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EDITOR'S CHOICE

Identification of chemical compounds regulating PD-L1 by introducing HiBiT-tagged cells

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Programmed death-ligand 1 (PD-L1) is a co-inhibitory molecule expressed on tumor cells. Immune checkpoint inhibitors focusing on the PD-L1 mechanism are now being studied for the treatment of various cancer types. However, the regulatory mechanism of PD-L1 is yet to be fully clarified, and a high-throughput system for comparing the abilities of small compounds in regulating PD-L1 has not yet been established. Therefore, we created a HiBiT-tagged lung adenocarcinoma cell line, PC9-KI, for easier and faster detection of changes in PD-L1 protein expression. Using PC9-KI cells, we screened 1280 chemical compounds from the Library of Pharmacologically Active Compounds and identified microtubule polymerization inhibitors and thapsigargin as PD-L1 upregulators and a p97 inhibitor as a PD-L1 downregulator.

Keywords: chemical compound; endogenous protein; HiBiT; high-throughput screening; PD-L1

The tumor microenvironment is composed of not only tumor cells but also various immune cells such as infiltrated T cells, tumor-associated macrophages, and natural killer cells. In the tumor microenvironment, tumor cells express various cytokines, chemokines, and ligands, which help them survive [1]. Programmed death-ligand 1 (PD-L1) is a factor considered to be a promising target for tumor therapy. High expression of PD-L1 by tumor cells induces anergy by directly binding to programmed cell death 1 (PD-1) receptors, making it possible for the tumor cells to evade the immune system [2]. Immune checkpoint inhibitors such as anti-PD-1 and anti-PD-L1 antibodies are now used for many cancer types, which reflects on the clinical importance of the PD-L1 regulatory mechanisms [3]. Cytokines dependent on signaling pathways have already been reported to be important for the regulation of PD-L1 [4–9]. Currently, no techniques exist to identify chemical compounds that regulate PD-L1 protein expression by using chemical compound libraries in a cross-sectional manner. Thus, a high-throughput screening system that detects changes in PD-L1 protein expressions is of vital importance.

Herein, we present a screening system that introduces HiBiT-tagged cells as a novel strategy for identifying chemical compounds that regulate PD-L1

Abbreviations
crRNA, CRISPR RNA; EC50, effective concentration 50; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HRP, horseradish peroxidase; IC50, inhibitory concentration 50; INF, interferon; LOPAC, library of pharmaceutically active compounds; PBST, phosphate-buffered saline-Tween 20; PD-1, programmed cell death 1; PD-L1, programmed death-ligand 1; qPCR, quantitative polymerase chain reaction; RIPA, radio-immunoprecipitation assay; RNA, ribonucleic acid; RPMI, Roswell Park Memorial Institute; siRNA, small interfering RNA; ssDNA, single-stranded DNA; TNF, tumor necrosis factor.

expression. The HiBiT tag is a peptide tag composed of only 11 amino acids that, upon insertion into the target proteins, functions as a nanoluciferase by forming a dimer with LgBiT. By measuring the luminescence changes after inserting the peptide tag, a relatively simple procedure can enable the rapid detection of expression changes in the target proteins [10]. Using genome editing, we modified the lung adenocarcinoma cell line PC9 with HiBiT tagged at the C terminus of PD-L1 to create PC9-KI cells. We then developed a chemical screening strategy by adding 1280 compounds from the chemical compound library LOPAC (library of pharmacologically active compounds; Sigma Aldrich, St. Louis, MO, USA). Here, we report our success in identifying several chemical compounds that regulate the expression of PD-L1.

Materials and methods

Cell culture

PC9 and PC9-KI cells were maintained in Roswell Park Memorial Institute (RPMI) (Corning, New York, NY, USA) medium supplemented with 10% FBS (Gibco, Waltham, MA, USA), and 1% penicillin/streptomycin (Wako, Osaka, Japan) at 37 °C with 5% CO_2. A375 and MDAMB231 cells were maintained in Dulbecco's modified Eagle medium (Corning) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Wako) at 37 °C with 5% CO_2.

Creating HiBiT knocked-in cells with genome editing

HiBiT-tagged PC9 cells were established using the CRISPR-Cas9 system. ALT-R XT CRISPR RNA (crRNA) (Integrated DNA Technologies, Coralville, IA, USA) was resuspended in Nuclease-free-Duplex Buffer (Integrated DNA Technologies) to a final concentration of 100 μM. Equal volumes of crRNA and trans-activating CRISPR RNA were mixed and heated for 5 min at 95 °C. After heating, the oligo complex was gradually cooled down to room temperature. The oligo complex was then incubated at room temperature for 20 min with ALT-R Cas9 Nuclease V3 (61 μM) (Integrated DNA Technologies) to form the Cas9 complex. The single-stranded DNA (ssDNA) oligo, including sequences of HiBiT, a complementary sequence to the C-terminal in the CD274 genome, and the Cas9 complex were then cotransfected into the PC9 cells using the NEPA21 Super Electroporator (NEPAGENE, Chiba, Japan). After incubating the cells for a few days, single cell cloning was performed to pick up the HiBiT-tagged cells. The sequences of crRNA and ssDNA oligo are shown in Table S1.

Validation of the targeted insertion of the HiBiT tag

Insertion of the HiBiT tag sequence in the genome of the knocked-in cells was confirmed by sequencing. For further confirmation, western blotting was performed as follows. SDS/PAGE was performed for ~ 50 μg of protein from wild-type (PC9) and HiBiT-tagged PC9 (PC9-KI) cells, which were collected using the radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). The proteins were then transferred to nitrocellulose membranes and blocked with Blocking-one (Nacalai Tesque, Kyoto, Japan) for 1 h. Two milliliters of LgBiT mix [PBST 2 mL and LgBiT (Promega, Madison, WI, USA) 8 μL] was added and reacted at 4 °C for overnight. The membrane was added three times in PBST for 10 min, and 1 mL of substrate mix [PBST 1 mL and substrate (Promega) 3 μL] was added and reacted for 10 min for detection. The chemical luminescence was captured using IMAGEQUANT LAS4000 (GE Healthcare, Chicago, MI, USA).

To compare the luminescence of wild-type PC9 cells and PC9-KI cells, 4×10^4 of PC9 and PC9-KI cells were seeded in each well of the 96-well plates per well, and incubated for 24 h. Media was discarded, then a detection mix [PBS 12.5 μL, lytic buffer 12.5 μL, substrate (Promega) 0.25 μL, LgBiT (Promega) 0.125 μL] was added and incubated for 10 min, and the luminescence was measured using ARVO X3 (PerkinElmer, Waltham, MA, USA).

For further validation, small interfering RNA (siRNA) knockdown was performed. Precisely 1.5×10^4 cells of PC9-KI cells were plated and incubated overnight. Following this, 1.5 pmol of siRNA for CD274 or the negative control (AllStars Hs Cell Death siRNA; Thermo Scientific, Waltham, MA, USA) was transfected with 0.5 μg of Lipofectamine RNA iMax (Thermo Scientific). The siRNA sequences are shown in Table S1. After 48 h, the medium was discarded and a detection mix [PBS 25 μL, lytic buffer 25 μL, substrate (Promega) 0.5 μL, and LgBiT (Promega) 0.25 μL] was added. The plate was incubated at room temperature in the dark for 10 min, and the luminescence was measured using ARVO X3.

