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Regulation of PD-L1 expression by matrix stiffness in lung cancer cells

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ABSTRACT

Expression of programmed death-ligand 1 (PD-L1) in tumor cells such as lung cancer cells plays an important role in mechanisms underlying evasion of an immune check point system. Lung cancer tissue with increased deposition of extracellular matrix is much stiffer than normal lung tissue. There is emerging evidence that the matrix stiffness of cancer tissue affects the phenotypes and properties of cancer cells. Nevertheless, the effects of substrate rigidity on expression of PD-L1 in lung cancer cells remain elusive. We evaluated the effects of substrate stiffness on PD-L1 expression in HCC827 lung adenocarcinoma cells by using polyacrylamide hydrogels with stiffnesses of 2 and 25 kPa. Expression of PD-L1 protein was higher on the stiffer substrates (25 kPa gel and plastic dish) than on the soft 2 kPa gel. PD-L1 expression was reduced by detachment of cells adhering to the substrate. Interferon-γ enhanced expression of PD-L1 protein cultured on stiff (25 kPa gel and plastic dishes) and soft (2 kPa gel) substrates and in the cell adhesion-free condition. As the stiffness of substrates increased, formation of actin stress fiber and cell growth were enhanced. Transfection of the cells with short interfering RNA for PD-L1 inhibited cell growth without affecting stress fiber formation. Treatment of the cells with cytochalasin D, an inhibitor of actin polymerization, significantly reduced PD-L1 protein levels. Taken together, a stiff substrate enhanced PD-L1 expression via actin-dependent mechanisms in lung cancer cells. It is suggested that stiffness as a tumor environment regulates PD-L1 expression, which leads to evasion of the immune system and tumor growth.

1. Introduction

The increased rigidity of the extracellular matrix (ECM) is one of characteristics of malignant phenotypes in solid tumors including lung cancer. The elastic modulus of solid tumors (20–30 kPa) is much stiffer than that of normal lung parenchyma (0.5–5 kPa) [1–3]. In cancer tissues, the surrounding cells, soluble factors, ECM, and mechanical cues, specifically matrix stiffness, form the tumor microenvironment [4]. In general, cells sense and respond to the stiffness of the substrates to which they are adhering. Motility, proliferation, gene expression, and differentiation of cells are critically regulated by substrate rigidity [5–7]. In cancer cells, stiff substrates corresponding to pathologically cancer-related fibrotic tissues induce differentiation to a highly synthetic and proliferative phenotype and epithelial-to-mesenchymal transition [8–10]. Thus, matrix stiffness contributes to the development and progression of cancer.

Programmed cell death-ligand 1 (PD-L1), also known as B7-H1 and CD274, is a ligand of programmed cell death (PD)-1, an immunoglobulin superfamily that regulates T cell antigens [11]. The expression of PD-L1 on cancer cells plays an important role in evasion of the immune system. Moreover, PD-L1 itself is related to growth and migration of cancer cells [10,12]. Recent clinical studies have shown the effectiveness of therapy targeting PD-1/PD-L1 signaling in patients with non-small cell lung cancer [13–15]. PD-L1 expression in cancer cells is regulated by multiple mechanisms such as PD-L1 genomic gains and release of interferon (IFN)-γ from T cells in the tumor microenvironment [16,17]. However, the effects of substrate stiffness on the expression of PD-L1 in tumor cells have not been elucidated.

This study was designed to determine whether substrate stiffness affects the regulation of PD-L1 in human lung cancer cells. We

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postulated that when cultured on soft substrates, cancer cells lose expression of PD-L1 protein. To this end, we used polyacrylamide gels with different stiffnesses as substrates [5].

