Identification of proteasomal catalytic subunit *PSMA6* as a therapeutic target for lung cancer

Tomohiko Kakumu,[1] Mitsuo Sato,[1] (iD) Daiki Goto,[1] Toshio Kato,[1] Naoyuki Yogo,[1] Tetsunari Hase,[1] Masahiro Morise,[1] Takayuki Fukui,[2] Kohei Yokoi,[2] Yoshitaka Sekido,[3,4] Luc Girard,[5] John D. Minna,[5] Lauren A. Byers,[6,7] John V. Heymach,[6,7] Kevin R. Coombes,[8] Masashi Kondo[1] and Yoshinori Hasegawa[1]

Departments of [1]Respiratory Medicine, Nagoya University Graduate School of Medicine, Nagoya; [2]Thoracic Surgery, Nagoya University Graduate School of Medicine, Nagoya; [3]Cancer Genetics, Nagoya University Graduate School of Medicine, Nagoya; [4]Division of Molecular Oncology, Aichi Cancer Center Research Institute, Nagoya, Japan; [5]Hamon Center for Therapeutic Oncology Research and the Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas; [6]Department of Thoracic/Head & Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas; [7]The University of Texas Graduate School of Biomedical Sciences, Houston, Texas; [8]Department of Biomedical Informatics, Ohio State University, Columbus, Ohio, USA

To identify potential therapeutic targets for lung cancer, we performed semi-genome-wide shRNA screening combined with the utilization of genome-wide expression and copy number data. shRNA screening targeting 5043 genes in NCI-H460 identified 51 genes as candidates. Pathway analysis revealed that the 51 genes were enriched for the five pathways, including ribosome, proteasome, RNA polymerase, pyrimidine metabolism and spliceosome pathways. We focused on the proteasome pathway that involved six candidate genes because its activation has been demonstrated in diverse human malignancies, including lung cancer. Microarray expression and array CGH data showed that *PSMA6*, a proteasomal subunit of a 20S catalytic core complex, was highly expressed in lung cancer cell lines, with recurrent gene amplifications in some cases. Therefore, we further examined the roles of *PSMA6* in lung cancer. Silencing of *PSMA6* induced apoptosis or G2/M cell cycle arrest in cancer cell lines but not in an immortalized normal lung cell line. These results suggested that *PSMA6* serves as an attractive target with a high therapeutic index for lung cancer.

Accumulating evidence from molecular biology, epidemiology and histopathology has suggested that human lung cancer develops through a multi-step carcinogenic process.[1–4] During this process, normal lung epithelial stem cells, presumably the origin of lung cancer, enhance their malignant potential step-wise by acquiring genetic or epigenetic changes in oncogenes or tumor suppressor genes associated with survival advantages before finally transforming to overt cancer cells.[5] Recent advances in high-throughput genetic analysis revealed that single lung cancer cells harbour a number of (~20 to ~200) genetic and epigenetic changes.[4] Nevertheless, findings from cancer epidemiology and the experimental models of the multi-step lung carcinogenic process, which were developed by our group and others, suggested that only a handful of changes are 'drivers' whereas others are only 'passengers'.[6,7] From these numerous altered genes, it is vital to sort out those that truly contribute to the oncogenic properties of cancer cells by performing functional screening because such genes could serve as valuable therapeutic targets.

A pooled shRNA library screening is emerging as a powerful tool for identifying genes that contribute to various malignant phenotypes, including enhanced ability of proliferation and survival as well as resistance to treatments.[8] For instance, using genome-wide shRNA screening, Luo *et al.* discovered that the knockdown of some genes selectively impaired the viability of *KRAS*-mutant cancer cells.[9] Another study has identified *MED12* as the gene that regulates the resistance of multiple types of human cancers to molecular-targeted drugs.[10] In search for new lung tumor suppressor genes (TSGs), a study used NIH3T3 cells for a shRNA screening and revealed new candidate TSGs, whose loss activated the pathway of the fibroblast growth factor (FGF) gene.[11] Collectively, these studies serve as a proof of principle for pooled shRNA-based screening to identify genes with important roles in the pathogenesis of cancer cells.

With this background, we performed a screening with a pooled shRNA library in search for genes that are critical for the survival and/or proliferation of lung cancer cells using a lung cancer cell line, NCI-H460. One inevitable issue with this type of analysis is that the genes identified by a screening would include numerous genes that are essential not only for

cancer cells but also for normal cells.[8] In addition, we cannot simply exclude such genes from the list of candidates because pathways essential for normal cells are sometimes activated in cancer cells and may serve as important therapeutic targets.[12,13] Therefore, we additionally used genomic data to strengthen the reliability of our screening process. We integrated our mRNA expression and gene copy number data into our shRNA screening results because we anticipated that amplified and/or highly expressed genes would be more likely to be associated with cancer-specific biology.

Through this integrative analysis we identified *PSMA6*, a subunit of the proteasome complex, as one of the most promising targets for lung cancer. We showed that *PSMA6* knockdown suppressed the viability of cancer cells through the induction of apoptosis or cell cycle arrest at G2/M, with only a minimal effect on normal lung epithelial cells; these suggested that the development of novel lung cancer therapies that target *PSMA6* holds great promise.

Materials and Methods

Cell cultures. Nineteen lung cancer cell lines and normal human bronchial epithelial cell line, NHBE and two *Cdk4/hTERT*-immortalised normal human bronchial epithelial cell lines, HBEC3 and HBEC4 were purchased from ATCC (Manassas, VA, USA) or obtained from the Hamon Center collection (University of Texas Southwestern Medical Center, Dallas, TX, USA).[14] 293FT was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Four cell lines (H460, H1299 H661, and HBEC3) were used for functional analyses. Lung cancer cell lines were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS); NHBE, HBEC3 and HBEC4 were cultured in KSFM (Life Technologies, Gaithersburg, MD, USA) supplemented with 50 ng/mL bovine pituitary extract and 5 ng/mL epidermal growth factor. 293FT cells were cultured in Dulbecco's modified eagle medium (DMEM) (Thermo Fishier Scientific) supplemented with 10% FCS.