Prescreening test for the HiBiT-tagged cells

To validate the effects of DMSO on the luminescence of PC9-KI, 5×10^3 PC9-KI cells were seeded in 384-well plates. After 24 h, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, or 0% of DMSO (final concentration) was added in RPMI medium with 10% FBS. After 24 h, a detection mix (PBS 5 μL, substrate 0.1 μL, LgBiT 0.05 μL) was added and incubated at room temperature in the dark for 10 min, and the luminescence was measured using ARVO X3.

To confirm the upregulation of PD-L1 by cytokines or growth factors, 4×10^4 of PC9-KI cells were seeded on

96-well plates. After 24 h, 1 $\mu g \cdot mL^{-1}$ of epidermal growth factor (EGF; WAKO), 100 $ng \cdot mL^{-1}$ of tumor necrosis factor (TNF) alpha (R&D, Minneapolis, MN, USA), 20 $ng \cdot mL^{-1}$ of interferon gamma (IFNγ) (R&D), or 0.1% of DMSO was added in RPMI with 10% FBS. After 24 h, the medium was discarded, and a detection mix [PBS 12.5 μL, lytic buffer (Promega) 12.5 μL, substrate (Promega) 0.25 μL, LgBiT (Promega) 0.125 μL] was added and incubated at room temperature in the dark for 10 min. Luminescence was measured using ARVO X3.

To further validate the dose-dependent changes in PD-L1 expression, 5×10^3 PC9-KI cells were seeded on a 384-well plate. Twenty-four hours later, 20, 10, 5, 2.5, 1.25, 0.625, or 0 μM of SB203580 (Sigma Aldrich) was added to RPMI medium with 10% FBS. After 24 h, the medium was discarded, and a detection mix [PBS 5 μL, substrate (Promega) 0.1 μL, LgBiT (Promega) 0.05 μL] was added and incubated at room temperature in the dark for 10 min. Luminescence was measured using ARVO X3.

Chemical compound screening with the LOPAC compound library

At first, 4×10^4 PC9-KI cells were seeded on 96-well plates. Then, after 24 h, 1 μM of compounds in the LOPAC chemical compound library (Sigma Aldrich) was added to each well. Following 24 h, the medium was discarded, and a detection mix [PBS 12.5 μL, lytic buffer 12.5 μL, substrate (Promega) 0.25 μL, and LgBiT (Promega) 0.125 μL] was added to each well. The plates were incubated at room temperature for 10 min in the dark, and the luminescence was measured using ARVO X3.

Effective concentration 50 assay with HiBiT activity measurement

First, 4×10^4 of PC9-KI cells were seeded on 96-well plates. After 24 h, 0.1% of DMSO or 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 μM of SB203580 (Sigma Aldrich), brefeldin A (BioLegend, San Diego, CA, USA), PD153035 (Selleck, Houston, TX, USA), colchicine (WAKO), vincristine (Cayman, Ann Arbor, MI, USA), thapsigargin (WAKO), and ML-240 (Sigma Aldrich) were added. After 24 h, the medium was discarded, and detection mix [PBS 12.5 μL, lytic buffer (Promega) 12.5 μL, substrate (Promega) 0.25 μL, and LgBiT (Promega) 0.125 μL] was added to each well.

Inhibitory concentration 50 assay with Cell Titer Glo assay

Initially, 5×10^3 of PC9-KI, A375, and MDAMB231 cells were seeded on 96-well plates. After 24 h, 0.1 % DMSO or 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 μM of colchicine (WAKO), vincristine (Cayman), thapsigargin (WAKO), and ML-240 (Sigma Aldrich) were added. After 48 h, 25 μL of Cell Titer Glo 2.0 (Promega) was added and incubated at 37 °C for 30 min. Absorbance at 490 nm was measured using ARVO X3.

Treating PC9-KI cells with colchicine for validating the results of the HiBiT assay

Initially, 4×10^5 of PC9-KI cells were seeded on six-well plates. After 24 h, 100 nM of colchicine was added to the medium and incubated at 37 °C overnight. Protein levels were analyzed as shown in Western blotting.

Treating cells with each compound for A375 and MDAMB231

Initially, 4×10^5 of A375 or MDAMB231 cells were seeded on six-well plates. After 24 h, 1 μM of colchicine, vincristine, thapsigargin, and ML-240 were added to the medium and incubated at 37 °C for 3 h. Proteins and RNA were analyzed as shown in Western blotting.

Western blotting

Proteins were collected with the RIPA lysis buffer. Proteins in the cell lysates were separated by SDS/PAGE followed by a semidry transfer to a polyvinylidene fluoride membrane. Membranes were blocked for 1 h with Blocking-One (Nacalai Tesque) and then reacted with PD-L1 primary antibodies (E1L3N; Cell Signaling Technology, Danvers, MA, USA) or antibeta-actin (AC-74; Sigma Aldrich or 010-27841; WAKO) at 4 °C overnight. Subsequently, the membrane was rinsed and reacted with enhanced chemiluminescence (ECL) mouse IgG horseradish peroxidase (HRP)-conjugated whole antibody (GE Healthcare) or rabbit IgG HRP-conjugated whole antibody (GE Healthcare). The blot was developed using the ECL Select Western Blotting Detection Reagent (GE Healthcare).

RNA extraction and quantitative PCR analysis

RNA was extracted using the ReliaPrep RNA Miniprep System (Promega, Wisconsin, Japan). RNA was reverse-transcribed with Prime Script (Takara, Kusatsu, Japan), and cDNA was analyzed by quantitative PCR (qPCR). Primers are shown in Table S1.

Results

In order to establish a simple assay system that can rapidly evaluate the endogenous expression of PD-L1, we knocked-in the HiBiT tag at the C terminus of the CD274 gene. We used the genome editing technology-