2. Materials and methods

2.1. Cells

Cells of the human lung cancer cell lines HCC827, H1975, and A549 were acquired from Prof. J. Minna, The University of Texas Southwestern Medical Center. Cells of the breast cancer cell line MCF-7 were acquired from the Center for Advanced Medicine and Clinical Research, Nagoya University Hospital. The cells were maintained in culture medium (RPMI-1640, Sigma-Aldrich, St. Louis, MO) containing 10% FBS and supplemented with 1% L-glutamate and sodium pyruvate (Thermo Fisher Scientific, Waltham, MA) in an atmosphere of 5% CO_2 and 95% air at 37 °C.

2.2. Polyacrylamide hydrogels

Commercially available polyacrylamide hydrogels of different stiffnesses (2 or 25 kPa) bound to 6-well polystyrene plates or polystyrene dishes coated with type I collagen were used (Softwell; Matrigen Life Technologies, Brea, CA) [5]. We used 2 and 25 kPa gels as substrates of normal (soft) human lung and cancer-associated (stiff) tissue [2,3].

2.3. Quantitative analysis of cell numbers

HCC827 cells were seeded on polyacrylamide gels and plastic dishes (Corning Costar TC-Treated Microplates, Sigma-Aldrich) for five days. Phase-contrast images of four randomly chosen fields of view per condition per experiment were taken using an inverted microscope (CKX41; Olympus, Tokyo, Japan). Cell numbers were counted manually at days 0, 1, 3, and 5.

2.4. Western blotting

Protein concentrations of cellular lysates were measured by using a protein assay reagent kit (Bio-Rad, Hercules, CA). Equal amounts of lysates, adjusted for protein concentration, were resolved by SDS-PAGE using a 5–20% linear gradient running gel (Wako, Osaka, Japan). Proteins were transferred to nitrocellulose membranes, and membranes were blocked in 5% skim milk for 1 h at room temperature. Immunoblotting was performed using antibodies against PD-L1 (dilution 1:1000, #13684; Cell Signaling Technology, Danvers, MA), and rabbit polyclonal anti-actin (dilution 1:10000, A2103; Sigma-Aldrich). Immunodetection was accomplished using a HRP-conjugated donkey anti-rabbit secondary antibody (dilution 1:2000, NA934; GE Healthcare, Buckinghamshire, UK). The intensity was quantified by using Quantity One software ver. 4.6.9 (BioRad).

2.5. Immunofluorescence staining

Cells grown on polyacrylamide gels or plastic dishes were fixed and permeabilized for 30 min with 4% formaldehyde and 0.2% Triton X-100 in PBS. This was followed by blocking with 1% bovine serum albumin (BSA) in PBS for 60 min [18–20]. Then, the cells were incubated with a rabbit monoclonal anti-PD-L1 antibody (dilution 1:500, #13684; Cell Signaling Technology) in PBS containing 1% BSA for 60 min, washed, and further incubated with a goat anti-mouse secondary antibody (dilution 1:1000, A-11001; Thermo Fisher Scientific) for 60 min at room temperature. Filamentous actin (F-actin) and nuclei were stained with rhodamine-phalloidin (dilution 1:1000, R415; Thermo Fisher Scientific) and 4,6-diamino-2-phenylindole (DAPI) (dilution 1:1000, D523; Dojin, Kumamoto, Japan) for 50 min at room temperature. The immunofluorescence images were obtained using an upright laser scanning confocal microscope (A1RMP; Nikon, Tokyo, Japan), with a ×25/1.2 NA Plan Apo violet-corrected water immersion objective.

2.6. Transfection with siRNA

RNA interference was performed using short interfering RNAs (siRNAs) specific for the PD-L1 gene (*CD274*) (s26547, s26548, s26549; Sigma-Aldrich) or control siRNA (#983657; Thermo Fisher Scientific). To minimize the possibility of off-target effects, three different siRNAs targeting *CD274* were tested. Transfection reagent–siRNA complexes were prepared by using Lipofectamine RNAiMAX reagent (#13778150; Thermo Fisher Scientific). The cells seeded on plastic dishes were transiently transfected with 10 nM siRNA sequences for PD-L1 (siPD-L1) or control siRNA. Extraction of cell lysates for Western blotting and other cell assays was performed 72 h after siRNA transfection.

