MN) were used in accordance with the manufacturer's instructions to quantify PDL-1 and HGF concentrations in CAF-derived CM.

2.10. Cytokine array

The cytokine secretion of CAFs, with or without cisplatin treatment, was assessed using Human cytokine array C1000 (Raybiotech, Norcross, GA) according to the manufacturer's instructions. Briefly, antibody arrays were incubated with blocking buffer for 30 mins and subjected to incubation with samples for 2 hrs. at room temperature. The arrays were then incubated with biotin-conjugated antibodies, followed by HRP-streptavidin. Lastly, membranes were visualized using a chemiluminescence imaging system.

2.11. PDL-1 surface marker analysis

The surface expression of PDL-1 on CAFs treated with or without chemotherapy was assessed by incubating the cells with APC anti-human CD274 antibody (BioLegend, San Diego) for 30 min at 4 °C in the dark. After incubation, cells were washed and fixed with 0.4% paraformaldehyde overnight at 4 °C (Thermo Fisher Scientific, Fair Lawn, NJ). PDL-1 cell surface expression was then detected with a BD LSRFortessa flow cytometer.

2.12. CRISPR/Cas9-mediated gene knockdown

For knockdown of PDL-1 expression, pLentiCRISPR v2 plasmids carrying a single guide RNA (sgRNA) targeting a specific gene CD274 (TACCGCTGCATGATCAGCTA) were purchased from Genscript (Piscataway, NJ). An empty pLentiCRISPR v2 vector was used as a negative control. The recombinant plasmid, together with packaging (pCMV-dR8.2 dvpr) and envelop plasmids (pCMV-VSV-G) (Addgene, Watertown, MA) were transfected into HEK-293 T cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) to generate lentiviral particles. The resulting lentivirus supernatant was collected and stored at − 80 °C for future use in the transduction of CAFs. In the presence of hexadimethrine bromide (polybrene; Sigma Aldrich, St. Louis, MO), CAFs were transduced with lentivirus to boost the transduction efficiency. Stable cell lines were generated by treating cells with puromycin (3 μg/ml; Gibco, New York, NY) for at least 2 weeks. The CAFs that were stably infected with CD274 lentivirus were referred to as sgPDL-1 and their controls were referred to as sgCtrl.

2.13. In vivo tumor studies

All animal experiments were performed in accordance with Guidelines for Animal Experiments at West Virginia University with the approval of the Institutional Animal Care and Use Committee (#1702005551_R1_1). Male athymic nude mice (4 weeks old) were purchased from Jackson Laboratory (ME, USA) and fed a standard chow diet. Mice were randomly assigned to three groups. In the control group, 1×10^6 luciferase-tagged H460 cells were injected subcutaneously into the flank of mice, whereas in the co-culture groups, mice were co-injected with 1×10^6 luciferase-tagged H460 cells and 3×10^6 CAFs transfected with sgCtrl or sgPDL-1 at a ratio of 1:3. When the tumor volume reached approximately 50 mm^3 in size, mice were randomly assigned to treated and control groups (n = 4–5). Mice in the treated group were injected with cisplatin (4 mg/kg every 3 days for 3 weeks) intraperitoneally, whereas the control group was injected with normal saline. The aforementioned doses of cisplatin were reported to be effective and safe by previous studies [24]. Until mice were sacrificed, tumor size was measured using a caliper every week. Tumor volume was calculated using the standard formula: tumor volume(mm^3) = $d^2 \times D/2$, where d represents the shortest diameter and D represents the longest diameter. Luciferase expression in mice was quantified with the Living Image software (PerkinElmer).

2.14. Immunohistochemistry imaging

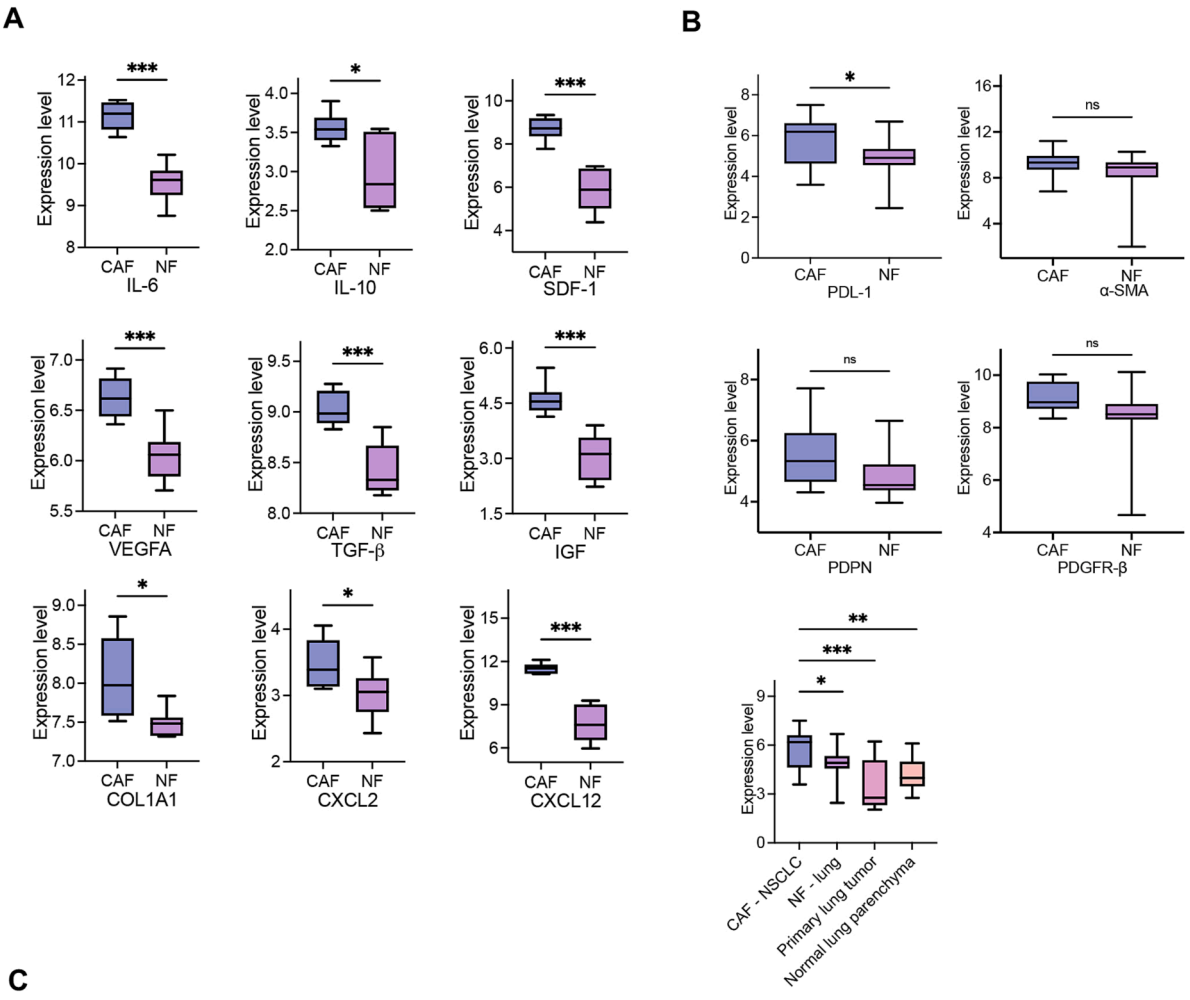
Excised tumors from cisplatin-treated and control mice were fixed in 4% formalin and embedded in paraffin. Sections were counterstained with hematoxylin to observe pathological differences in tissue sections. The slides were immunohistochemically stained according to the manufacturer's instructions. Ki-67 (Invitrogen, Carlsbad, CA), ABCG2 (Abcam, Cambridge, UK), and HGF (Thermo Fisher Scientific, Fair Lawn, NJ) antibodies were used in the experiments. Immunohistochemical signal intensity and positively stained field of tissue sections were analyzed by Image J.