Packaging of lentiviral pool. DECIPHER library human Module 1 (#DHPAC-M1-P) was obtained from Cellecta Inc. (Mountain View, CA, USA). For packaging into lentiviral particles, plasmid DNA from Module 1 was co-transfected into 293FT cells together with Packaging Plasmid Mix (Cellecta Inc.). One day before transfection, cells were seeded on four 75-cm^2 flasks at 8×10^4 cells/cm^2 in standard DMEM. On the day of transfection, 6 μg shRNA library was mixed with 30 μg Packaging Plasmid Mix and 60 μL PLUS Reagent (Thermo Fisher Scientific) to make a total volume of 1.3 mL DMEM. The plasmid mix was incubated for 15 min at room temperature before mixing with 1.3 mL of Opti-MEM containing 90 μL Lipofectamine 2000 (Thermo Fishier Scientific). The complete transfection mix was incubated for another 15 min at room temperature before adding 600 μL to each cell culture flask containing 293FT cells. At 24 h post-transfection the medium in each culture flask was replaced by 9 mL DMEM. At 24 h after medium change, the supernatant containing lentivirus was harvested and passed through a 0.45-μm filter. Lentiviral pools were centrifuged at 106,750 *g* for 2 h in an ultracentrifuge (Optima XE-100; Beckman Coulter, Brea, CA, USA). The supernatant was replaced with 300 μL PBS and stored at −80°C until further use.

Pooled RNAi screening. H460 cells were seeded 24 h prior to transduction. In total, of 4×10^7 cells were seeded for transduction with the shRNA pool into 24 culture flasks measuring

75 cm^2. Cells were transduced with the shRNAs packaged with lentivirus at low multiplicity of infection (MOI = 0.2) in RPMI. As a result, we anticipated that each of the 27 500 shRNA expression plasmids was integrated into the genome of 200 individual cells. After 24 h, the viral supernatant was replaced with standard RPMI culture medium containing 2 μg/mL cell culture that was tested on puromycin dihydrochloride. Selection was continued for 3 more days, following which the cells were transferred into fresh standard RPMI medium without antibiotics and grown for 24 h. Cells were transferred into RPMI containing 1% FCS and incubated for 5 more days without treatment. Cells were passaged after reaching 80% confluence. Finally, cells were harvested, shock-frozen and kept at −80°C. Genomic DNA was prepared from cell pellets; polymerase chain reaction amplification of barcodes and barcode quantitation by next-generation sequencing were performed at Cellecta.

Construction of *PSMA6*-expressing lentiviral vector and viral transduction in HBEC3. A custom-ordered pUC57 plasmid vector containing a full-length fragment of *PSMA6*, designated as pUC57-*PSMA6*, was purchased from Genescript (Piscataway, NJ, USA). An *Eco*RI/*Sal*I-digested *PSMA6* insert from pUC57-*PSMA6* was cloned into *Eco*RI/*Xho*I-digested pLenti6/V5-GW/*lacZ*, generating pLenti6-*PSMA6* vector. Lentiviral transduction of *PSMA6* in HBEC3 was performed as described previously.[15]

Microarray expression analysis. 163 non-small cell lung cancer (NSCLC) cell lines and 59 normal control cell lines were used (Table S1). Using the Illumina TotalPrep RNA Amplification Kit (Cat# IL1791; Ambion, Austin, TX, USA), 500 ng of total RNA from each sample was used to label the cRNA probes. Amplified and labelled cRNA probes (1.5 μg) were hybridised to Illumina Human WG-6 v3.0 Expression Bead-Chip (Cat# BD-101-0203; Ambion) before being, washed, blocked and detected by streptavidin-Cy3, following the manufacturer's protocol; thereafter, the chips were scanned using the Illumina iScan System (Ambion). Bead-level data were obtained and pre-processed using the R package Model-Based Background Correction for background correction and probe summarization. Pre-processed data were then quantile-normalized and log-transformed.

DNA copy number analysis. 108 NSCLC cell lines were used (Table S2). Illumina BeadStudio was used to extract the "Log R Ratio" for each SNP. The package *DNA* copy for the R statistical software environment was used to segment the data. Final copy number variation was interpreted qualitatively as deleted, unchanged, or amplified.

Proteasome activity assay. Proteasome activity was measured using a 20S proteasome activity assay kit (Merck Millipore, Temecula, CA, USA), according to the manufacturer's protocol. In short, total cell lysates prepared from each cells were incubated with the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC. The free AMC fluorescence can be quantified using a 380/460 nm filter set in a fluorometer.

Western blot analysis. Western blot analysis was performed using whole cell lysates, as described previously.[15] The primary antibodies used were rabbit polyclonal anti-actin (Sigma-Aldrich), rabbit monoclonal anti-PSMA6, rabbit monoclonal anti-cleaved poly(ADP-ribose) polymerase (PARP), rabbit polyclonal anti-p53, and rabbit monoclonal anti-p21[WAF1/CIP1] (Cell Signaling Technology, Boston, MA, USA). Actin protein levels were measured as a control for equality of protein loading. Anti-rabbit antibody or anti-mouse antibody (GE Healthcare, Tokyo, Japan) was used at 1:2000 dilution as a secondary antibody.

Transfection of short interfering RNA. In total, 5×10^5 of cells were plated in 10-cm plates. On the next day, the cells

were transiently transfected with either 10 nM predesigned short interfering RNA (siRNA) (MISSION siRNA; Sigma-Aldrich) that targeted *PSMA1*, *PSMA2*, *PSMA3*, *PSMA6*, *PSMA7* and *PSMD13* or control siRNA (Sigma-Aldrich) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. After 48 h, the transfected cells were harvested for further analyses or were re-plated for cell growth assays.

Immunohistochemistry. Surgically resected lung cancer samples were obtained from patients at the Nagoya University Hospital. Before tissue sample collection, ethics committee approval and fully informed written consent were obtained from all patients. Tissue sections (4-μm-thick) were cut, deparaffinized in xylene, rehydrated in graded alcohols and blocked with 5% skim milk for 10 min. Rabbit monoclonal anti-PSMA6 antibody (Cell Signaling Technology) was used. Staining was performed manually.

Tissue samples were divided into four groups, according to the percentage of tumour cells that were strongly stained: immunohistochemistry (IHC) scores were 0, 1+, 2+ and 3+ for, 0%, <33%, 33–66% and >66% staining, respectively.