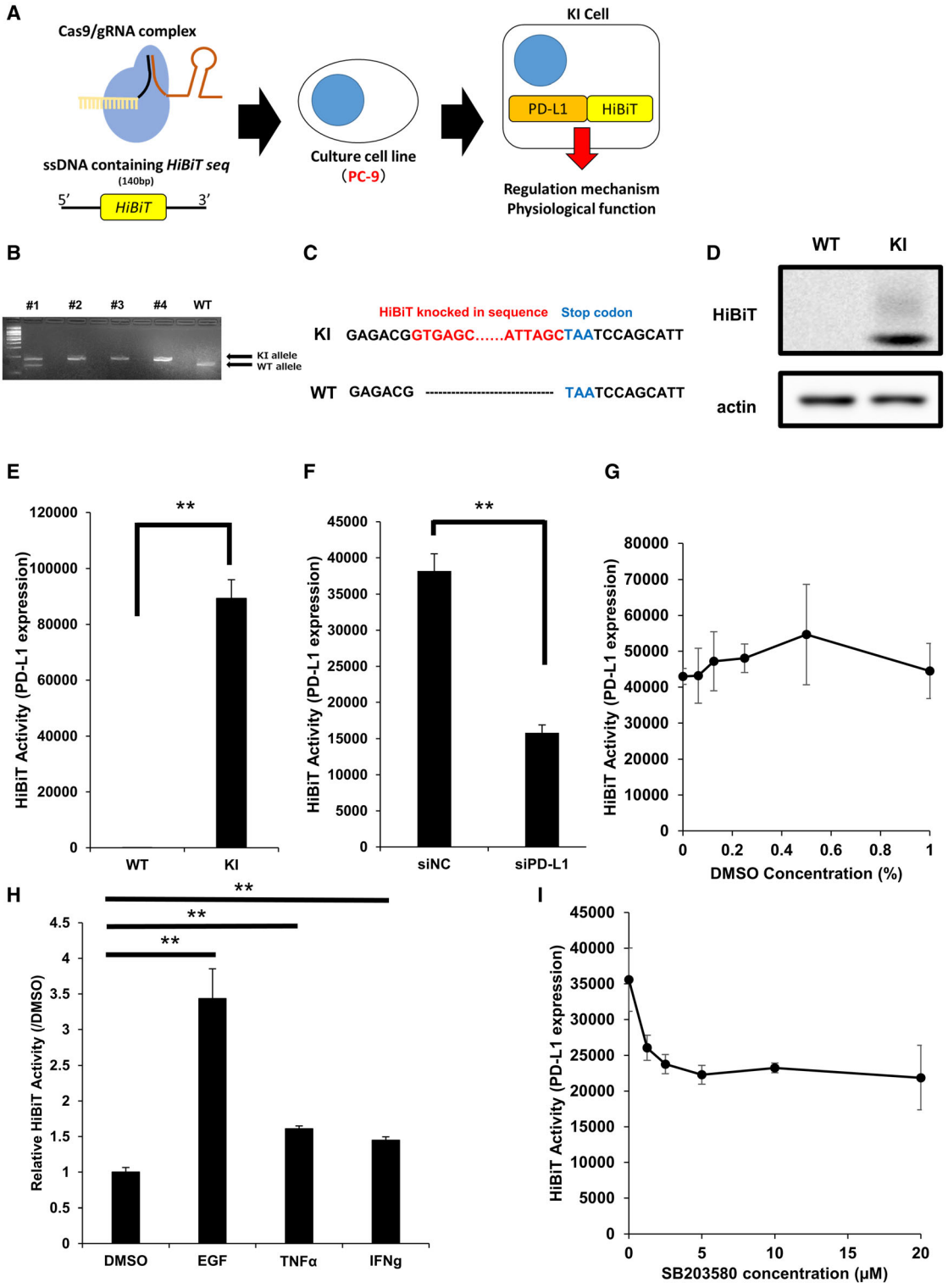
CRISPR-Cas9 and oligo ssDNA that were complementary to genome sequences in the lung adenocarcinoma cell line PC9 (Fig. 1A). We selected four clones from single cell cloning and confirmed the integration of HiBiT sequences by PCR. In our analysis, we used clone #3, which was homozygous for the targeted insertion of the HiBiT tag, in our analysis (Fig. 1B). With the help of sequencing, we confirmed the targeted insertion of the HiBiT tag of the knocked-in cells (PC9-KI) (Fig. 1C). We also confirmed the tagging with western blotting using LgBiT (Fig. 1D). By measuring the reporter activity of the PC9-KI cells, we confirmed the highly specific luminescence activity of PC9-KI compared with that of the wild-type PC9 (Fig. 1E). Furthermore, by knocking-down endogenous PD-L1 using siRNA in PC9-KI cells, we confirmed a distinct decrease in luminescence, which reflected changes in the endogenous expression (Fig. 1F). In this cell-based assay system, we confirmed that there were no changes in PD-L1 expression with 0%, 0.0625%, 0.125%, 0.25%, 0.5%, and 1% DMSO (Fig. 1G). In addition, we found that adding EGF, TNFα, and IFNγ, which have been previously reported as PD-L1 upregulators, increased the HiBiT activities compared with when 0.1% DMSO was added (Fig. 1H). Moreover, treatment with the p38 inhibitor, already reported as a downregulator of PD-L1, decreased the HiBiT activity in a dose-dependent manner (Fig. 1I). Based on these results, we performed a chemical compound screening using PC9-KI cells.

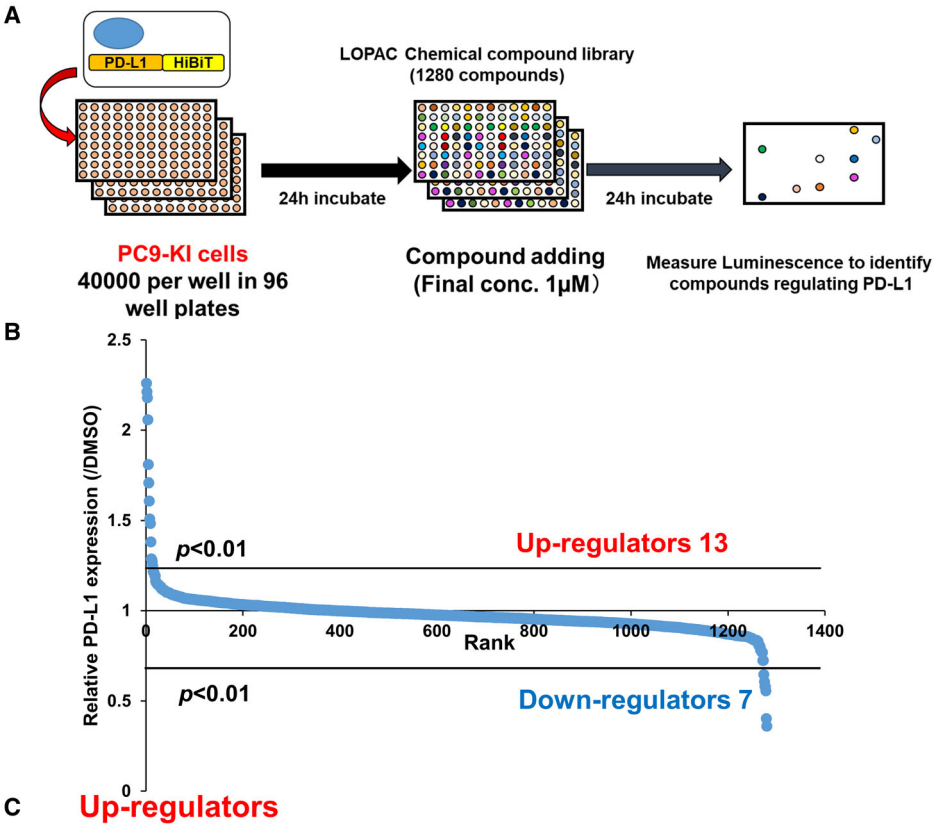
For the chemical screening, we seeded 4×10^4 PC9-KI cells on 96-well plates, incubated them for 24 h, and added 1280 chemical compounds from the LOPAC chemical compound library. At a final concentration of 1 μM, we incubated the cells for another 24 h and then measured the HiBiT activity (Fig. 2A). We defined regulators as compounds that regulated the expression of PD-L1 in the range of $P < 0.01$. As a result, we identified thirteen upregulators and seven downregulators (Fig. 2B, Table S2). Colchicine and vincristine, currently known for targeting microtubules and thapsigargin, which can increase intracellular calcium levels were newly identified as upregulators of PD-L1 (Fig. 2C). We identified seven chemical compounds as candidate downregulators (Fig. 2D). To exclude the possibility of overestimating the candidate downregulators due to their cytotoxicity, we performed an inhibitory concentration (IC50) assay for PC9-KI cells. As a result, brefeldin A and epidermal growth factor receptor (EGFR) inhibitor had a strong cytotoxicity at a dose of 1 μM, and their relative cell viabilities were < 60% as compared to the treatment with DMSO. On the other hand, treatment with the p38 inhibitor and ML-240 showed milder cell toxicity, and their relative cell viabilities were more than 60% as compared to the treatment with DMSO (Fig. S1). From these results, we regarded the p38 inhibitor, an already known downregulator of PD-L1, and ML-240, an AAA ATPase p97 inhibitor, as downregulators of PD-L1.