2.15. Data collection

A systemic search of gene expression profiles of multiple tissues in NSCLC was carried out using two gene datasets, GSE22874[25] and GSE164750[26], which are publicly available in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds). The expression levels of genes that regulate tumor-promoting properties of CAFs in NSCLC were identified using GEO2R tool with GSE164750 data set. The GSE22874 data set was used to determine the expression intensities of genes that encode traditional fibroblast biomarkers between CAFs and NFs, and novel PDL-1 biomarker between fibroblasts and NSCLC tissues. To perform a more robust survival analysis, forest plots were constructed based on the summary of data obtained from previous studies [19,27]. These plots depict the hazard ratios (HR) of fibroblast markers for recurrence-free survival (RFS), along with the associated 95% CI and P value. A univariate cox proportional hazards model was utilized in the aforementioned studies to estimate the association between fibroblast marker expression and NSCLC patients' prognosis. A hazard ratio (HR) > 1 is considered as a risk factor for poor survival.

2.16. Statistical analysis

Statistical significance was calculated with GraphPad Prism 7.0. Non-parametric tests (*t*-test and one-way ANOVA) were performed to



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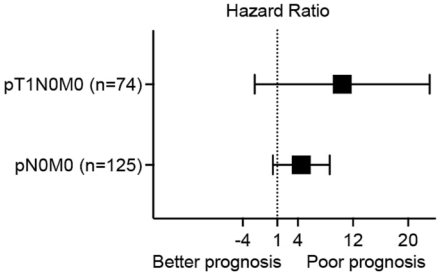
	Marker	Hazard ratio (95% CI)	P value
Teramoto et al. 2019	PDL-1	5.275 (1.118 – 24.899)	0.036
		3.225 (1.144 - 9.086)	0.027



	Marker	Hazard ratio (95% CI)	P value
Irvine et al. 2021	FAP	2.25 (1.39–3.63)	0.001
	α-SMA	5.38 (3.34–8.67)	< 0.001
	PDPN	2.19 (1.54–3.12)	< 0.001

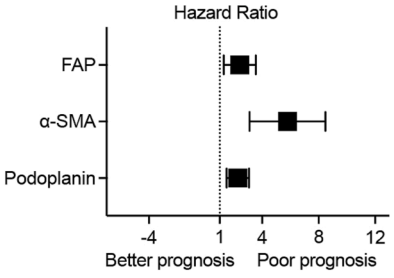


Fig. 1. PDL-1 is overexpressed in CAFs and associated with poor prognosis of NSCLC. (A) Boxplots of differential expression levels between CAFs and NFs on genes that encode secreted proteins. Results show increased pro-tumorigenic cytokines levels in CAFs compared to NFs. *p < 0.1, ***p < 0.001. (B) Boxplots represent the expression of CAF biomarkers at the gene level in CAFs compared to NFs (upper panel), primary tumor, and normal lung parenchyma (lower panel). *p < 0.1, **p < 0.01, ***p < 0.001. (C) Forest plot describing the summary results of the adjusted prognostic effect (HR) of PDL-1 protein, as extracted from the study by Teramoto and colleagues[19] (upper panel). The results are shown based on the "multivariate analysis on relapse-free survival after surgery". Forest plot displaying the summary results for univariate analysis of the RFS/DFS outcome group for CAF biomarkers (lower panel). The reported data was taken from the review of Irvine and colleagues[27]. The current data suggest that PDL-1 was linked to poor prognosis and could serve as a prognostic marker for NSCLC patients. HR: Hazard Ratio RFS/DFS: recurrence-free survival/disease-free survival.