Cell growth assays. A colorimetric proliferation assay was performed using the WST-1 Assay Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. Liquid colony formation and soft agar colony formation assays were performed, as described previously.[15]

Cell cycle analysis. Cells were harvested 48 h after the transfection of siRNAs and washed in ice-cold PBS. Following centrifugation at 437 *g* for 3 min, cells were suspended in 300 μL of cold PBS under gentle vortex before fixing by dropwise addition of 700 μL ice-cold ethanol. Fixed cells were stored at 4°C for at least 2 h. For staining, pelleted cells were washed twice with cold PBS and re-suspended in 1 mL PBS containing 200 μg/mL RNase before staining with 20 μg propidium iodide. Cells were incubated at 37°C for 30 min and maintained at 4°C before analysis. Cells were filtered through a 40-μm nylon mesh and analyzed using a flow FACS Gallios flow cytometer (Beckman Coulter).

Statistical analysis. IBM SPSS version 23 software (International Business Machines Corp., Armonk, NY, USA) was used for all statistical analyses in this study. The Mann–Whitney *U*-test was used to analyze differences between two groups.

Results

Semi-genome-wide screening with a pooled shRNA library identified the genes essential for the proliferation and/or survival of the lung cancer cell line NCI-H460. To systemically identify genes indispensable for lung cancer cell survival and/or proliferation, we performed semi-genome-wide dropout viability analysis using a pooled shRNA library that targeted 5043 genes. The library was transduced in the NCI-H460 lung cancer cell line by lentiviral infection (Fig. 1a). We used this cell line because of the following reason. The cell line had been shown to be highly invasive and metastatic[16] but to have wild-type p53,[17] which is a key player in both apoptosis and

cell cycle arrest. Therefore, it seemed to be suitable for screening of aberrantly, oncogenically activated genes whose knockdown causes growth suppression mainly through apoptosis and/or cell cycle arrest. The abundance of individual shRNA constructs for each gene was quantified by sequencing the associated barcode sequences with next-generation sequencing. The suppressive effects on cell viability were determined by dividing the normalized barcode abundance by that of the baseline reference. The significance of the suppressive effects was determined by performing *t*-test to compare replicates of shRNA with a given gene with those of luciferase. The result is shown as a volcano plot (Fig. 1b). We selected 51 genes as potential candidates on the basis of significant average suppressive effects ($P < 0.05$) below a $\log^2$ of -2.

To identify the pathways overrepresented in the 51 genes, we performed gene-annotation enrichment analysis using a web-based online pathway tool, NIH-DAVID.[18,19] We found that the 51 genes were significantly enriched for the five pathways, including ribosome, proteasome, RNA polymerase, pyrimidine metabolism and spliceosome pathways (Table 1). All five pathways were essential for survival and/or proliferation, ensuring the reliability of our screening procedure. We focused on the proteasome pathway because its activation has been demonstrated in multiple types of human cancers and a drug targeting proteasome, bortezomib, has been clinically used for multiple myeloma.[20] In the proteasome pathway, there were five potential candidate genes (*PSMA1*, *PSMA2*, *PSMA3*, *PSMA6* and *PSMD13*) that encoded subunits of the 26S proteasome complex; *SHFM1* encoded a multifunctional protein involved in DNA repair and proteasome assembly. We excluded *SHFM1* from our subsequent analysis because its oncogenic roles have already been demonstrated in several types of cancers such as gastric, ovarian and breast cancers.[21,22] In addition, in our analysis, we included *PSMA7*, another subunit of the 26S proteasome complex, which was included in our 51 candidate genes but did not appear in the proteasome pathway after our gene-annotation enrichment analysis. Therefore, we selected six 26S proteasome subunits genes for validation and functional analyses. To validate our screening results, we individually silenced these six genes with two independent synthesized siRNA oligos for each gene and evaluated the effects on cell viability. The analysis revealed that the knockdown of proteasome subunit genes suppressed the viability of H460 in all cases, confirming our screening results (Fig. 1c).

Genome-wide gene expression and copy number data suggested that *PSMA6* is one of the most attractive targets. To choose the most promising targets from the candidate proteasome subunit genes, we integrated our data on gene expression analysis and gene copy number analysis of a panel of normal and lung cancer cell lines. We expected that genes with increased expression and/or increased copy number may have more tumor-specific biology; therefore, such genes would be more likely to serve as better therapeutic targets. Data of our gene expression analysis using Illumina chips in 163 cell lines revealed that compared with 59 normal controls, *PSMA3* and