To measure the effective concentration 50 (EC50) of colchicine for maximum upregulation of PD-L1, we performed a concentration dilution assay on PC9-KI cells using the HiBiT system. We determined that the EC50 of colchicine was ~ 50 nM, and it upregulated PD-L1 protein expression 1.8 times as much as DMSO (Fig. 3A). The IC50 for maximum suppression of cell viabilities of PC9-KI cells by colchicine was ~ 1 nM (Fig. 3B). Moreover, we used western blotting with antibodies for endogenous PD-L1 to validate the results of the HiBiT assay, and we confirmed the upregulation of PD-L1 in PC9-KI cells treated with 1 μM of colchicine overnight (Fig. 3C). Furthermore, to show that the upregulation of PD-L1 by colchicine was a common phenomenon, we validated the effects of colchicine in other cell lines. By adding 1 μM of colchicine to the melanoma cell line A375 for 3 h, we confirmed the upregulation of PD-L1 protein and RNA (Fig. 3D,E). The IC50 for the maximum suppression of cell viability of A375 by colchicine was ~ 50 pM (Fig. 3F). Adding 1 μM of colchicine to the breast cancer cell line MDAMB231 for 3 h also showed the upregulation of PD-L1 protein and RNA (Fig. 3G,H). The IC50 for the maximum suppression of viability of MDAMB231 cells by colchicine was ~ 1 nM (Fig. 3I).

Fig. 1. Creating HiBiT knocked-in PC9 cells. (A). Schema for constructing HiBiT knocked-in PC9 cells using CRISPR Cas9 system. (B) Results of electrophoresis for PCR products of genome extracted from picked up clones. (C) Sequencing check for HiBiT tagging revealed the targeted insertion of HiBiT tag. (D) Western blotting confirmation for the targeted insertion of HiBiT tag in PC9-KI cells. (E) HiBiT assay for PC9 cells and PC9-KI cell to confirm the specificity of luminescence; $n = 3$. (F) HiBiT assay for PC9-KI cells transfected with siRNA for negative control or PD-L1 for 48 h; $n = 4$. (G) HiBiT assay for PC9-KI cells with various concentration of DMSO; $n = 4$. (H) HiBiT assay for PC9-KI cells stimulated with EGF, TNFα, and IFNγ; $n = 4$. (I) HiBiT assay for various concentration of p38 inhibitor SB203580; $n = 4$. Error bars represent the standard error mean (**$P < 0.01$, two-tailed Student's *t*-test).

We also measured the EC50 of vincristine for the maximum upregulation of PD-L1 on PC9-KI cells. The EC50 of vincristine for PC9-KI cells was ~ 100 nM, and the maximum upregulation of PD-L1 was twice than that with DMSO (Fig. 4A). The IC50 for the maximum suppression of the viabilities of PC9-





C **Up-regulators**

Name	Class	Action	Selectivity	Ratio(/DMSO)
Emetine dihydrochloride hydrate	Apoptosis	Activator		2.260
Vincristine sulfate	Cytoskeleton and ECM	Inhibitor	Tubulin	2.212
Podophyllotoxin	Cytoskeleton and ECM	Inhibitor	Tubulin	2.180
Vinblastine sulfate salt	Cytoskeleton and ECM	Inhibitor	Tubulin	2.057
Colchicine	Cytoskeleton and ECM	Inhibitor	Tubulin	1.809
Thiocolchicine	Apoptosis	Inducer		1.708
Thapsigargin	Intracellular Calcium	Releaser		1.609
Phorbol 12-myristate 13-acetate	Phosphorylation	Activator	PKC	1.509
Gemcitabine hydrochloride	Apoptosis and Cell Cycle			1.484
Ouabain	Ion Pump	Inhibitor	Na+/K+ ATPase	1.382
RepSox	Neuroscience	Inhibitor		1.287
SB743921 hydrochloride	Cell Cycle	inhibitor	KSP	1.283
Brequinar sodium salt hydrate	DNA-RNA transcription regulators	Inhibitor	DHODH	1.272

D **Down-regulators**

Name	Class	Action	Selectivity	Ratio(/DMSO)
Brefeldin A from Penicillium brefeldianum	Cytoskeleton and ECM	Inhibitor	Golgi apparatus	0.360
SB 202190	Phosphorylation	Inhibitor	p38 MAPK	0.403
PD153035 hydrochloride	Phosphorylation	Inhibitor	EGFR	0.557
Tyrphostin AG 1478	Phosphorylation	Inhibitor	EGFR	0.580
PD 169316	Phosphorylation	Inhibitor	p38 MAPK	0.581
ML240	Cell Signaling and Neuroscience	Inhibitor	AAA ATPase p97	0.608
GW2974	Phosphorylation	Inhibitor	EGFR / ErbB-2	0.647

Fig. 2. Compound screening using HiBiT-KI cells. (A) Schema for chemical compounds screening with LOPAC chemical compound library. (B) Results of chemical compound screening. (C) Candidate upregulators in chemical compound screening. (D) Candidate downregulators in chemical compound screening.

KI cells by vincristine was ~ 1 nM (Fig. 4B). Furthermore, to show that the upregulation of PD-L1 by vincristine was a common phenomenon, we validated the effects of vincristine in other cell lines. By adding 1 μM of vincristine to A375 for 3 h, we confirmed the upregulation of PD-L1 protein and RNA (Fig. 4C,D). The IC50 for the maximum suppression of cell viability of A375 cells by vincristine was ~ 500 pM (Fig. 4E). Adding 1 μM of vincristine to MDAMB231 cells for 3 h also showed moderate upregulation of PD-L1 protein and RNA (Fig. 4F,G). The IC50 for the maximum suppression of cell viability of MDAMB231 cells by vincristine was ~ 500 pM (Fig. 4H).