2.7. Cell proliferation assay

Cells (1×10^3) were seeded on 96-well plates (Corning 96-well TC-Treated Microplates, Sigma-Aldrich). A colorimetric viability assay was performed using the WST-1 Assay Kit (Roche, Basel, Switzerland) [21]. Absorbance was measured at a wavelength 450 nm with a reference wavelength of 650 nm using a Synergy HTX Multimode plate reader (Biotek, Winooski, VT).

2.8. Statistical analysis

Data are expressed as means ± SD. Unpaired *t*-test or analysis of variance (ANOVA) followed by Bonferroni's or Games-Howell's *post hoc* test was used to evaluate the statistical significance. $P < .05$ was considered statistically significant. Statistical analyses were performed using SPSS ver. 24 (SPSS Inc., Chicago, IL).

3. Results

3.1. Expression of PD-L1 protein in human lung cancer cells

First, we determined the levels of PD-L1 protein expression in three different lung cancer cell lines, HCC827, H1975, and A549, cultured on plastic dishes for five days. Consistent with the findings in the previous literature [22], the expression of PD-L1 proteins was observed in HCC827 and H1975 cells (Fig. 1A). There was little constitutive expression of PD-L1 protein in A549 cells. Application of IFN-γ (100 ng/ml) for 24 h significantly increased PD-L1 protein levels in all three cell types (Fig. 1A and B).

Because serum in a cell culture medium contains various kinds of growth factors and mediators, the effects of serum on PD-L1 expression were examined. Levels of PD-L1 proteins were not different between HCC827 cells with and without 10% FBS both in IFN-γ (100 ng/ml)-treated and untreated conditions (Fig. 1C and D). Based on these results, HCC 827 cells, which express the most PD-L1 among the three different cell types, were chosen, and the culture medium containing 10% FBS were used for the subsequent experiments.

3.2. Substrate stiffness regulates expression of PD-L1

The effects of substrate stiffness on PD-L1 expression were examined. HCC827 cells were cultured on polyacrylamide gels or a plastic dish for five days. The PD-L1 protein levels/actin ratio of the