Fig. 1. Semi-genome-wide screening with a pooled shRNA library identifies genes essential for the proliferation and/or survival in the lung cancer cell line NCI-H460. (a) A schematic summary of shRNA screening for identifying genes indispensable for lung cancer cells to survive and/or proliferate. The screen involves three steps: (i) cell transduction with a library of shRNA agents; (ii) 96-h incubation; (iii) quantification of shRNA abundance by barcode sequencing. Depleted (blue), unchanged (green) or enriched (red) amount of shRNA means suppressive, none or promoting effects on the proliferation and/or survival of each cell during the screening, respectively. (b) Screening results are presented as a volcano plot, with 5043 genes being ranked by fold-change and significance. (c) Cell viability assay for H460 cells transfected with two independent synthesized siRNA oligos targeting *PSMA1*, *PSMA2*, *PDSMA3*, *PSMA6*, *PSMA7* or *PSMD13*.
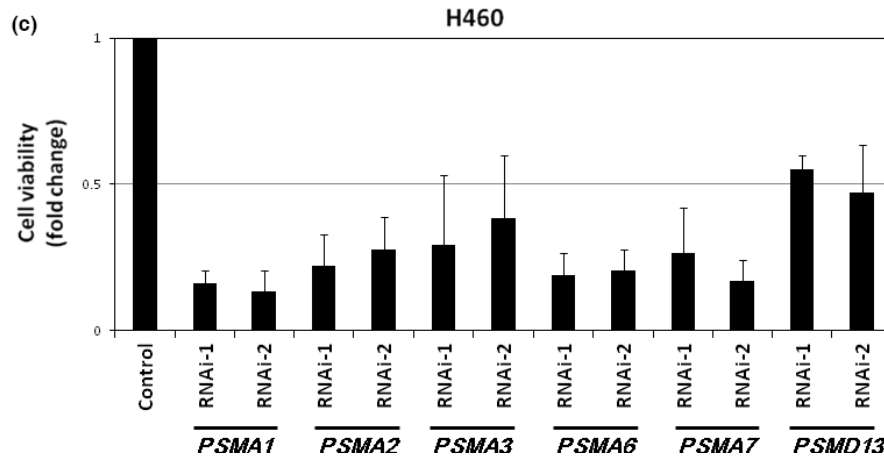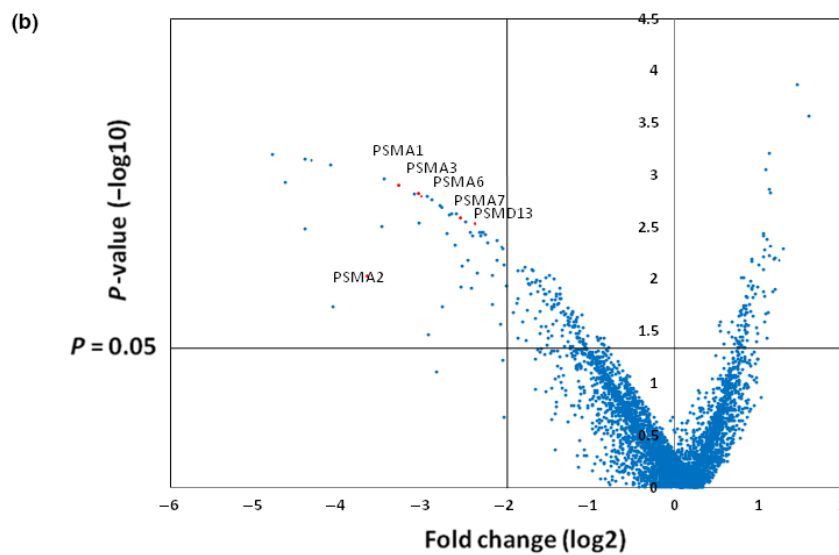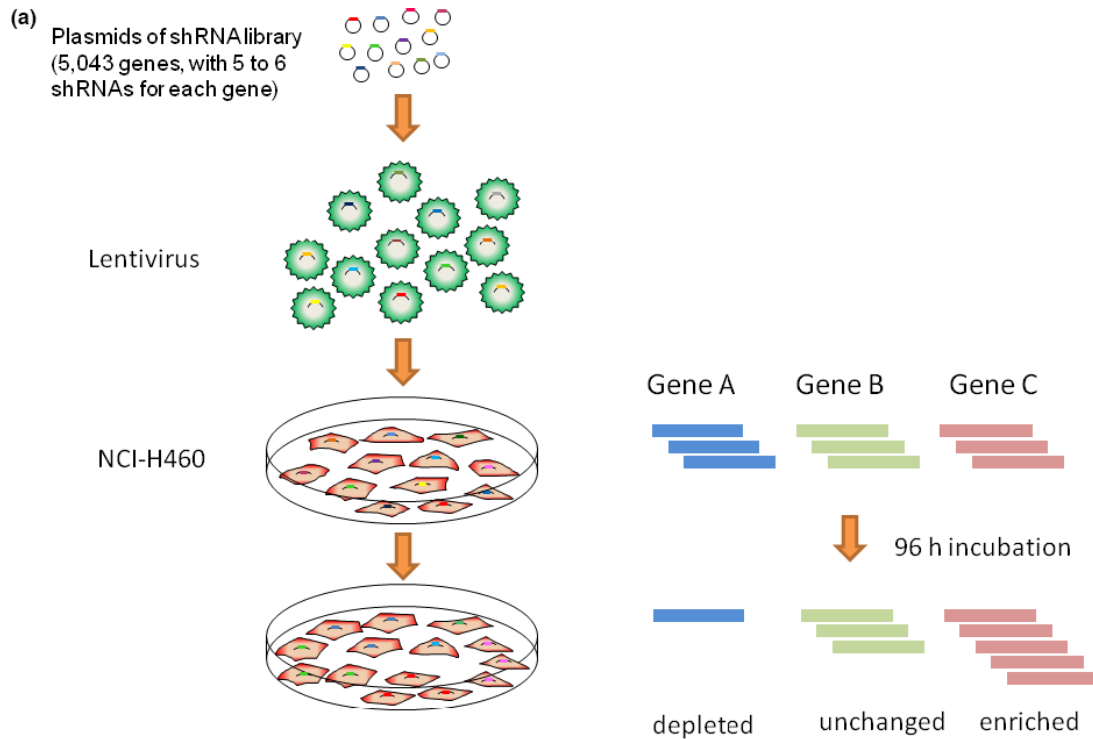
(a) Plasmids of shRNA library
(5,043 genes, with 5 to 6
shRNAs for each gene)

Lentivirus

NCI-H460

Gene A   Gene B   Gene C

96 h incubation

depleted   unchanged   enriched

(b)

P-value (−log10)

P = 0.05

PSMA1
PSMA3
PSMA6
PSMA7
PSMD13

PSMA2

Fold change (log2)

(c)   H460

Cell viability
(fold change)

Control | RNAi-1 | RNAi-2 | RNAi-1 | RNAi-2 | RNAi-1 | RNAi-2 | RNAi-1 | RNAi-2 | RNAi-1 | RNAi-2 | RNAi-1 | RNAi-2

PSMA1   *PSMA2*   *PSMA3*   *PSMA6*   *PSMA7*   *PSMD13*

Table 1. Pathways overrepresented with 51 target genes identified through shRNA screening

Pathway name	No. genes	% of the target genes	*P*-value	Benjamini
Ribosome	13	27.1	7.80E-13	3.2E-11
Proteasome	6	12.5	2.50E-05	5.1E-4
RNA polymerase	3	6.2	1.90E-02	2.3E-1
Pyrimidine metabolism	4	8.3	3.60E-02	3.1E-1
Spliceosome	4	8.3	7.10E-02	4.5E-1

PSMA6 were upregulated in cancer cell lines (Mann–Whitney *U*-test, $P < 0.001$), whereas *PSMD13* was downregulated in cancer cells (Fig. 2a). Expression data for *PSMA7* were not available. Copy number analysis by array CGH showed that *PSMA6* (7.3%; cut-off was set as > fivefold increase) was amplified in a subset of cancer cell lines, whereas the other five genes were not amplified (Fig. 2b). Cell lines with a high *PSMA6* copy number expressed significantly higher levels of *PSMA6* mRNA, indicating that *PSMA6* amplification resulted in its overexpression (Fig. 2c).

From these results, we concluded that *PSMA6* is one of the most attractive targets for lung cancer and decided to focus on it for further analysis.