Subsequently, we validated the upregulation of PD-L1 by thapsigargin. The EC50 of thapsigargin for PC9-KI cells was ~ 5 nM, and the maximum upregulation of PD-L1 was 1.5 times higher than DMSO (Fig. 5A). The IC50 for maximum suppression of cell viability of PC9-KI cells by thapsigargin was ~ 5 nM (Fig. 5B). Furthermore, to demonstrate that the upregulation of PD-L1 by thapsigargin was a common phenomenon in other cell lines, we validated the effects of thapsigargin in A375 cells. By adding 1 μM of thapsigargin to A375 cells for 3 h, we confirmed the upregulation of PD-L1 protein and RNA (Fig. 5C,D). The IC50 for the maximum suppression of cell viability of A375 cells treated with thapsigargin was ~ 500 pM (Fig. 5E).

Finally, we validated the downregulation of PD-L1 by the AAA ATPase p97 (VCP) inhibitor -ML-240. The EC50 of ML-240 for PC9-KI cells was ~ 1 μM, and the maximum downregulation of PD-L1 was suppressed by 10% as compared to DMSO (Fig. 6A). Furthermore, to show that the downregulation of PD-L1 by ML-240 was a common phenomenon in multiple cell lines, we validated the effects of ML-240 on other cell lines. By adding 1 μM of ML-240 to A375 cells overnight, we confirmed the moderate downregulation of the PD-L1 protein, although no significant change was detected in PD-L1 RNA (Fig. 6B,C). The IC50 for maximum suppression of cell viability of A375 cells by ML-240 was ~ 5 μM (Fig. 6D). Adding 1 μM of ML-240 to MDAMB231 cells overnight also showed the downregulation of the PD-L1 protein and RNA (Fig. 6E,F). The IC50 for the maximum suppression of cell viability of MDAMB231 cells by ML-240 was ~ 5 μM (Fig. 6G).

Discussion

We created the HiBiT-tagged lung adenocarcinoma cell line, PC9-KI, using CRISPR Cas9 genome editing technology, which made it possible to detect the PD-L1 protein expression changes quickly and easily. Working on PC9-KI cells, we performed chemical compound screening using the LOPAC chemical compound library, and we identified two new PD-L1 upregulators (microtubule polymerization inhibitors and the calcium releasing inducer thapsigargin) and a novel PD-L1 downregulator (a p97 inhibitor ML-240). Furthermore, we confirmed that the regulation of PD-L1 by each compound could be observed in a melanoma cell line, A375, and a breast cancer cell line MDAMB231, which suggests the potential of PD-L1 regulation in various cell lines.

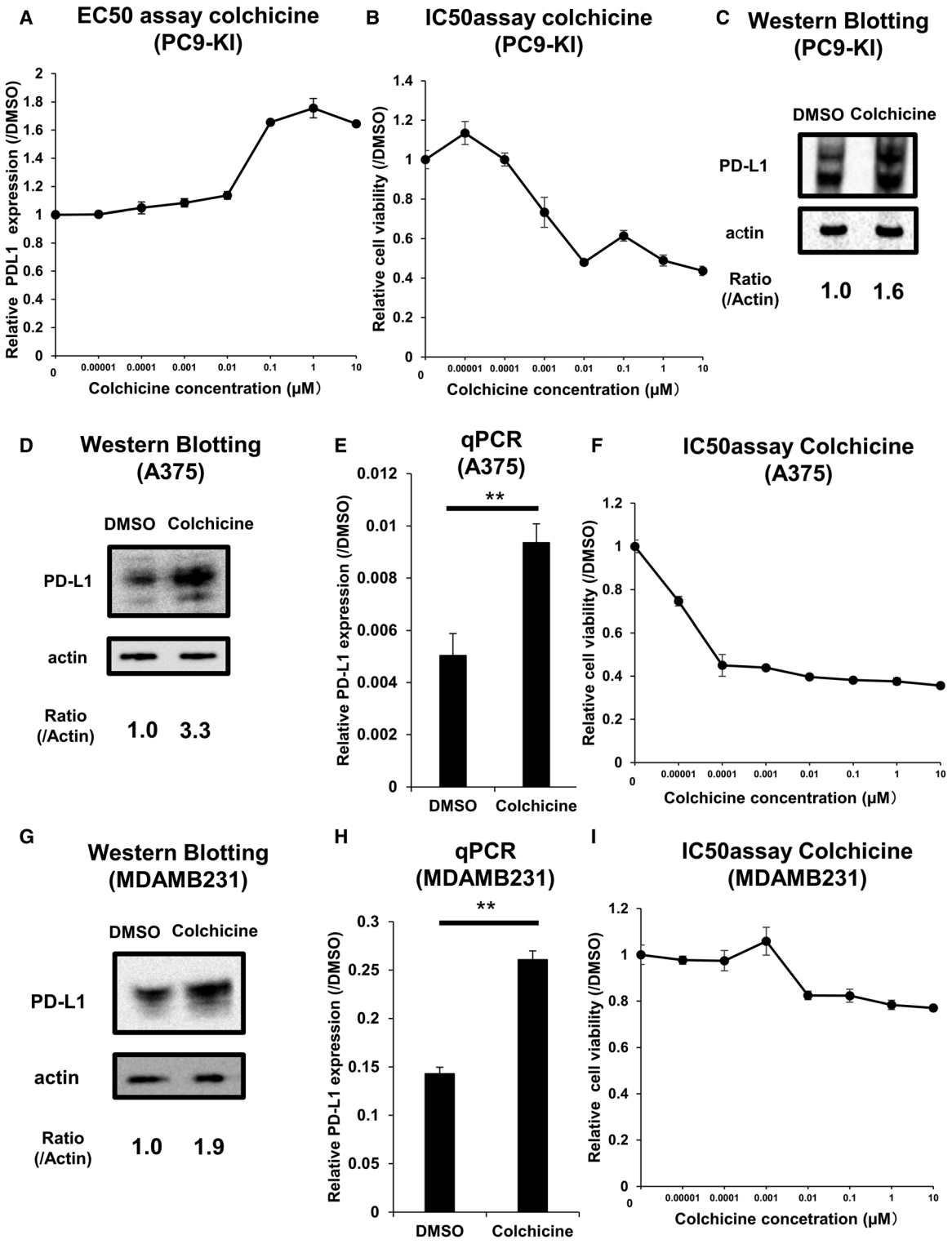
Chemical screening system using a HiBiT-tagged cell line

Although chemical screening using HiBiT-tagged cells has been previously reported, our study is the first to perform chemical screening using HiBiT for detecting endogenous protein expression changes for cancer research [11–13]. We used ssDNA for homologous recombination, taking advantage of the characteristics of the HiBiT tag. Using this method, we could easily prevent off-target tagging. Compared with the classic protein detection methods such as western blotting, ELISA, and fluorescence-activated cell sorting, the HiBiT system represents a quicker and streamlined method for detecting changes in protein expression. We expect this technology to be applied to screening systems for detecting important endogenous protein expression changes in various types of cancers in the future.