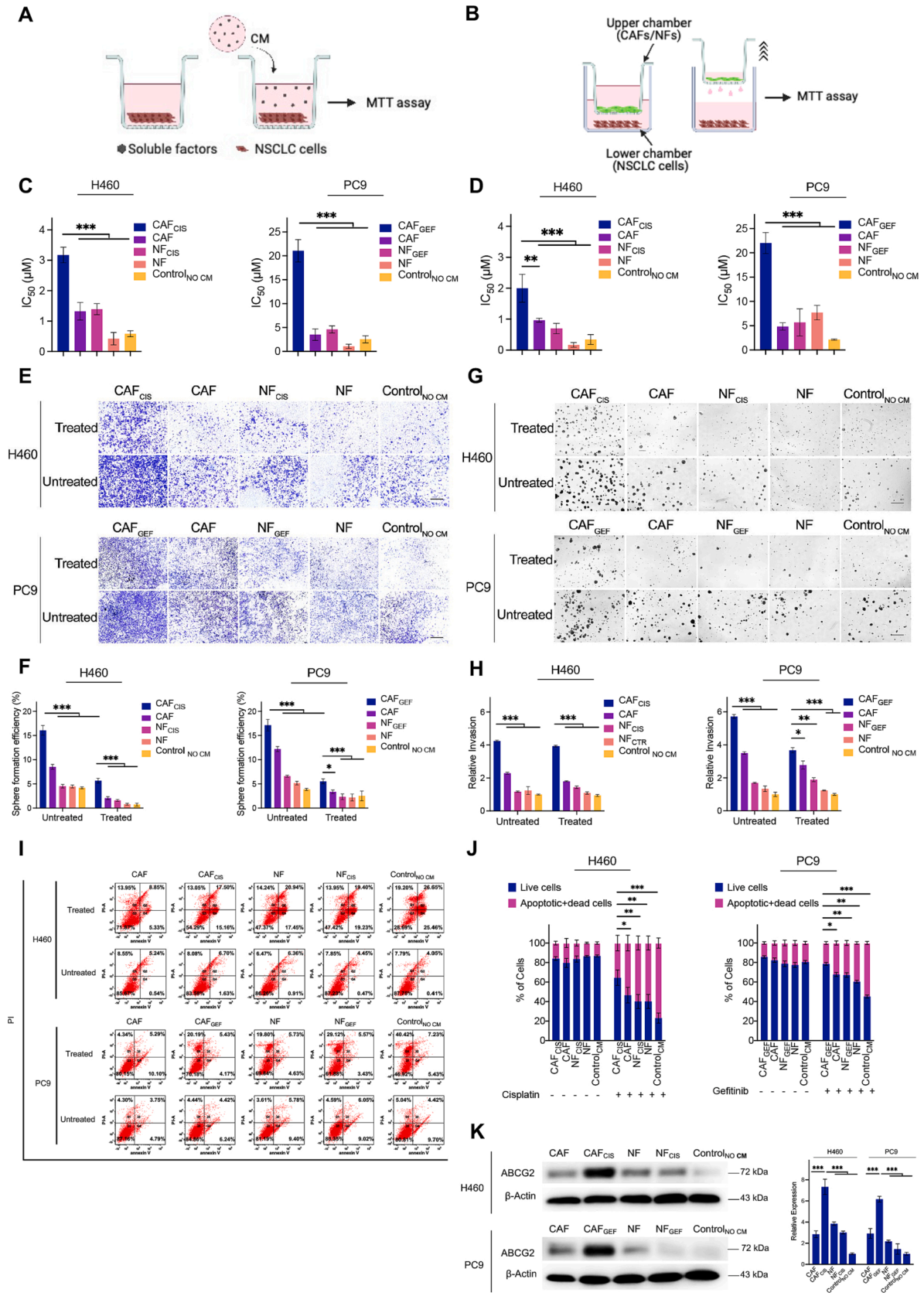


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Fig. 2. Chemotherapy-treated CAFs induce chemoresistance in NSCLC cells. (A) Schematic image of the conditioned medium (CM) assay. (B) Schematic image of the Transwell assay. (C) H460 and PC9 cells displayed increased IC_{50} value and chemoresistance for CAF_{CIS} and CAF_{GEF}. (D) The bar charts display increased IC_{50} value and resistance to cisplatin and gefitinib when co-cultured with chemotherapy-treated CAFs. Cell viability was assessed by MTT assay and IC_{50} values were calculated with GraphPad Prism 7 software. n = 3. **p < 0.01, ***p < 0.001. (E) Sphere forming assay was performed to analyze the effect of chemotherapy-treated CAFs on stem-cell properties of H460 and PC9 cells. Scale bars = 100 μm. (F) The bar charts show that CAF_{CIS} and CAF_{GEF} significantly increased the number of formed spheres for NSCLC cells with or without treatment. n = 3. *p < 0.1, ***p < 0.001. (G) Transwell assays analyzed the invasion of NSCLC cells that were pre-incubated with chemotherapy-treated CAFs CMs. Scale bars = 100 μm. (H) The bar charts show that cell invasion of treated and untreated NSCLC cells is enhanced by CAF_{CIS} and CAF_{GEF}. n = 3. *p < 0.1, **p < 0.01, ***p < 0.001. (I) Apoptotic cells were detected by Annexin V-FITC and PI double staining and analyzed by flow cytometry. (J) The bar charts show that co-culture with CAF_{CIS} and CAF_{GEF} reduced cisplatin and gefitinib-induced apoptosis, respectively. n = 3. *p < 0.1, **p < 0.01, ***p < 0.001. (K) Western blot results showed increased expression of ABCG2 in H460 and PC9 cells that were incubated with CAF_{CIS} or CAF_{GEF} CM. n = 3. ***p < 0.001. CAF_{CIS}: cisplatin-treated CAFs; CAF_{GEF}: gefitinib-treated CAFs.

compare the difference between multiple groups. Data are presented as means ± standard deviation. Differences were statistically significant when the p value was <0.05 (*p < 0.05, **p < 0.01, and ***p < 0.001).

3. Results

3.1. CAFs promote tumor progression and PDL-1-positive CAFs are associated with poor prognosis in NSCLC patients

Our analysis using GEO2R revealed that CAFs secrete elevated levels of stimulating growth factors (TGF-β, IGF), proinflammatory cytokines (IL-6, IL-10, vascular endothelial growth factor (VEGFA), collagen type I alpha 1 chain (COL1A1)), and chemokines (CXCL2, CXCL12, stromal cell-derived factor 1 (SDF-1)) compared to NFs. (Fig. 1A). Though CAFs implicate a strong tumor-modulating effect, a precise molecular definition of CAFs is still ill-defined due to the lack of CAF-specific markers [14]. To address this, we assessed the expression level of genes that encode fibroblast biomarkers in CAFs compared to NFs. Traditional biomarkers of CAFs, such as α-SMA, PDPN, and PDGFR-β [17], were highly expressed in both CAFs and NFs (Fig. 1B upper panel). However, PDL-1 biomarker was significantly increased in CAFs compared to NFs, primary lung tumor cells, and normal lung parenchyma (Fig. 1B lower panel). Also, our findings indicated that there was no significant difference in PD1 expression between CAFs and NFs (Supplementary Fig. 1). CAF biomarkers were further analyzed for prognostic information in NSCLC. Multivariate analysis of RFS for different CAF markers was reported by Irvine *et al.* 2021. Results showed that as traditional CAF biomarkers, a high level of PDL-1-positive CAFs was correlated with worse prognosis in NSCLC patients [19] (pN0M0; HR: 3.225,95% CI: 1.144–9.086, P = 0.027, pT1N0M0; HR:5.275,95% CI: 1.118–24.899, P = 0.036) (Fig. 1C). This gene expression data of CAFs suggests that PDL-1 is an important prognostic biomarker for lung cancer, and it may be correlated with CAF-induced chemoresistance.

3.2. Chemotherapy-treated CAFs induce chemoresistance of NSCLC cells in vitro

We investigated the impact of chemotherapy-treated CAFs on chemoresistance of NSCLC cells by performing key functional assays. We established a model by incubating NSCLC cells (H460 and PC9) with CM from CAFs treated with cisplatin (CAF_{CIS}), gefitinib (CAF_{GEF}), or their corresponding vehicles (CAF: 0.9% NaCl or DMSO) (Fig. 2A). In parallel, RPMI 1640 medium or CM from NFs treated with chemotherapy or vehicle were used as controls. IC_{50} results indicated that chemotherapy-treated CAFs increased chemoresistance of NSCLC cells compared to vehicle-treated CAFs, NFs, or RPMI 1640 medium (Fig. 2C). Similar results were observed with the Transwell co-culture assay of CAFs and NSCLC cells. Both H460 and PC9 cells exhibited a modest increase in IC_{50} when co-cultured with chemotherapy-treated CAFs (Fig. 2B, D), demonstrating a positive impact of chemotherapy-stimulated CAFs on tumor cell fate. One major contributor to chemoresistance is the existence of cancer stem cells [28,29]. To test the potential role of chemotherapy-treated CAFs in regulating stem cell-like properties of NSCLC cells, we performed tumorsphere formation and cell invasion assays. In concordance with cell viability results, chemotherapy-treated CAFs significantly boosted the number of tumorspheres formed in H460 and PC9 cells regardless of their exposure to chemotherapy (Fig. 2E, F). Consistent with the previous findings, chemotherapy-treated CAFs significantly enhanced the invasiveness of NSCLC cells regardless of being treated with or without chemotherapy (Fig. 2G, H). The annexin-V binding assay was performed to determine the apoptotic nature of chemotherapy-induced cell death. The results showed that chemotherapy-induced apoptosis of NSCLC cells was significantly reduced when the cells were pre-incubated with chemotherapy-treated CAFs as compared to other control groups (Fig. 2I, J). Protein expression of lung CSC marker ABCG2 was analyzed using Western blot assay. The expression of ABCG2 was dramatically increased in H460 and PC9 cells when they were pre-incubated with chemotherapy-treated CAFs, whereas other control groups showed no apparent changes after incubation (Fig. 2K). Taken together, these results in two exposure models illustrate that chemotherapy-stimulated CAFs induce chemoresistance of NSCLC cells and promote their stem cell-like properties that could contribute to chemoresistance.