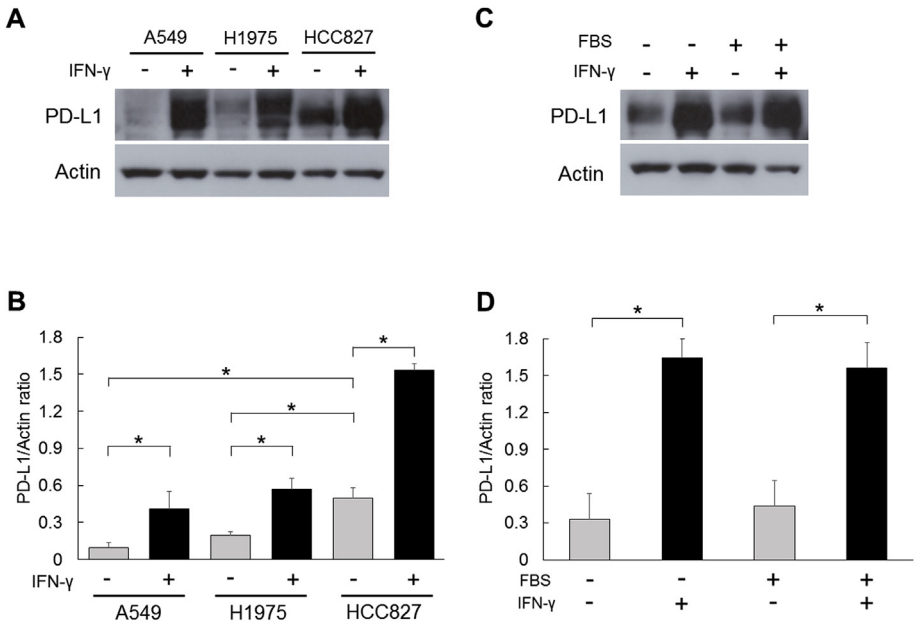

Fig. 1. Expression of PD-L1 in lung cancer cells.
(A) Assessment of protein expression of PD-L1 and actin in A549, H1975, and HCC827 cells by Western blotting. **(B)** Comparison of protein levels of PD-L1 to actin ratio. Cells were cultured on plastic dishes with or without IFN-γ (100 ng/ml) (n = 3). **(C)** Effects of serum on PD-L1 expression. HCC827 cells were cultured on plastic dish with or without 10% FBS. **(D)** Comparison of protein levels of PD-L1 to actin ratio (n = 4). Values are means ± SD. *Significantly different ($P < .05$) between the groups.

cells grown on 2 kPa gels were significantly lower than those grown on 25 kPa gels and plastic dishes (Fig. 2A and B). Similar to the finding in HCC827 cells, expression of PD-L1 protein in H1975 cells was reduced on 2 kPa gel (data not shown). Fig. 2C shows immunofluorescent images of PD-L1, fluorescent F-actin, and merged images of HCC827 cells cultured on 2 and 25 kPa gels and plastic