We selected PC9, an EGFR-mutated lung adenocarcinoma cell line, to establish this screening system. This was because of the moderate expression of PD-L1 and the ease of applying the existing CRISPR-Cas9 gene editing technology.

Immune checkpoint inhibitors that target PD-L1 and PD-1 have been used in clinical practice in many cancer types, including melanoma and lung adenocarcinoma [3,14–17]. However, although recent studies have revealed the regulation of PD-L1 expression through signaling pathways, ubiquitination, and glycosylation, small molecule compounds that regulate PD-L1 expression have not been identified [4–9,15,18–21]. Moreover, PD-L1 expression changes based on drugs used in combination with immune checkpoint inhibitors were not defined clearly; therefore, we believe that our strategy provides a simple and quick method to confirm and validate PD-L1 protein expression changes.

Compounds that can negatively regulate PD-L1 have the potential to directly regulate tumor immunity.



In addition, it has been reported that the clinical efficacy of immune checkpoint inhibitors is enhanced when PD-L1 expression increases, suggesting that compounds that positively regulate PD-L1 expression have the potential to enhance the effects of immune checkpoint inhibitors [22]. Our study is significant

Fig. 3. Colchicine functions as a PD-L1 upregulator. (A) EC50 assay of colchicine for maximum upregulation of PD-L1 in PC9-KI cells; $n = 3$. (B) IC50 assay of colchicine for minimum downregulation of PD-L1 in PC9-KI cells; $n = 3$. (C) Western blotting for endogenous PD-L1 of PC9-KI cells treated with 1 μM colchicine for overnight. (D) Western blotting for endogenous PD-L1 of A375 cells treated with 1 μM colchicine for 3 h. (E) qPCR of endogenous PD-L1 of A375 cells treated with 1 μM colchicine for 3 h; $n = 3$. (F) IC50 assay of colchicine for minimum downregulation of PD-L1 in A375 cells; $n = 3$. (G) Western blotting for endogenous PD-L1 of MDAMB231 cells treated with 1 μM colchicine for 3 h. (H) qPCR of endogenous PD-L1 of MDAMB231 cells adding 1 μM colchicine for 3 h; $n = 3$. (I) IC50 assay of colchicine for minimum downregulation of PD-L1 in MDAMB231 cells; $n = 3$. Error bars represent the standard error mean (**$P < 0.01$, two-tailed Student's *t*-test).

because the potential of regulating PD-L1 for each drug in the chemical compound library was obtained in a cross-sectional manner by our screening system.

Newly identified compounds regulating PD-L1 from the chemical screening

Microtubule polymerization inhibitors such as colchicine and vincristine were identified by chemical screening as compounds that specifically upregulate PD-L1 expression. The cross talk between microtubule regulation and the immune system has been reported for its importance in the inflammasome induction [23]. On the other hand, the relationship between PD-L1 regulation and microtubules is yet to be reported, which suggests the existence of a novel microtubule-dependent mechanism for the regulation of PD-L1 expression. In a previous report, PD-L1 expression was upregulated during the M phase of the cell cycle, and CUL3-SPOP induced by cyclin D reduced PD-L1 expression [21]. We infer from these reports that microtubule inhibitors stop the cell cycle in the M phase, which induces the upregulation of PD-L1 expression.

Although colchicine and vincristine are compounds with cytotoxic activity and anticancer potential, it is worth noting that microtubule-targeted compounds specifically upregulated PD-L1 expression, while other frequently used anticancer drugs with cytotoxic activity, such as cisplatin and fluorouracil, did not upregulate PD-L1 [24–26]. This result suggests a mechanism in tumor cells that detects the cellular stress caused by inhibition of microtubule polymerization, and thereby increasing the expression of PD-L1. Moreover, according to the results of the IC50 assay in our study, vincristine and colchicine increased PD-L1 expression in the cytotoxic concentration range. These results suggest that using immune checkpoint inhibitors with microtubule inhibitors may be therapeutically useful.

Another compound identified as an upregulator was thapsigargin, a noncompetitive inhibitor of sarco/endoplasmic reticulum (ER) calcium transport ATPase for ER calcium uptake. Thapsigargin has the ability to increase cytosolic calcium levels and induce ER stress [27,28]. Our study showed that thapsigargin increased the expression of PD-L1 in PC9-KI and A375 cells. The results of our screening indicated that compounds that induce extracellular calcium influx did not increase PD-L1 expression; therefore, there may be a mechanism of PD-L1 expression dependent on calcium influx from the ER (Table S1).

We also identified ML-240, an inhibitor of p97, as a compound that reduces PD-L1 expression. P97 is an AAA ATPase with multiple functions including cell cycle regulation and proteasome and DNA damage repair [29,30]. Our study is the first to report possible links between PD-L1 regulation and p97. ML-240 has also been reported to have a promising potential as an antitumor drug [31,32]. Therefore, our reports suggest the potential of ML-240 as an antitumor drug, which effectively prevents tumors from evading the immune system.

Limitation

In this study, we did not conduct *in vivo* experiments with immune checkpoint inhibitors. Therefore, further validation with *in vivo* experiments is essential for making the results clinically significant. In addition, the PC9 cell line used in this study was an EGFR-mutated cell line, which was not indicated for use with immune checkpoint inhibitors. Thus, it is necessary to evaluate its reproducibility in multiple cancer types. In our study, we confirmed the reproducibility of the breast cancer cell line MDAMB231 and the melanoma cell line A375, both of which showed similar tendencies as PC9-KI cells. Based on this information, the results of this study should be interpreted with caution with respect to clinical application.

Conclusion

We established a lung adenocarcinoma cell line (PC9-KI), with HiBiT-tagged into the C terminus of PD-L1 by genome editing technology, and used it in chemical