***PSMA6* was highly expressed in lung cancer.** Next, we evaluated PSMA6 protein expression in lung cancer cell lines as well as in clinical specimens. Western blot of PSMA6 for a panel of 19 lung cancer cell lines exhibited that compared with normal culture of lung epithelial cells, NHBE, most of lung cancer cell lines tended to express higher levels of PSMA6 protein (Fig. 3a). IHC of PSMA6 on 20 matched NSCLC and normal lung specimens revealed that PSMA6 protein expression was increased in 90% (18/20) of cases (Fig. 3b,c). To examine whether this increased PSMA6 protein in clinical samples was associated with *PSMA6* amplification, we analyzed *PSMA6* copy numbers in lung cancer samples as well as in the corresponding normal lungs, but we found no amplifications in the samples. We examined the correlation between the expression levels of PSMA6 protein and the clinicopathological characteristics, including gender, smoking history, mutation status of epidermal growth factor receptor, pathological stage and overall survival. However, none of these was associated with PSMA6 protein expression (Fig. S1, Table S3). We also looked at the public database of *PSMA6* mRNA expression that was associated with the survival of lung cancer patients.[23] Patients with high *PSMA6* expression exhibited a tendency for shorter overall survival than those with low *PSMA6* expression ($P = 0.118$) (Fig. 3d).

***PSMA6* knockdown induced apoptosis or cell cycle arrest in lung cancer cells but not in normal bronchial epithelial cell line.** Next, we wanted to clarify the molecular mechanisms underlying *PSMA6* knockdown-induced inhibition of cell viability. In the following functional analyses, in addition to H460, we used two lung cancer cell lines, H1299 and H661 as well as HBEC3 cdk4-hTERT immortalized normal human bronchial epithelial cell line as a control. We chose H661 because it had 13 copies (6.5-fold increase) of *PSMA6* and thus seemed to be suitable for examining whether cell lines with high *PSMA6* amplification are hypersensitive to *PSMA6* knockdown. We obtained efficient *PSMA6* knockdown in H460, H1299, and HBEC3. However, only modest *PSMA6* knockdown was

obtained in H661 possibly because of high *PSMA6*-amplification in the cell line. We performed cell cycle and apoptosis analyses. Apoptotic analysis with western blot of cleaved PARP revealed that *PSMA6* knockdown increased levels of apoptosis clearly in H460 and weakly in H1299 but not in others (Fig. 4a). Cell cycle analysis with propidium iodide staining followed by flow cytometry revealed that *PSMA6* knockdown induced apoptosis in H460 and G2/M arrest in H1299 but did not apparently affect HBEC3 or H661 (Fig. 4b). By measuring proteasome activity in the cells, we examined whether such large phenotypic differences in response to *PSMA6* knockdown may result from differences in the effects of the knockdown on proteasome activity. We found that *PSMA6* knockdown equally inhibited proteasome activity by approximately 50% of the control in all the three cell lines except H661; this excluded the possibility that the difference in the *PSMA6* knockdown-induced phenotypic changes resulted from different effects of *PSMA6* knockdown on proteasome activity at least between H460 or H1299 versus HBEC3 (Fig. 4c).

In addition, we examined the effects of *PSMA6* knockdown on the expression of p21^Cip1/Waf1, which plays important roles in the cell cycle and whose expression is controlled by the proteasome pathway. We found that the protein expression levels of p21^Cip1/Waf1 were equally upregulated by knockdown of *PSMA6* in H460, H1299 and HBEC3 but not in H661 (Fig. 4a).

These results suggested that the high specificity of viability suppression by *PSMA6* knockdown occurs through higher dependency on proteasome activity in cancer cells than in normal cells. Nevertheless, we were unable to judge whether cancer cell lines with high *PSMA6* amplification are hypersensitive to *PSMA6* knockdown because of inefficient *PSMA6* knockdown in H661.

Next, we examined whether the knockdown of the other five candidate proteasome genes also induced apoptosis or cell cycle arrest in cancer cells. We silenced all the candidate proteasome genes in H460 and H1299, and looked at levels of induced apoptosis as well as effects on cell cycle. Efficient knockdown of all the six genes was obtained (Fig. S2). Apoptosis analysis revealed that in H460, knockdown of the other five genes also induced apoptosis, albeit to a less extent compared with that of *PSMA6* (Fig. 4d) while in H1299, knockdown of all the candidate proteasome genes except *PSMA7* induced comparable levels of apoptosis (Fig. 4d). Cell cycle analysis revealed that in H460, knockdown of *PSMA1* or *PSMA3* induced G2/M arrest while in H1299, knockdown of all the candidate proteasome genes induced G2/M arrest to some extent (Fig. 4e). These induction of apoptosis or cell cycle arrest did not seem to correlate with degrees of inhibition of proteasome activity (Fig. 4f). These results suggest that *PSMA6* knockdown does not necessarily induce the greatest levels of apoptosis or cell cycle arrest but instead that resulting phenotypic responses due to the knockdown of the candidate proteasome genes occur in a cell context dependent manner, possibly not necessarily correlating with proteasome activity.