3.3. Chemotherapy upregulates PDL-1 expression in CAFs and stimulates CAFs to release elevated levels of pro-tumorigenic cytokines

There has been a growing interest in PDL-1-positive CAFs as a potential prognostic factor for NSCLC [19]. To our knowledge, PDL-1 expression on CAFs in response to chemotherapy has never been investigated in NSCLC and its roles in chemoresistance and tumor recurrence remain to be clarified. As a first step towards exploring the role of PDL-1 in NSCLC chemoresistance, we performed Western blot analysis to evaluate PDL-1 expression in CAFs at baseline level and after chemotherapy treatment. PDL-1 expression was highly upregulated in CAFs after chemotherapy (Fig. 3A). ELISA assay confirmed PDL-1 protein secretion from CAFs in response to chemotherapy (Fig. 3B). The surface expression of PDL-1 on chemotherapy-treated CAFs was further analyzed by flow cytometry. In concordance with the Western blot and ELISA results, the majority of chemotherapy-treated CAFs expressed PDL-1, whereas vehicle-treated CAFs less commonly expressed PDL-1 (Fig. 3C), thus supporting PDL-1 as a CAF biomarker for chemotherapy response. The data from co-culture experiments indicated that CAFs secrete biologically important cytokines or soluble factors in response to chemotherapy. Therefore, we conducted a comparative analysis of cytokines in CM from chemotherapy-treated CAFs, using a multiplex human cytokine array. As expected, several cytokines, including HGF, monocyte chemoattractant protein-1 (MCP-1), IL-1α, SDF-1, and angiogenin were upregulated (>2-fold increase) in chemotherapy-treated CAFs. Of the top upregulated cytokines, a high level of HGF was noted in the CM from chemotherapy-treated CAFs compared to vehicle-treated CAFs (Fig. 3D). Taken together, these findings suggest that CAFs respond to chemotherapy by elevating PDL-1 expression and secreting pro-tumorigenic cytokines that promote tumor growth and chemoresistance of NSCLC.

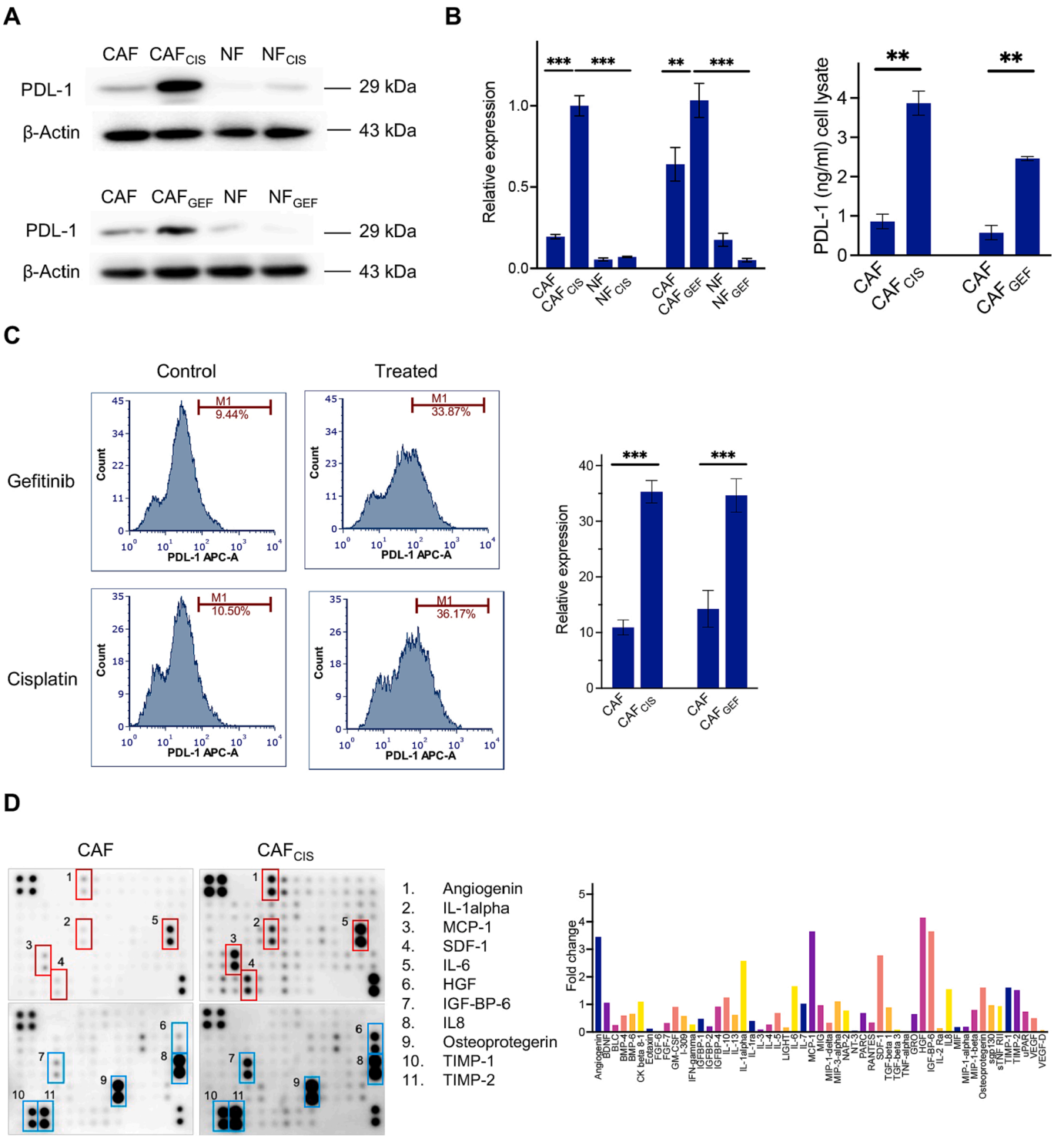

Fig. 3. The expression of PDL-1 is upregulated in chemotherapy-treated CAFs, and elevated levels of pro-tumorigenic cytokines are released from cisplatin-treated CAFs. (A) Western blot assay showing the protein expression level of PDL-1 in chemotherapy-treated CAFs and NFs. The bar chart shows the higher PDL-1 expression in CAFs treated with cisplatin and gefitinib. n = 3. **p < 0.01, ***p < 0.001. (B) ELISA analysis of PDL-1 protein expression. The bar charts show increased PDL-1 expression in CAFs treated with cisplatin and gefitinib. n = 3. **p < 0.01. (C) Flow cytometry analysis of cell-surface protein expression. Untreated CAFs were used as the control. The bar charts show increased PDL-1 cell-surface expression in CAFs treated with cisplatin and gefitinib. n = 3. ***p < 0.001. (D) Human cytokine array analysis of CM from cells treated with cisplatin and vehicle (0.9% NaCl). Only factors with at least 1.5-fold increased expression upon cisplatin treatment are represented with boxes.

3.4. Silencing PDL-1 inhibits chemoresistance induced by chemotherapy-treated CAFs

Our earlier results demonstrated that PDL-1-positive CAFs may promote chemoresistance of NSCLC cells by inducing stem cell-like properties. To further clarify the role of PDL-1-positive CAFs on chemoresistance, we knocked down PDL-1 (sgPDL-1) in CAFs using a lentivirus-based CRISPR knockdown (Fig. 4A). NSCLC cells were co-