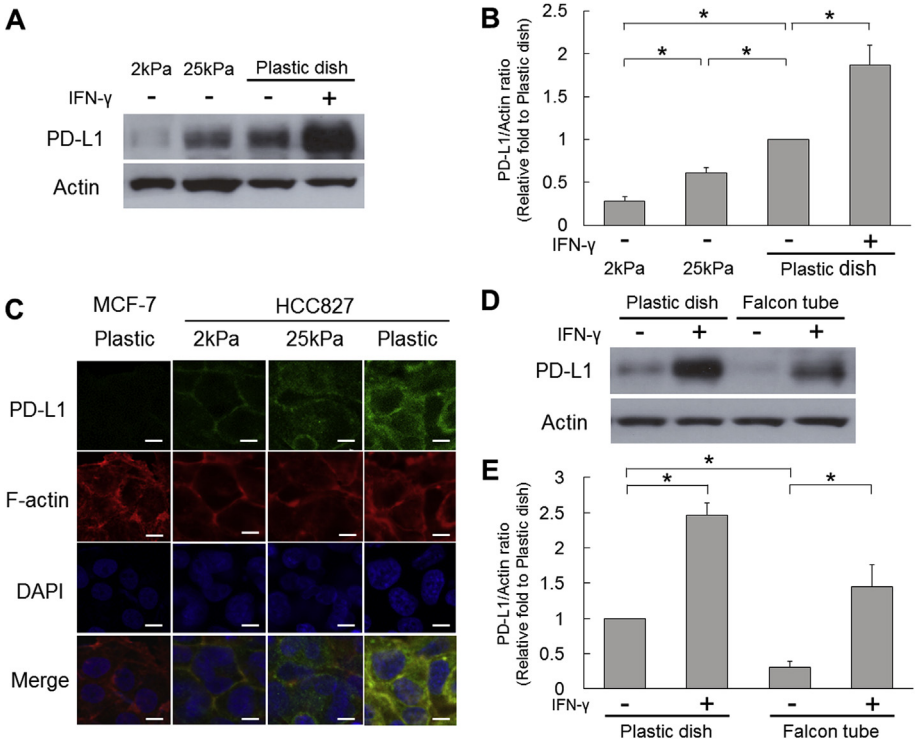

Fig. 2. Regulation of substrate stiffness and cell adhesion expression by PD-L1.
(A) The effects of substrate stiffness on expression of PD-L1 protein in HCC827 cells. Cells were cultured on polyacrylamide gels (2 and 25 kPa) and plastic dishes. **(B)** Comparison of protein levels of PD-L1 to actin ratio (n = 4). **(C)** Representative immunofluorescence images of HCC827 cells cultured on increasing substrate stiffnesses with or without IFN-γ (100 ng/ml) for four days, stained for PD-L1 (green), F-actin (red), and nuclei (blue), and then merged. Images were obtained using a confocal microscopy with a 25× objective. MCF-7 cells were used as a negative control for PD-L1. **(D)** Roles of cell adhesion on PD-L1 expression. Cells were cultured on plastic dishes or in 50 ml Falcon tubes with or without IFN-γ (100 ng/ml). **(E)** Comparison of protein levels of PD-L1/actin ratio (n = 4). Values are means ± SD. *Significantly different ($P < .05$) between the groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dishes. MCF-7 cells were used as a negative control without PD-L1 expression [23]. Expression of PD-L1 on plasma membrane and peripheral formation of F-actin stress fibers were observed on 25 kPa gels and plastic dishes and were decreased on 2 kPa gels (Fig. 2C).

3.3. Role of cell adhesion in PD-L1 expression

Next, we examined whether cell adhesion affects PD-L1 expression using Falcon® tubes (#352070; Corning Inc., Corning, NY) in which cells cannot adhere to substrates. The cells lost PD-L1 proteins when cultured in Falcon tubes for 24 h (Fig. 2D and E). Similar to the finding on plastic dishes, expression of PD-L1 protein was enhanced by treatment with IFN-γ (100 ng/ml) even in Falcon tubes (Fig. 2D and E).

3.4. Effects of substrate stiffness on cell morphology and proliferation

Supplementary Fig. S1A shows representative morphological images of HCC827 cells cultured on 2 and 25 kPa gels and plastic dishes for five days. The cells grew as various-sized colonies consisting of tightly packed small cells. Cell growth depended on substrate stiffness, and the numbers of cells cultured on 2 kPa gel were the smallest (Supplementary Fig. S1B). Application of IFN-γ (100 ng/ml) to the cells on 2 kPa gel altered colony formation and significantly inhibited cell growth (Supplementary Figs. S1A and B).

3.5. Role of PD-L1 in cell proliferation and F-actin formation

Next, we examined whether PD-L1 is involved in the mechanisms of stiff substrate-induced cell proliferation. HCC827 cells seeded on plastic dishes were transfected with siPD-L1 or control siRNA. The PD-L1 protein/actin ratios of the cells transfected with three different siPD-L1s were significantly lower than the control cells (Fig. 3A and B). Immunofluorescent images showed that expression of PD-L1 protein was reduced but that F-actin formation was preserved in the HCC827 cells transfected with siPD-L1 on plastic dishes (Fig. 3C). Fig. 3D shows representative morphological images of HCC827 cells transfected with siPD-L1 cultured on plastic dishes for three days. Colonies of cells were fewer in the siPD-L1-transfected condition than in plastic dishes. In quantitative cell proliferation analysis, absorbance of WST-1 assay of the cells transfected with siPD-L1 was significantly lower than that of the control cells (Fig. 3E).

3.6. Role of F-actin in PD-L1 expression induced by stiff substrate

We further examined whether the actin cytoskeleton is involved in the mechanisms of PD-L1 expression induced by stiff substrate. HCC827 cells seeded on plastic dishes were treated with cytochalasin D (Sigma-Aldrich), an inhibitor of actin polymerization or its solvent DMSO (Sigma-Aldrich) for 24 h. Levels of PD-L1 protein of the cells treated with cytochalasin D (0.1 and 1 μM) were significantly lower than the control cells (Fig. 4A and B). Immunofluorescent images show that both expression of PD-L1 protein and F-actin formation were reduced in the HCC827 cells treated with cytochalasin D (Fig. 4C).

4. Discussion

The main findings of the present study are that: (1) protein expression of PD-L1 in HCC827 cells on soft substrates or without cell adhesion to substrates was significantly lower than that on stiff substrates, (2) as the substrate stiffness increased, the cells proliferated with the formation of actin stress fibers, (3) transfection with siPD-L1 inhibited cell proliferation without affecting stress fiber formation, and (4) expression of PD-L1 was inhibited by disruption of actin polymerization with cytochalasin D. To our knowledge, we demonstrated for the first time that matrix stiffness as a tumor microenvironment regulates PD-L1 expression in cancer cells.