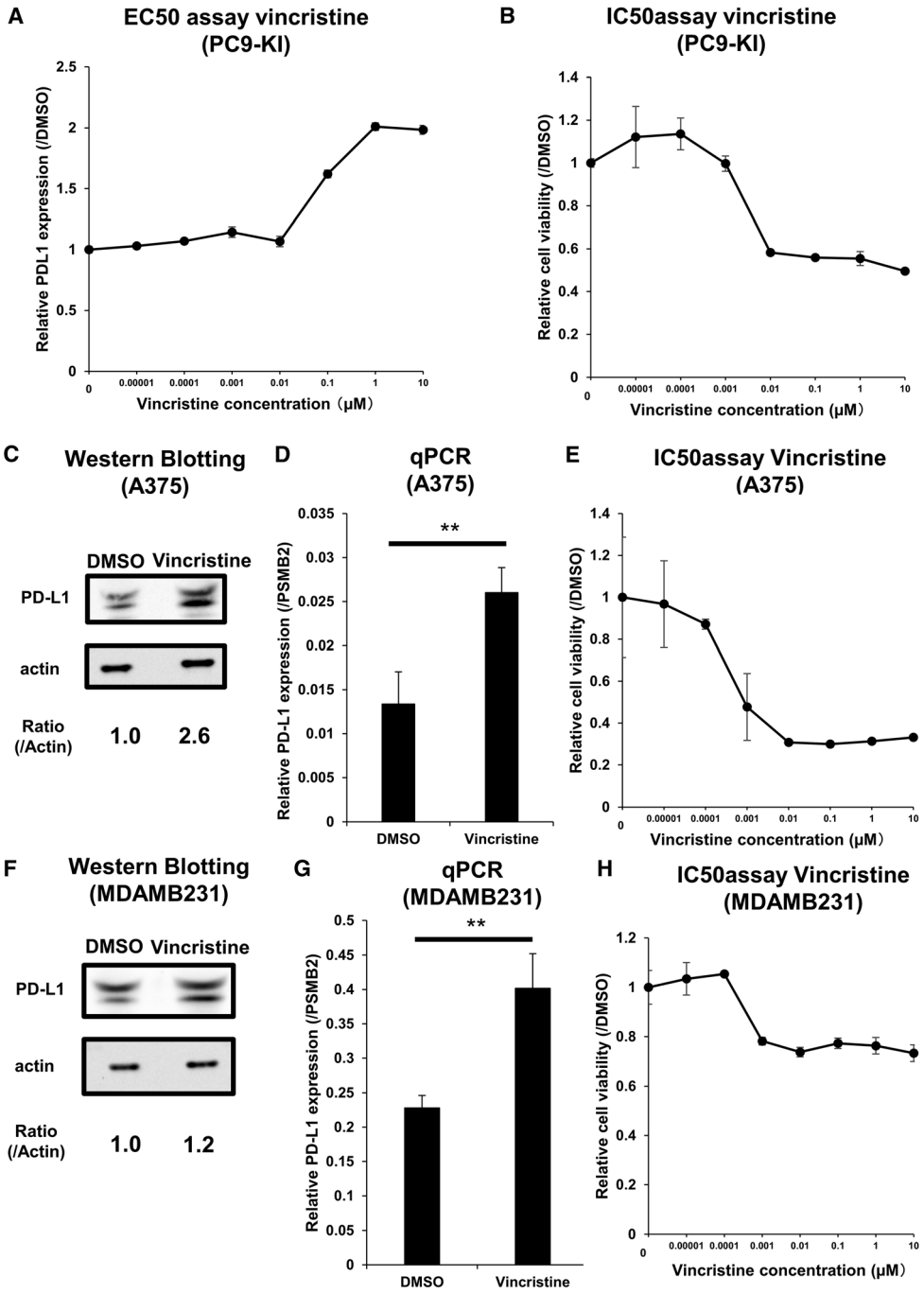


Fig. 4. Vincristine functions as a PD-L1 upregulator. (A) EC50 assay of vincristine for maximum upregulation of PD-L1 in PC9-KI cells; $n = 3$. (B) IC50 assay of vincristine for minimum downregulation of PD-L1 in PC9-KI cells; $n = 3$. (C) Western blotting for endogenous PD-L1 of A375 cells treated with 1 μM vincristine for 3 h. (D) qPCR of endogenous PD-L1 of A375 cells treated with 1 μM vincristine for 3 h; $n = 3$. (E) IC50 assay of vincristine for minimum downregulation of PD-L1 in A375 cells; $n = 3$. (F) Western blotting for endogenous PD-L1 of MDAMB231 cells treated with 1 μM vincristine for 3 h. (G) qPCR of endogenous PD-L1 of MDAMB231 cells treated with 1 μM vincristine for 3 h; $n = 3$. (H) IC50 assay of vincristine for minimum downregulation of PD-L1 in MDAMB231 cells; $n = 3$. Error bars represent the standard error mean (**$P < 0.01$, two-tailed Student's *t*-test).