***PSMA6* overexpression in HBEC3 did not affect proteasome activity or proliferation.** Finally, to evaluate the potential of *PSMA6* overexpression in oncogenic transformation, we examined whether *PSMA6* overexpression in normal bronchial epithelial cells affected their proliferation rate. Lentivirally introduced expression of *PSMA6* was confirmed by western blotting (Fig. 5a). *PSMA6* overexpression in HBEC3 did not affect proteasome activity or enhance proliferation (Fig. 5b,c,d).
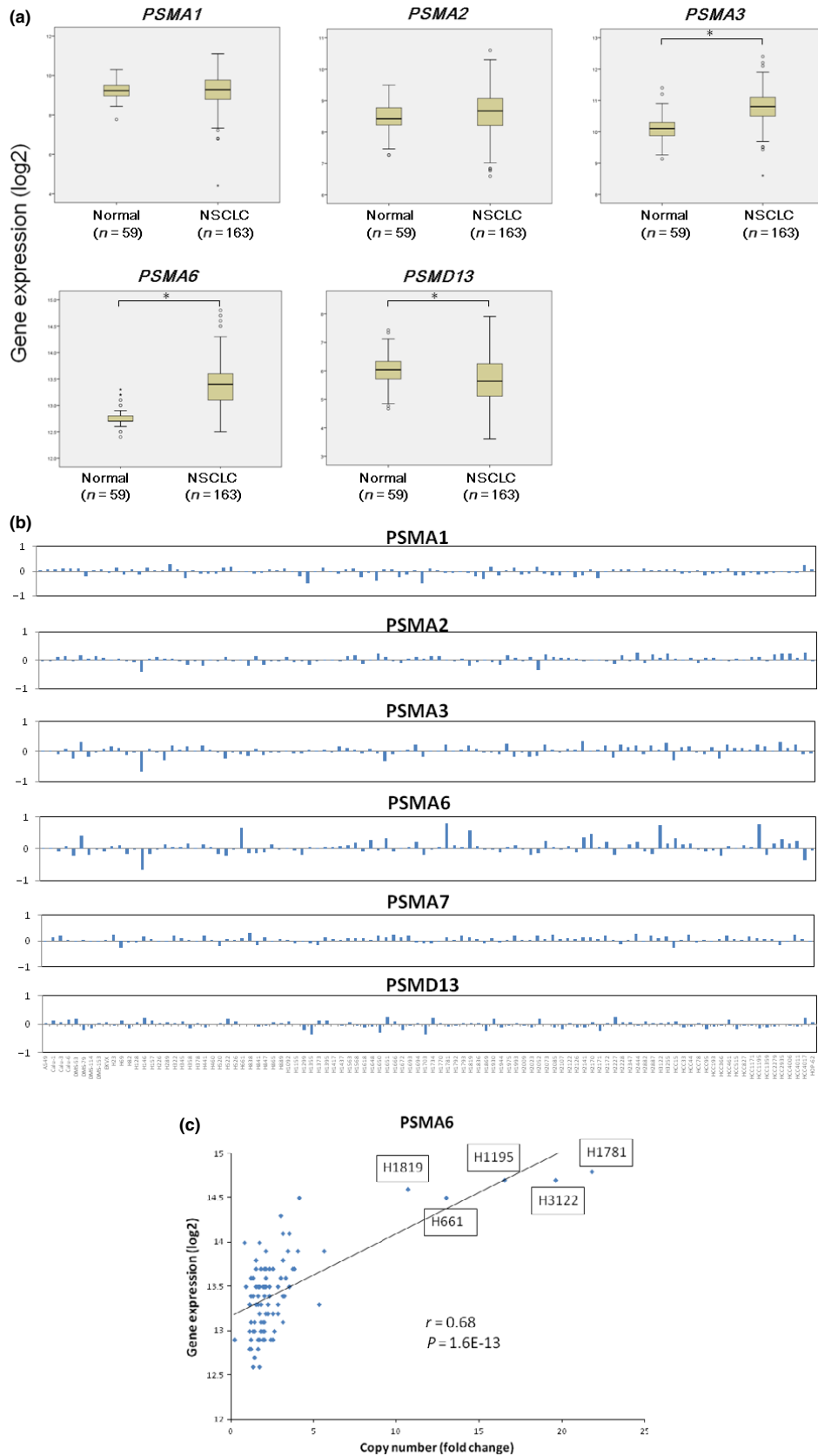
Fig. 2. Genome-wide gene expression and copy number data suggest that *PSMA6* is one of the most attractive targets. (a) Microarray expression analysis of *PSMA1*, *PSMA2*, *PDSMA3*, *PSMA6* and *PSMD13* mRNA. The bottom and top of the box are lower and upper quartiles, respectively. The band near the middle of the box is the median. *$P < 0.001$(Mann–Whitney *U*-test). (b) Array CGH analysis of *PSMA1*, *PSMA2*, *PSMA3*, *PSMA6*, *PSMA7* and *PSMD13* showing increases in the copy number of *PSMA6* in a small fraction of NSCLC cell lines. (c) Correlation between copy numbers and mRNA expression of *PSMA6* in lung cancer cell lines.
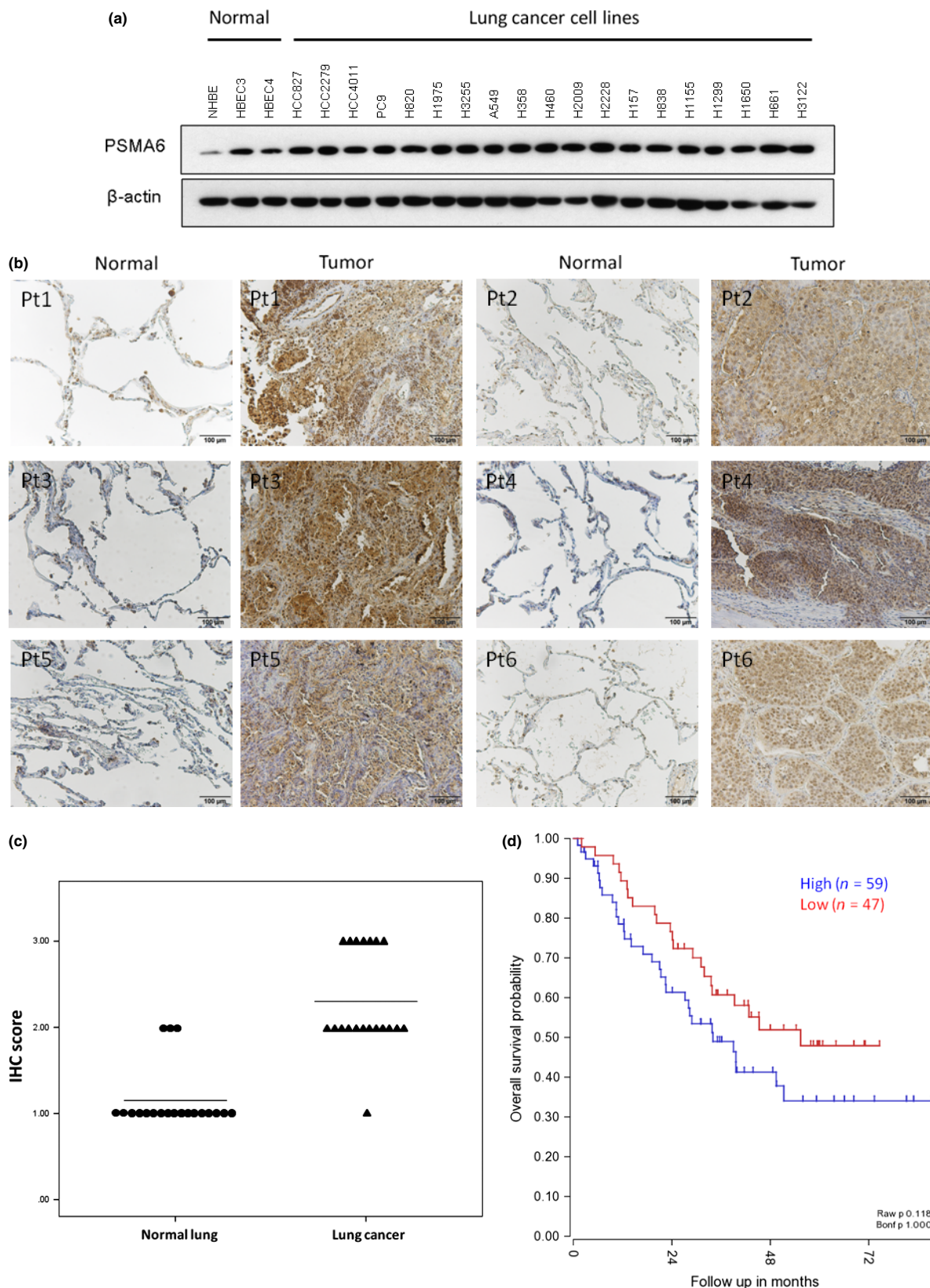
Fig. 3. *PSMA6* is highly expressed in lung cancer. (a) Western blot of PSMA6 in three normal controls (one primary culture NHBE and two immortalized normal bronchial epithelial cell lines, HBEC3 and HBEC4) and 19 lung cancer cell lines. (b) Immunohistochemical (IHC) staining of PSMA6 in surgically resected lung cancer and matched normal lung specimens. Scale bars: 100 μm. Pt, patient. (c) Immunohistochemical staining scores in surgically resected lung cancer and matched normal lung specimens. 0 = none, 1 = weak, 2 = positive, 3 = strong. (d) The lung cancer set from Bild *et al.*[23] was analyzed to generate Kaplan–Meier plots of OS according to *PSMA6* expression levels using the R2 genomics analysis and visualization platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi).