Expression of PD-L1 protein was significantly reduced when HCC827 cells were cultured on soft 2 kPa gel (Fig. 2), demonstrating that substrate stiffness regulates production of PD-L1 in lung cancer cells. Moreover, PD-L1 proteins were abolished when the cells were cultured in Falcon tubes (Fig. 2). Our results further suggest that adhesion of the cells to the matrix is essential for the stiff matrix-mediated PD-L1 expression. Surprisingly, expression of PD-L1 was restored by application of IFN-γ, even in the cells on soft gels and under the cell adhesion-free condition (Fig. 2). Moreover, PD-L1 protein was newly induced by IFN-γ in A549 cells, which do not constitutively express PD-L1 (Fig. 2). Thus, other mechanisms independent of adhesion and mechanical cues also exist for regulation of PD-L1 production.

The actin cytoskeleton is known as the major mechanosensor as well as a component regulating cellular mechanotransduction [19,24,25]. In the present study, F-actin formation was decreased in HCC827 cells on 2 kPa gel (Fig. 2). Moreover, disruption of the actin cytoskeleton with cytochalasin D inhibited expression of the PD-L1 protein induced by the stiff condition (Fig. 4). These results suggest that the polymerized actin is involved in the mechanisms of stiffness-induced PD-L1 expression in lung cancer cells. It has been proposed that various pathways such as MAPKs, Akt, microRNAs, and transcriptional factors play key roles in the regulation of constitutive and induced PD-L1 expression [17,26–29]. Further studies are needed to elucidate the molecular mechanisms by which mechanical cues and the actin cytoskeleton induced PD-L1.

Recent clinical studies have demonstrated that PD-L1 expression is related to tumor microenvironments in lung cancer. Saruwatari et al. reported that PD-L1 expression levels of solid-type tumors are significantly higher than those of other tumor types in surgically resected lung adenocarcinoma with EGF receptor mutation [30]. Toyokawa et al. reported that rates of PD-L1 were higher in radiologically and pathologically invasive tumors than those in non-invasive tumors in resected stage I lung adenocarcinoma [31]. Taken together, substrate stiffness as a biomechanical property of the tumor microenvironment together with other factors, such as IFN-γ, may contribute to PD-L1 expression of lung cancer cells and evasion of the anti-cancer immune system *in vivo*. Further clinical and translational studies are necessary.

In the morphological study, colony formation was different among the cells cultured on substrates of different stiffnesses (Fig. S1). Consistent with the findings in cancer cells [10,12], proliferation of HCC827 cells was affected by the rigidity of substrates (Fig. S1). As we postulated, reductions of PD-L1 proteins by siRNA transfection inhibited cell proliferation (Fig. 3). These findings demonstrated that PD-L1 is partially involved in the mechanisms underlying cell growth mediated by matrix stiffness in HCC827 cells. Importantly, IFN-γ, a strong inducer of PD-L1, inversely inhibited proliferation of HCC827 cells on 2 kPa gel (Fig. S1). Moreover, it is well-known that PD-L1 is constitutively expressed only in certain cancer cell types [22]. The findings in our study suggest that PD-L1-independent pathways also exist in the growth of HCC827 cells and that the regulation of PD-L1 expression and role of PD-L1 in proliferation depends on cell types and stimuli.