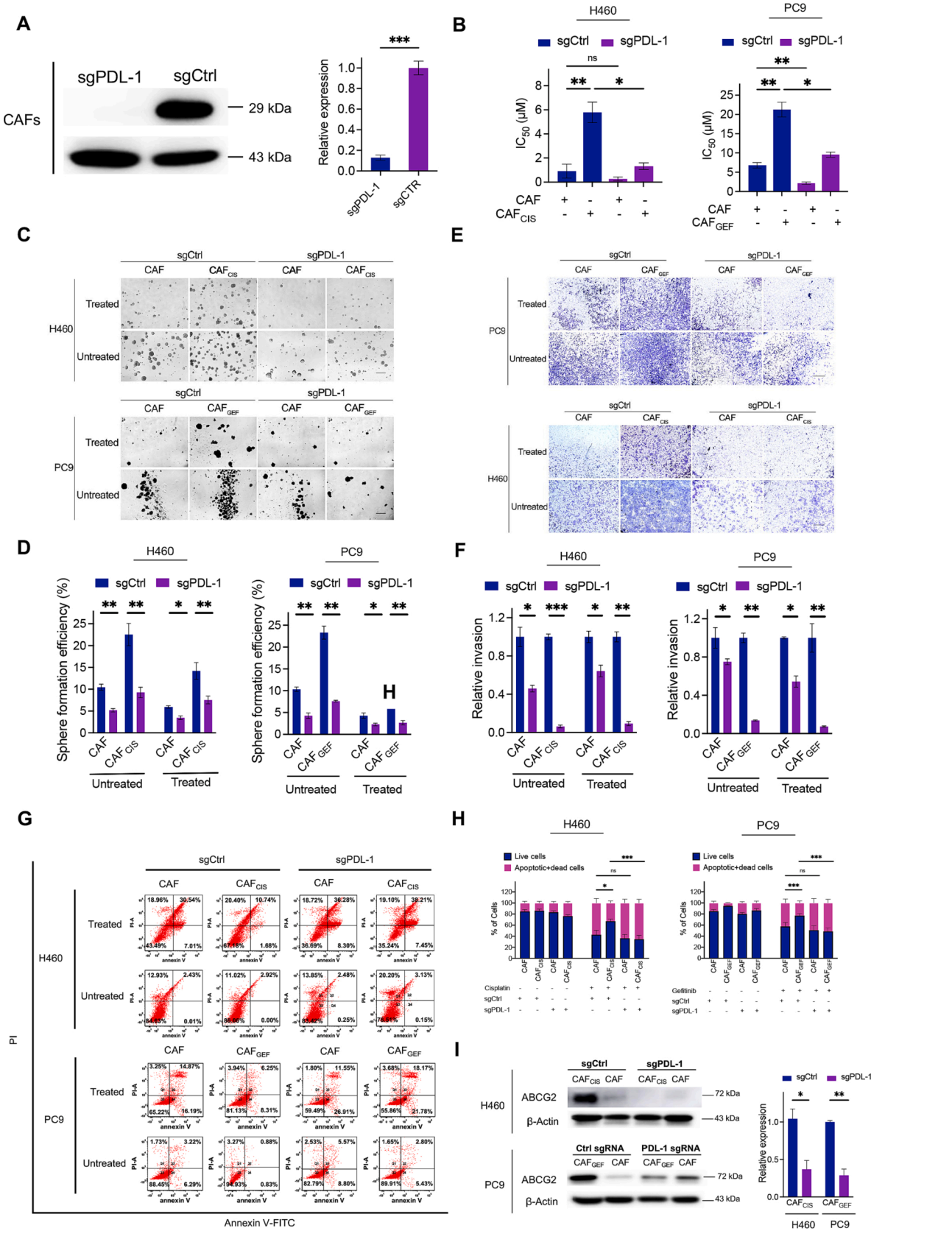

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Fig. 4. Knockdown of PDL-1 in CAFs inhibits cell viability, sphere formation, cell invasion, chemotherapy-induced apoptosis, and stemness of lung cancer cells. (A) validation of the PDL-1 knockdown efficiency in CAFs using Western blot analysis. Band density was quantified in Fiji and normalized to β-actin. A significant PDL-1 knockdown efficiency was detected in CAFs transfected with the CRISPR/Cas9 plasmids. n = 3. ***p < 0.001. (B) MTT assay showed that chemotherapy-treated CAFs with sgPDL-1 decreased the IC_{50} values of H460 and PC9 cells. n = 3. *p < 0.1, **p < 0.01. (C) Sphere formation assays were performed to analyze the effect of PDL-1 knockdown on stem cell properties of H460 and PC9 cells. Scale bars = 100 μm. (D) The bar charts show that the number of spheres decreased with PDL-1 knockdown. n = 3. *p < 0.1, **p < 0.01. (E) Transwell invasion assays were performed to evaluate the role of PDL-1 in regulating the invasion of lung cancer cells. Scale bars = 100 μm. (F) The bar charts show that silencing PDL-1 expression downregulated the invasion ability of H460 and PC9 cells. n = 3. *p < 0.1, **p < 0.01,***p < 0.001. (G) Annexin V/PI assay was performed by flow cytometry to analyze the effects of PDL-1 knockdown on apoptosis of NSCLC cells. (H) The bar charts show that downregulation of PDL-1 increased the apoptotic rate in H460 and PC9 cells. n = 3. *p < 0.1, ***p < 0.001. (I) Western blot assay detected the lower ABCG2 protein expression in lung cancer cells with PDL-1 knockdown of chemotherapy-treated CAFs. n = 3. *p < 0.1, **p < 0.01. sgPDL-1: CAFs with PDL-1 knockdown, sgCtrl: Mock transfected CAFs.

cultured with CM from chemotherapy-treated CAFs with sgPDL-1 to assess the sensitivity of NSCLC cells to chemotherapy. Chemotherapy-treated mock-transfected CAFs were used as their corresponding controls. Fig. 4B shows that PDL-1 knockdown attenuated chemoresistance of NSCLC cells, as indicated by the reduction in IC_{50} values of H460 and PC9 cells when incubated with CM from chemotherapy-treated CAFs with sgPDL-1. In line with this, NSCLC cells incubated with the CM from chemotherapy-treated mock-transfected CAFs exhibited a higher IC_{50}. In concordance with PDL-1 being an important regulator of stem cell phenotype and chemoresistance, we found that chemotherapy-treated CAFs with sgPDL-1 greatly reduced the ability of NSCLC cells to form tumor spheres, regardless of whether the cells had undergone chemotherapy (Fig. 4C, D). Also, the results showed that CAF-induced tumor cell invasion depends on PDL-1. Loss of PDL-1 in CAFs significantly decreased the ability of NSCLC cells to invade, regardless of their exposure to chemotherapy (Fig. 4E, F). To determine the effects of PDL-1 knockdown on cell apoptosis of NSCLC cells, we performed an Annexin V-FITC assay using flow cytometry. The results demonstrated that chemotherapy-treated CAFs with sgPDL-1 markedly increase chemotherapy-induced apoptosis of NSCLC cells (Fig. 4G, H). Furthermore, knockdown of PDL-1 resulted in a decrease in the expression of stem cell marker ABCG2 in cancer cells (Fig. 4I). PDL-1 suppression in CAFs also augmented the sensitivity to chemotherapy, resulting in reduced chemoresistance of NSCLC cells. These findings confirm the positive regulatory role of PDL-1 in chemoresistance and suggest this molecule as a therapeutic target for resistant NSCLC.