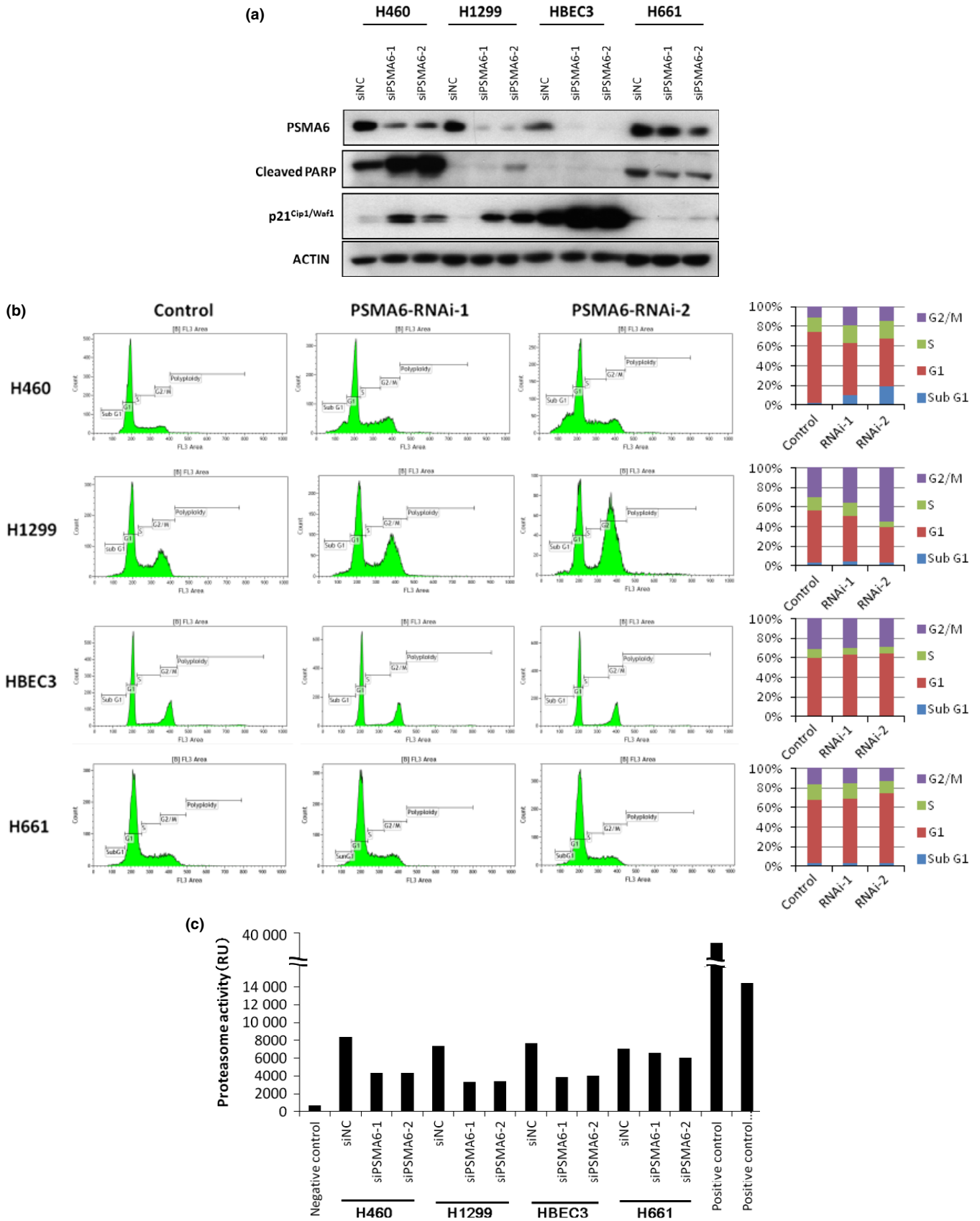
Fig. 4. *PSMA6* knockdown induces apoptosis or cell cycle arrest in lung cancer cells but not in normal bronchial epithelial cell line. (a) Western blot analysis of cleaved PARP and p21$^{Cip1/Waf1}$ in H460 and H1299, HBEC3 and H661 transfected with *PSMA6* siRNA or control oligos. (b) Flow cytometry analysis of cell cycle progression in H460, H1299, HBEC3, and H661 transfected with *PSMA6* siRNA or control oligos. Cells were harvested for the analysis 48 h after siRNA treatment. (c) Effects of *PSMA6* knockdown on proteasome activity in H460, H1299, HBEC3, and H661. Intracellular proteasome activity was measured using cell lysates treated with 10 nM siRNAs for 48 h. Proteins were adjusted for equal concentration and subjected to a proteasome assay using the *N*-succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosyl-7-amido-4-methylcumarin (Suc-LLVY-AMC) substrate. Proteasome specificity was determined by the difference in AMC formation in the absence and presence of lactacystin. The results are expressed as specific AMC formation. (d) Western blot analysis of cleaved PARP, p53 and p21$^{Cip1/Waf1}$ in H460 and H1299 transfected with *PSMA1*, *PSMA2*, *PSMA3*, *PSMA6*, *PSMA7*, *PSMD13* RNAi or control oligos. Western blot of p53 was not shown in H1299 because p53 is deleted in it. (e) Flow cytometry analysis of cell cycle progression in H460, H1299, and H661 transfected with *PSMA1*, *PSMA2*, *PSMA3*, *PSMA6*, *PSMA7*, *PSMD13* RNAi or control oligos. Cells were harvested for the analysis 48 h after siRNA treatment. (f) Proteasome activity in H460, and H1299 transfected with *PSMA1*, *PSMA2*, *PSMA3*, *PSMA6*, *PSMA7*, *PSMD13* RNAi or control oligos.