In summary, expression of PD-L1 and cell growth were affected by matrix stiffness in human lung cancer cells. Moreover, disruption of the actin cytoskeleton inhibited the stiffness-induced PD-L1. Our findings suggest that activation of lung cancer cells by a stiff

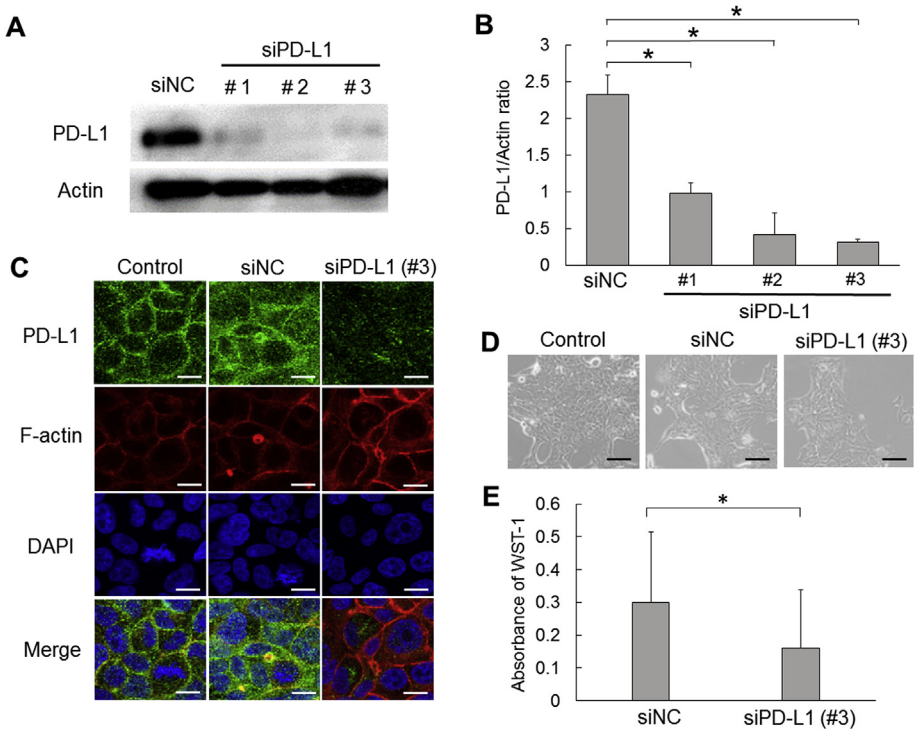

Fig. 3. Role of PD-L1 in regulation of cell proliferation in HCC827 cells.
(A) Western blotting of PD-L1 and actin to evaluate the knockdown efficiency of three different PD-L1 siRNAs (siPD-L1) or control siRNA (siNC) on plastic dishes. **(B)** PD-L1 protein/actin protein ratios were compared (n = 3). **(C)** Representative immunofluorescence images of HCC827 cells stained for PD-L1 (green), F-actin (red), and nuclei (blue), and then merged. Images were obtained using a confocal microscope with a 25× objective. Bar = 200 μm. **(D)** Representative phase-contrast images of HCC827 cells cultured on plastic dish for five days. Bar = 200 μm. **(E)** The numbers of cells were assessed by absorbance of WST-1 cell proliferation assay (n = 3). Values are means ± SD. *Significantly different ($P < .05$) between the groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