3.5. HGF secreted by PDL-1-positive CAFs promotes cancer chemoresistance

To gain a better insight into PDL-1-mediated cytokine secretion and chemoresistance, a comparative analysis of cytokines secreted from CAFs was carried out following PDL-1 knockdown. The analysis showed that HGF level was substantially downregulated in CM from chemotherapy-treated CAFs with sgPDL-1 compared to that from chemotherapy-treated mock-transfected CAFs (sgCtrl) (Fig. 5A). ELISA results confirmed that concentration of HGF was greatly reduced upon PDL-1 knockdown. (Fig. 5B). The results also indicated that HGF secretion from chemotherapy-stimulated CAFs significantly correlated with their PDL-1 expression. Furthermore, HGF has been reported to induce stem cell-like properties [13] and chemoresistance of NSCLC cells [30]. To test the functional role of HGF in our system, we added exogenous HGF to HGF-suppressed CM from chemotherapy-treated CAFs with sgPDL-1 and examined its effect on chemoresistance of NSCLC cells. Such addition was shown to reverse the sensitizing effect of PDL-1 knockdown as indicated by the increase in IC_{50} of lung cancer cells upon the addition of HGF (Fig. 5C). We also evaluated the impact of HGF on CAF-regulated cancer stemness. Regardless of cancer cells treated with chemotherapy or not, the addition of HGF was found to rescue tumor sphere formation to the level observed with CM from chemotherapy-treated CAFs with sgCtrl (Fig. 5D, E). As HGF may contribute to tumor invasion, we evaluated and found that exogenous HGF increased the invasiveness of NSCLC cells (Fig. 5H, I). To validate the importance of HGF in chemotherapy-induced apoptosis, pre-incubated NSCLC cells were exposed to chemotherapy with or without added HGF. The addition of HGF to CM from chemotherapy-treated CAFs with sgPDL-1 markedly decreased apoptosis of NSCLC cells (Fig. 5F, G, H, I). Western blot analysis showed an upregulation of ABCG2 in cancer cells in the presence of HGF, and this effect was significantly decreased after incubating the cells with chemotherapy-treated CAFs with sgPDL-1 (Fig. 5J). To further study the impact of CAF-secreted HGF, cell viability was performed by adding neutralizing anti-HGF antibody (HGF NAb) (R&D Systems, Germany) to chemotherapy-treated CAF CM. The addition of HGF NAb resulted in decreased chemoresistance of NSCLC cells (Supplementary Fig. 2). Collectively, these results suggest that PDL-1-positive CAFs promote chemoresistance of NSCLC cells by upregulating HGF secretion in response to chemotherapy.

3.6. Knockdown of PDL-1 inhibits CAF-induced tumor growth and chemoresistance of NSCLC cells in vivo

To determine the effect of PDL-1 on tumor growth in response to chemotherapy, we subcutaneously injected H460 cells alone or in combination with PDL-1 knockdown or mock-transfected CAFs into athymic nude mice (Fig. 6A). Consistent with the *in vitro* results, co-injection of H460 and mock-transfected CAFs in mice resulted in an increase in tumor size and weight, regardless of mice being treated with or without chemotherapy. However, xenografted tumors grown in mice co-implanted with H460 alone or combined with CAFs with sgPDL-1 had a significantly smaller tumor size and weight (Fig. 6B, C). As expected, mock-transfected CAFs accelerated tumor growth in mice compared to H460 alone or with PDL-1 knockdown CAFs. Consistent with the *in vitro* findings, even after chemotherapy, the average tumor volume of H460 combined with mock-transfected CAFs was significantly higher than that of H460 alone. However, chemotherapy led to a significant reduction in tumor volume when mice were co-implanted with H460 and PDL-1 knockdown CAFs (Fig. 6D). Co-injection of H460 and mock-transfected CAFs exhibited strong luciferase activity in mice even after chemotherapy. However, co-injection of H460 with PDL-1 knockdown CAFs resulted in a significant reduction in luciferase activity (Fig. 6E). Accordingly, we examined the markers of tumor cell proliferation (Ki-67) and lung cancer stemness (ABCG2) in H460 cells by immunohistochemistry. Histopathology results indicated that the general architecture of the tumor remained unaffected upon the knockdown of PDL-1 in CAFs. Even after chemotherapy, Ki-67 expression in lung cancer cells remained significantly high in mice co-implanted with H460 and mock-transfected CAFs. Also, no significant difference was observed in Ki-67 expression between sgCtrl-CAFs and sgPDL-1-CAFs (Supplementary Fig. 3), as determined by immunohistochemistry analysis of the CAF population regions. However, silencing PDL-1 expression drastically reduced Ki-67 expression in lung cancer cells, suggesting a correlation between PDL-1 expression and reduction of tumor cell proliferation. Furthermore, the expression of ABCG2 was much lower in lung cancer cells when the mice were injected with H460 alone or co-implanted with H460 and CAFs with sgPDL-1, which further confirmed that PDL-1 expression was closely correlated with the induction of stem cell-like properties in lung cancer cells. Lastly, we assessed the possible impact