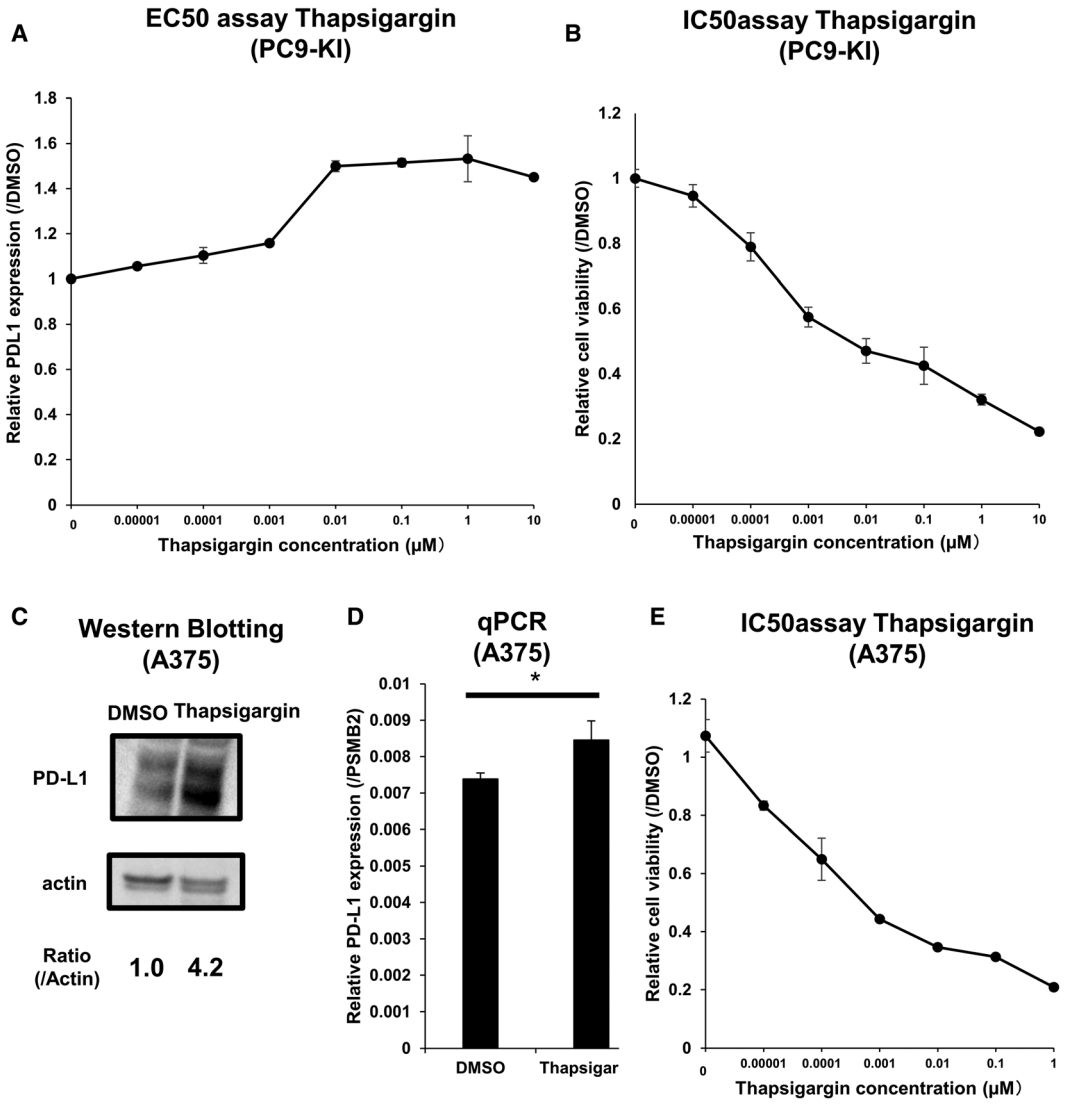


Fig. 5. Thapsigargin functions as a PD-L1 upregulator. (A) EC50 assay of thapsigargin for maximum upregulation of PD-L1 in PC9-KI cells; $n = 3$. (B) IC50 assay of thapsigargin for minimum downregulation of PD-L1 in PC9-KI cells; $n = 3$. (C) Western blotting for endogenous PD-L1 of A375 cells treated with 1 μM thapsigargin for 3 h. (D) qPCR of endogenous PD-L1 of A375 cells treated with 1 μM vincristine for 3 h; $n = 3$. (E) IC50 assay of vincristine for minimum downregulation of PD-L1 in A375 cells; $n = 3$. Error bars represent the standard error mean (*$P < 0.05$, two-tailed Student's *t*-test).

screening to identify compounds that regulate PD-L1 expression. As a result, we newly identified microtubule polymerization inhibitors and thapsigargin as upregulators, and a p97 inhibitor as a downregulator of PD-L1. These compounds regulate PD-L1 not only in lung adenocarcinoma, but also in breast cancer and melanoma.

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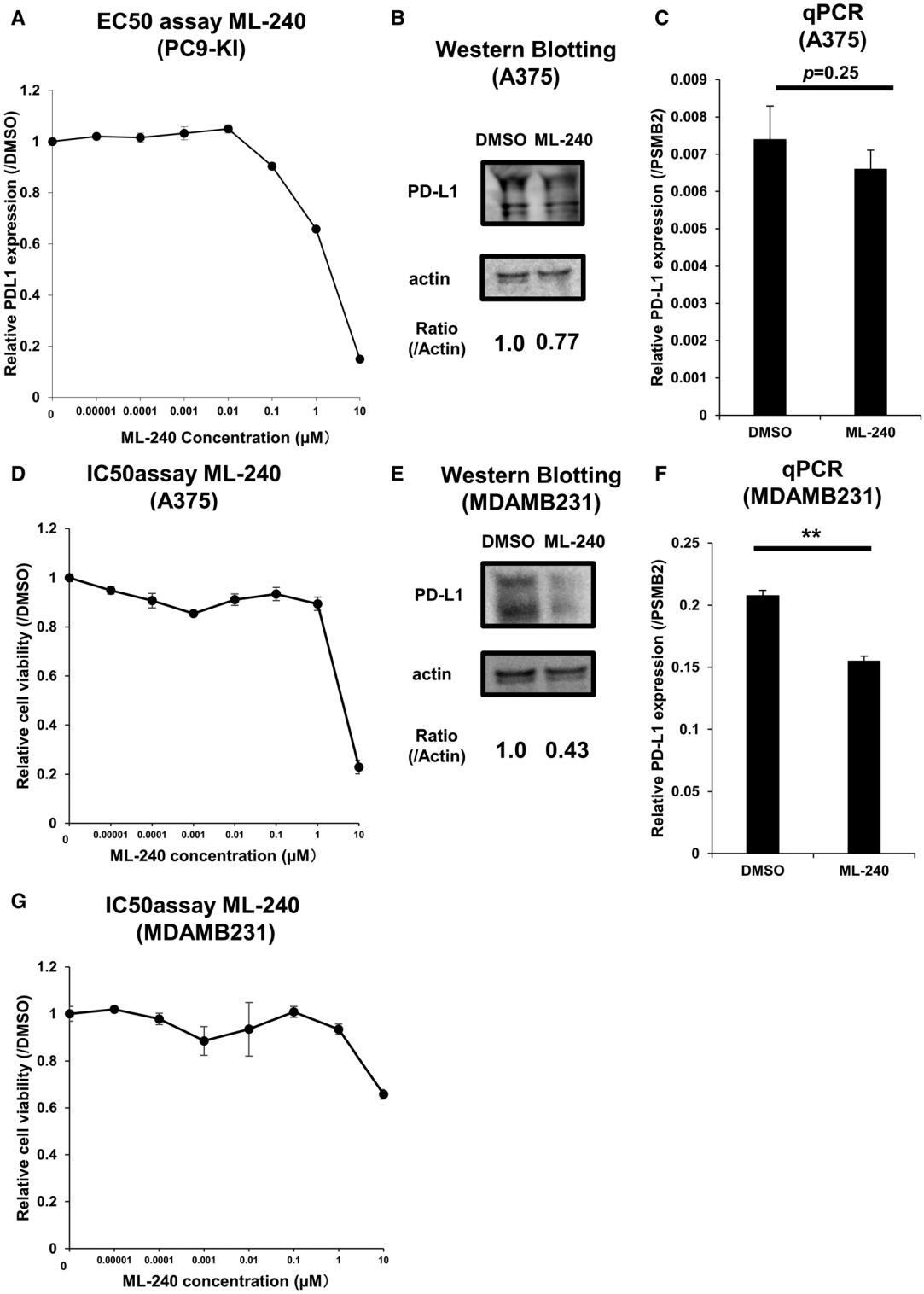

Fig. 6. ML-240 functions as a PD-L1 suppressor. (A) EC50 assay of ML-240 for minimum downregulation of PD-L1 in PC9-KI cells; $n = 3$. (B) Western blotting for endogenous PD-L1 of A375 cells treated with 1 μM ML-240 for 3 h. (C) qPCR of endogenous PD-L1 of A375 cells treated with 1 μM ML-240 for 3 h; $n = 3$. (D) IC50 assay of ML-240 for minimum downregulation of PD-L1 in A375 cells; $n = 3$. (E) Western blotting for endogenous PD-L1 of MDAMB231 cells treated with 1 μM ML-240 for 3 h. (F) qPCR of endogenous PD-L1 of MDAMB231 cells treated with 1 μM ML-240 for 3 h; $n = 3$. (G) IC50 assay of ML-240 for minimum downregulation of PD-L1 in MDAMB231 cells; $n = 3$. Error bars represent the standard error mean (**$P < 0.01$, two-tailed Student's *t*-test).

Author contributions

YU designed the study, analyzed the data, and wrote the manuscript. TM designed the study and provided critical advice on the experiments. RK analyzed the data and provided critical advices on the data analysis and writing of the manuscript. TC provided critical advices. YI performed the experiments. HA conceptualized the study and was in charge of the overall direction and planning.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. IC50 Assay for candidate downregulators of PD-L1.

Table S1. Sequences of oligos, primers, siRNAs and guide RNAs.

Table S2. Results of chemical compounds screening.