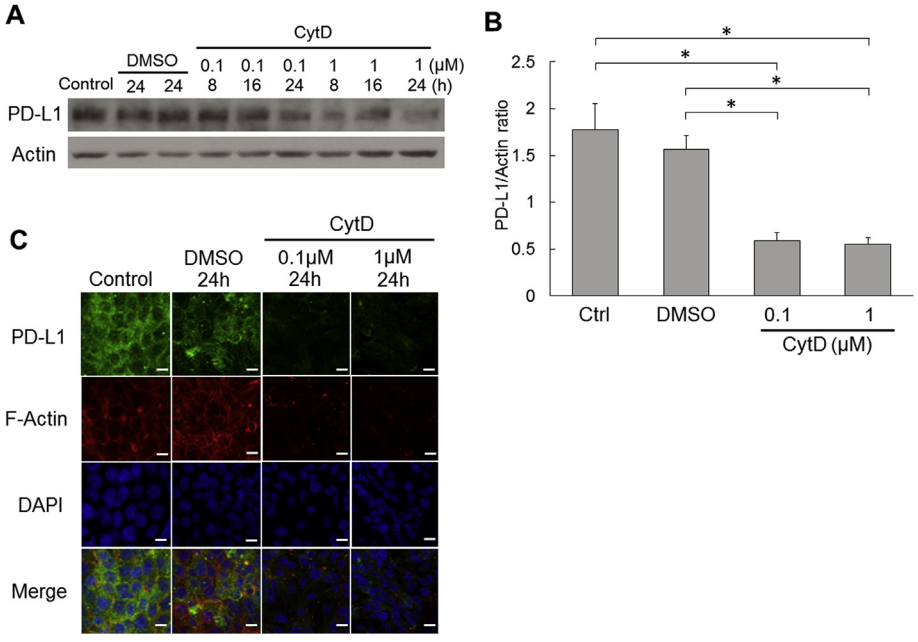

Fig. 4. Role of actin cytoskeleton in PD-L1 expression induced by stiff substrate.
(A) Western blotting of PD-L1 and actin to evaluate the effects of cytochalasin D (CytD), an inhibitor of actin polymerization. HCC827 cells cultured on plastic dishes were treated with cytochalasin D (0.1 and 1 μM) or solvent DMSO for 24 h. **(B)** PD-L1 protein/actin protein ratios were compared (n = 3). Values are means ± SD. *Significantly different ($P < .05$) between the groups. **(C)** Representative immunofluorescence images of HCC827 cells stained for PD-L1 (green), F-actin (red), and nuclei (blue), and then merged. Images were obtained using a confocal microscope with a 25× objective. Bar = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

substrate as a cancer tissue microenvironment may be involved in evasion of the immune system and the mechanisms of progression of lung cancer.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.12.115.

Transparency document

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Appendix A. Supplementary data

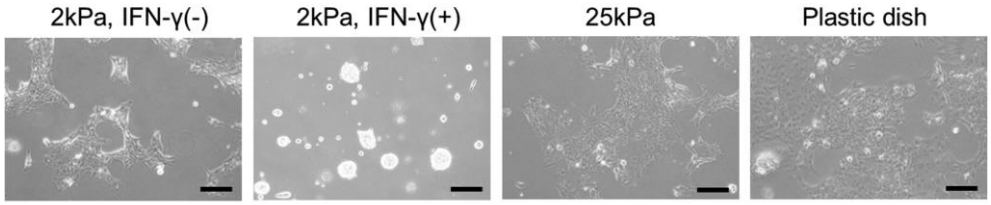
Figure legend

Supplementary Fig. S1.

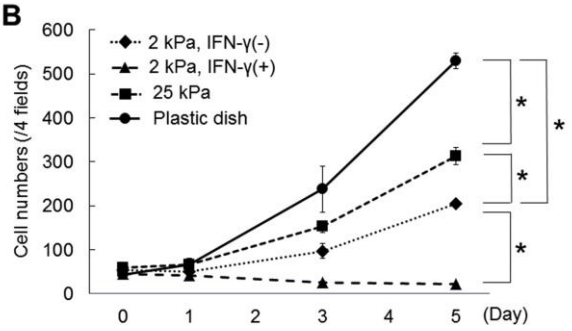
Effects of substrate stiffness on morphology and proliferation of HCC827 cells.

(A) Representative phase-contrast images of HCC827 cells cultured on polyacrylamide hydrogels (2 and 25 kPa) or plastic dish for five days. The cells were also cultured on 2 kPa gel with IFN-γ (100 ng/ml) for five days. **(B)** The numbers of cells in four randomly chosen fields were manually counted. Values are means±SD (n=4). *Significantly different (P<0.05) between the groups. Bar=200 μm.

S1A


2kPa, IFN-γ(-)
2kPa, IFN-γ(+)
25kPa
Plastic dish

S1B


2 kPa, IFN-γ(-)
2 kPa, IFN-γ(+)
25 kPa
Plastic dish
Cell numbers (/4 fields)
600
500
400
300
200
100
0
0
1
2
3
4
5
(Day)
*
*
*