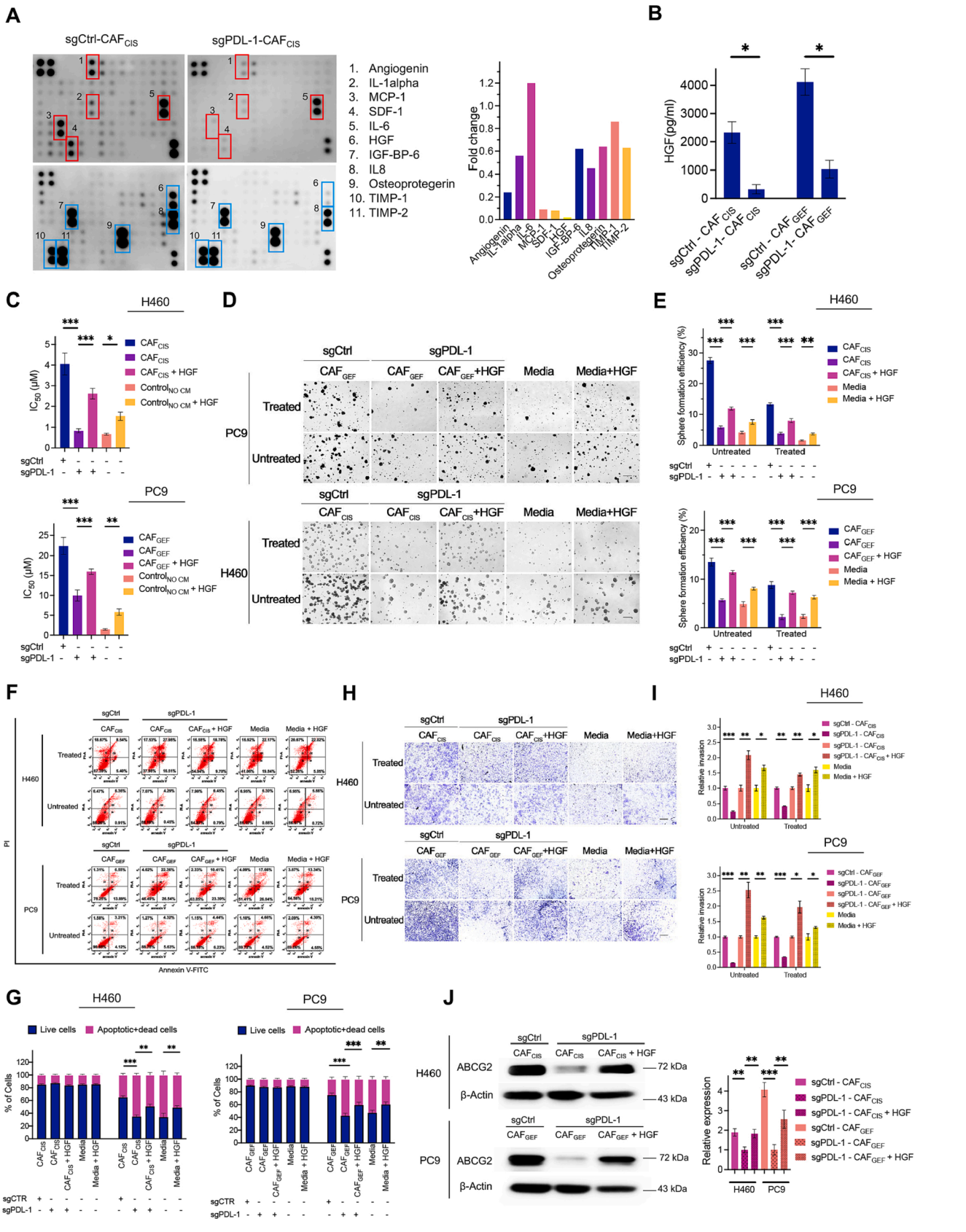

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Fig. 5. PDL-1 positive CAFs promote chemoresistance and tumorigenesis of NSCLC cells by secreting HGF. (A) Cytokine array analysis in CMs from cisplatin-treated CAFs with or without sgPDL-1. A significant decrease in HGF level was observed upon knockdown of PDL-1. (B) By ELISA, the concentration of HGF was confirmed to be lower in chemotherapy-treated CAFs with PDL-1 knockdown. n = 3. *p < 0.1. (C) IC_{50} values in H460 and PC9 cells were analyzed with GraphPad Prism 7 after performing a MTT assay under various concentrations of cisplatin and gefitinib. IC_{50} of NSCLC cells was decreased with PDL-1 knockdown. The suppression of cell growth was rescued by the addition of HGF. n = 3. *p < 0.1,**p < 0.01,***p < 0.001. (D, E) Sphere formation assay showed that chemotherapy-treated CAFs with sgPDL-1 suppressed the sphere forming ability of NSCLC cells which was rescued by the addition of HGF to CMs. n = 3. **p < 0.01, ***p < 0.001. Scale bars = 100 μm. (F, G) The effect of HGF on chemotherapy-induced apoptosis of NSCLC cells was analyzed by Annexin V-FITC apoptosis assay. Chemotherapy-treated CAFs with sgPDL-1 showed increased cell apoptosis which was partially inhibited by the addition of HGF to CM. n = 3. **p < 0.01, ***p < 0.001. (H, I) Cell invasion assays showed that knocking down PDL-1 in CAFs significantly compromised the invasiveness of lung cancer cells which was rescued by the addition of HGF to the CM from chemotherapy-treated CAFs with sgPDL-1. Scale bars = 100 μm. n = 3. *p < 0.1,**p < 0.01,***p < 0.001. (J) Western blotting shows that ABCG2 expression of lung cancer cells was partially increased when the cells were pre-incubated with HGF-added CM from chemotherapy-treated CAFs with PDL-1 knockdown. **p < 0.01, ***p < 0.001.

of PDL-1 expression on CAF-induced HGF secretion in response to chemotherapy. Consistent with the *in vitro* findings, analysis of HGF expression in CAFs showed that PDL-1 knockdown significantly reduced HGF expression in CAFs. (Fig. 6F, G). Taken together, these findings demonstrated that PDL-1 in CAFs plays a vital role in regulating tumor growth and chemoresistance of NSCLC.

4. Discussion

NSCLC, a subtype of lung cancer, is often distinguished by inflammatory and highly active tumor microenvironment [31]. Using GEO2R analysis, we found that CAFs secrete elevated levels of pro-tumorigenic cytokines. These soluble factors have been suggested to promote tumor progression and chemoresistance [13,17,32]. Previous epidemiological and preclinical studies have shown that chemotherapy actively induces cellular and physical changes in non-cancerous cells in the TME to favor cancer progression [33]. However, very little is known about the impact of chemotherapy on CAFs and the role of chemotherapy-stimulated CAFs secretome in cancer chemoresistance.

Several molecular mechanisms underlying CAF-mediated tumor progression and chemoresistance following chemotherapy have been proposed. Chemotherapy significantly eradicates CAFs and stimulates them to secrete pro-survival and pro-tumorigenic cytokines [14]. For example, Lotti *et al.* found that chemotherapy-stimulated CAFs enhance self-renewal of cancer-initiating cells and tumor progression with increased secretion of interleukin-17A (IL-17A)[34]. Che et al. reported that elevated secretion of plasminogen activator inhibitor (PAI-1) from cisplatin-treated CAFs promoted esophageal squamous cell carcinoma [35]. Another study reported that cisplatin-stimulated CAFs exhibited a significant upregulation of IL-11, which promotes chemoresistance in lung adenocarcinoma patients [12].

In the current study, we demonstrated similar pro-tumorigenic effects from chemotherapy-treated CAFs. They promote cancer stem cell-like properties and invasiveness of NSCLC cells, regardless of their exposure to chemotherapy. We also discovered that chemotherapy-stimulated CAFs induce a high level of stemness-associated protein ABCG2 compared to untreated control. This protein has been identified as a prominent biomarker for cancer stem cells in NSCLC [36]. ABCG2 belongs to the ATP-binding cassette (ABC) transporter superfamily and is responsible for the multidrug resistance phenotype of cancer [37]. Studies have demonstrated that overexpression of ABCG2 is correlated with tumor recurrence and chemoresistance in NSCLC [37,38]. Furthermore, the cytokine array analysis indicated that chemotherapy-stimulated CAFs secrete elevated levels of several pro-tumorigenic cytokines, such as HGF [39–41], MCP-1[36,42], and SDF-1[43,44] which have been shown to be associated with cancer stemness, tumor angiogenesis, tumor progression, and chemoresistance. Therefore, our findings and the previous studies postulated that chemotherapy-stimulated CAF-secretome promotes chemoresistance of NSCLC cells by inducing their stem cell-like properties. Targeted intervention of this crosstalk between CAFs and cancer cells could provide a means to overcome chemoresistance and improve the effectiveness of current chemotherapies.

PDL-1 has emerged as a potential new biomarker for CAFs although detailed studies are lacking. To the best of our knowledge, this is the first detailed analysis of PDL-1 in CAFs, its role in chemoresistance, and its potential as a CAF biomarker for chemotherapy response. Gene expression analysis demonstrated that unlike other traditional CAF biomarkers CAFs exhibited a significantly higher level of PDL-1 than NFs. According to our results, PDL-1 expression in CAFs was significantly upregulated when exposed to chemotherapy, suggesting that PDL-1 could also serve as a potential drug delivery target to CAFs, i.e., for enhanced and targeted cancer therapies. The mechanism by which PDL-1 is upregulated in CAFs remains to be further elucidated and is one of the limitations of this study.

PDL-1 is a crucial immune checkpoint protein that promotes anti-tumor immune response [21]. It has also been reported to be highly expressed in NSCLC. Overexpression of PDL-1 was found to be associated with tumor growth, metastasis, and poor prognosis of NSCLC [23,45]. It has also been linked to chemoresistance and stem-like features in NSCLC [46,47]. Our findings support the previous findings and further indicate the functional role and underlying mechanisms of chemoresistance induced by PDL-1-positive CAFs. Using gene silencing and various molecular techniques and functional assays, we demonstrated that PDL-1 knockdown in CAFs suppresses cell viability, stem-like properties, and invasiveness of NSCLC cells. *In vivo* studies support the *in vitro* findings and indicate the pro-tumorigenic and chemoresistant roles of PDL-1 on CAFs. Our results further suggest that CAF-associated PDL-1 mediates its effects on NSCLC cells by promoting their stem-like properties, which results in chemoresistance of the cells.

As compared to many other pro-tumorigenic cytokines induced by chemotherapy, HGF was found to be highly upregulated in CAFs and its secretion by the cells was strongly suppressed by PDL-1 knockdown, suggesting their linkage and the potential role of HGF as a key mediator of PDL-1-mediated chemoresistance. HGF is a well-known cytokine that predominantly originated from CAFs [39]. It enhances tumor progression [40], chemoresistance [41,48,] and metastasis [39] of NSCLC. Our gene manipulation and functional studies support the previous findings and indicated that the elevated levels of HGF in CAFs contributed to the chemoresistance of NSCLC cells. We also demonstrated that the increase in chemoresistance is likely mediated through the induction of cancer stem-like cells since the addition of exogenous HGF reversed the inhibitory effect of PDL-1 knockdown on chemoresistance and cancer stem cell-like properties of NSCLC cells. HGF addition exhibited stronger features of stemness and increased tumorigenesis. The c-mesenchymal–epithelial transition (c-Met), a kinase receptor for HGF, and the aberrant HGF/c-Met signaling leads to tumorigenesis in lung cancer [40]. Notably, our results showed PDL-1 knockdown markedly decreased c-Met expression in lung cancer cells, suggesting that PDL-1 positive CAFs may promote NSCLC chemoresistance through PDL-1/HGF signaling pathway (Supplementary Fig. 4). It is likely that multiple mechanisms of HGF activation of cancer cells are involved in PDL-1-mediated chemoresistance. For instance, Ying *et al.* reported that CAF-derived HGF promotes chemoresistance of NSCLC cells by upregulating phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) pathway [41]. Other signaling pathways may also be involved and are activated

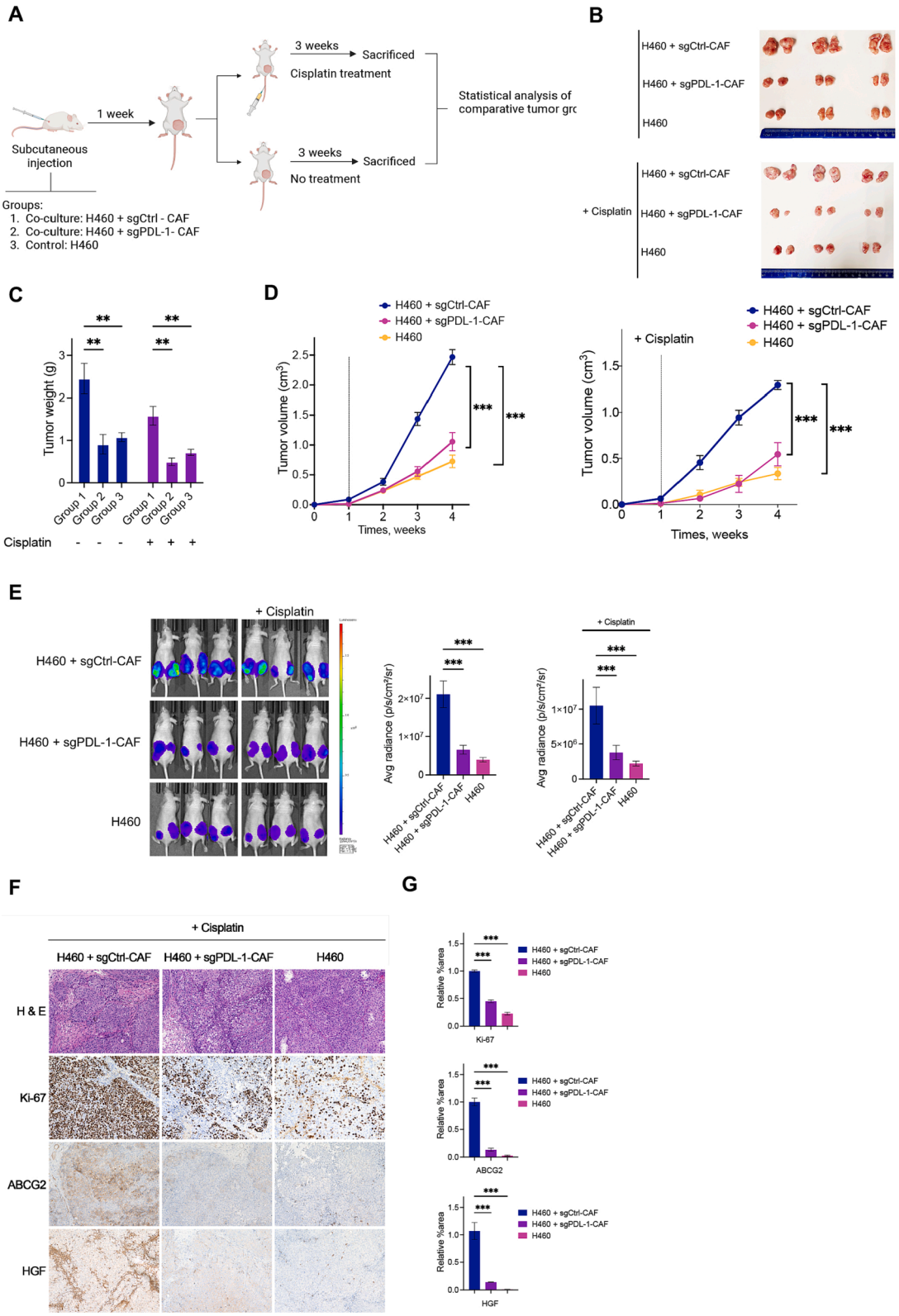

Fig. 6. PDL-1-expressed CAFs induce chemoresistance of lung cancer cells *in vivo*. (A) Schematic illustration of the treatment protocol of cisplatin administration at the dose of 4 mg/kg in tumor-bearing mice by intraperitoneal injection. (B) Tumors derived from H460 cells alone or in combination with CAFs transfected with sgCtrl or sgPDL-1 are shown. (C, D) The bar chart and tumor growth curves showed that xenografted tumors grown in mice co-implanted with H460 alone or combined with mock-transfected CAFs had a larger tumor weight and volume. $^{**}p < 0.01$,$^{***}p < 0.001$. Meanwhile, tumors grew significantly slower in mice receiving H460 cells and sgPDL-1-CAFs. (E) Representative images and quantification of luciferase signal in mice injected with H460 alone or in combination with CAFs transfected with sgCtrl or sgPDL-1. $^{***}p < 0.001$. (F) Representative hematoxylin and eosin (H&E) staining, and immunohistochemistry of Ki-67, ABCG2, and HGF. Scale bars = 100 μm. (G) Quantification of Ki-67 and ABCG2 in lung cancer cells, and HGF in CAFs. n = 3. $^{***}p < 0.001$.

in cancer cells during their adaptive transformation, which need further investigations. In summary, our findings indicated that chemotherapy induces upregulation of PDL-1 expression in CAFs. PDL-1-positive CAFs modulate stem cell-like properties of NSCLC cells by secreting an elevated level of HGF, thereby promoting chemoresistance. Our findings highlight the importance of PDL-1 as a valuable prognostic biomarker for CAFs and as a potential drug delivery and therapeutic target for resistant NSCLC.

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CRediT authorship contribution statement

Gayathri Heenatigala Palliyage: Conceptualization, Data curation, Methodology, Software, Formal analysis, Investigation, Validation, Visualization, Project administration, Writing – original draft, Writing – review & editing. **Parinya Samart:** Software, Validation, Formal analysis, Writing – review & editing. **Sharan Bobbala:** Supervision, Resources, Writing – review & editing. **Liying W. Rojanasakul:** Supervision, Resources, Writing – review & editing. **Jayme Coyle:** Software, Writing – review & editing. **Karen Martin:** Resources, Writing – review & editing. **Patrick S. Callery:** Resources, Writing – review & editing. **Yon Rojanasakul:** Conceptualization, Methodology, Resources, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lungcan.2023.107258.

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