Based on these results, we suggest that *PSMA6* overexpression alone does not have oncogenic ability.

Discussion

Several studies have reported the roles of PSMA6 in the carcinogenesis of other types of cancer. Two previous shRNA screening studies identified *PSMA6* as an essential survival gene in malignant mesothelioma and glioblastoma cells.[24,25] In addition, proteomic analysis of transgenic mice developing hepatocellular carcinoma revealed that several proteasome subunits, including PSMA6, were upregulated in the tumor.[26] The findings of these studies, together with our results, suggest the oncogenic function of *PSMA6* in multiple types of human cancer; therefore, *PSMA6*-targeted therapy may have broad applications for different types of tumors.

It has been well acknowledged that proteasome inhibition exerts more cytotoxicity in cancer cells than in corresponding normal cells.[20,27] In agreement with this, we found that *PSMA6* knockdown shows greater growth suppressive effects in cancer cells than in immortalized normal cells. We also show that *PSMA6* knockdown induced greater apoptosis than knockdown of other five proteasome subunit genes in H460 although knockdown of these genes comparably inhibited proteasome activity. These results suggest that *PSMA6* knockdown may induce apoptosis through unknown factors that are not directly associated with proteasome activity. Such proteasome-independent biological functions of another proteasome subunit gene, *PSMD10* (also known as *Gankyrin* or *p28*) has been demonstrated.[28,29] It functions as an oncogene by increasing hyperphophorylation of Rb by CDK4 as well as promoting the degradation of p53 by MDM2. Further studies will be needed to clarify whether *PSMA6* also has such proteasome-independent biological functions.

We found different responses to *PSMA6* knockdown in three lung cancer cell lines, H460, H1299, and H661. We anticipated H661 with high *PSMA6* amplification to exhibit hypersensitivity to *PSMA6* knockdown; however it did not show apoptosis or cell cycle arrest. Nevertheless, because of inefficient *PSMA6* knockdown (~50% protein reduction), we cannot judge whether cell lines with high *PSMA6* amplifications are hypersensitive to *PSMA6* knockdown. H460 and H1299 differ in their p53 statuses; p53 is intact in H460 but is deleted in H1299. This may influence their apoptotic responses to *PSMA6* knockdown. Nevertheless, obviously, these two cell lines differ significantly in numerous other genes involved in the apoptosis pathway, and thus we cannot draw definite conclusions. Further studies using isogenic cell lines differing only in the p53 status will be needed.

Intriguingly, the involvement of PSMA6 in another common human adult disease, myocardial infarction has been reported.

Independent studies have reported that SNP in exon 1 in *PSMA6* with enhanced transcription is a risk factor for developing myocardial infarction, which is hypothesized to be attributable to enhanced inflammation resulting from upregulation of NF-kB due to enhanced activity of proteasome.[30,31] Because the involvement of NF-kB in carcinogenesis is well-acknowledged,[32] one can hypothesize that PSMA6 exerts its oncogenic ability through enhanced inflammation resulting from NF-kB upregulation. It would be interesting to examine whether SNP in exon1 is also a risk factor for developing cancer.

Several studies have reported tumor-promoting or suppressive roles of proteasome subunits, other than *PSMA6*, in lung cancer. For instance, Matsuyama *et al.* have reported that the knockdown of *PSMD2*, a subunit of the 19S regulatory unit, causes apoptosis and G1 cell cycle arrest in lung cancer cells and that its higher expression is correlated with shorter patient survival in patients with surgically treated lung adenocarcinoma, suggesting its oncogenic roles in lung cancer. Consistent with these results, our shRNA screening identified *PSMD2* as a gene required for viability of H460 cells (76th gene in the ranking of fold reduction). Another study analysed the expression of four 20S proteasome subunits (PSMA1, PSMA5 and PSMB4 and one 19S proteasome subunit) in several histological types of lung cancer specimens and found that all subunits were overexpressed in all histological types.[33] The authors showed that among the five genes studied, only PSMB4 expression, as measured by Ki67 expression, was positively correlated with proliferation activity in neuroendocrine tumors, suggesting its involvement in the pathogenesis of lung cancer. We did not observe a significant decrease in *PSMB4* shRNAs. However, the H460 cell line that was used in our screening was not a neuroendocrine type of lung cancer; it is possible that *PSMB4* acts as an oncogenic gene only in neuroendocrine tumors. Altogether, these findings suggested that the deregulation of the proteasome pathway plays pivotal roles in the pathogenesis of lung cancer.

We found increased expression of PSMA6 at both mRNA and protein levels in lung cancer cell lines as well as in clinical specimens. We also found that *PSMA6*, located at the 14q13.3 region, was amplified in approximately 7% of lung cancer cell lines, which was associated with increased *PSMA6* expression, indicating that increased *PSMA6* in lung cancer is at least in part due to gene amplification. A prior study has reported that the 14q13.3 region is amplified in approximately 15% of lung cancer specimens. In addition, by further analyzing a large number of other tumor types and performing functional experiments, the study identified three developmental genes (TTF1/NKX2-1, NKX2-8 and PAX9) located in the 14.q13.3 region as oncogenic genes in lung cancer.[34] In particular, the oncogenic roles of TTF1/NKX2-1 have been demonstrated by several lines of evidence.[35–37] Our results
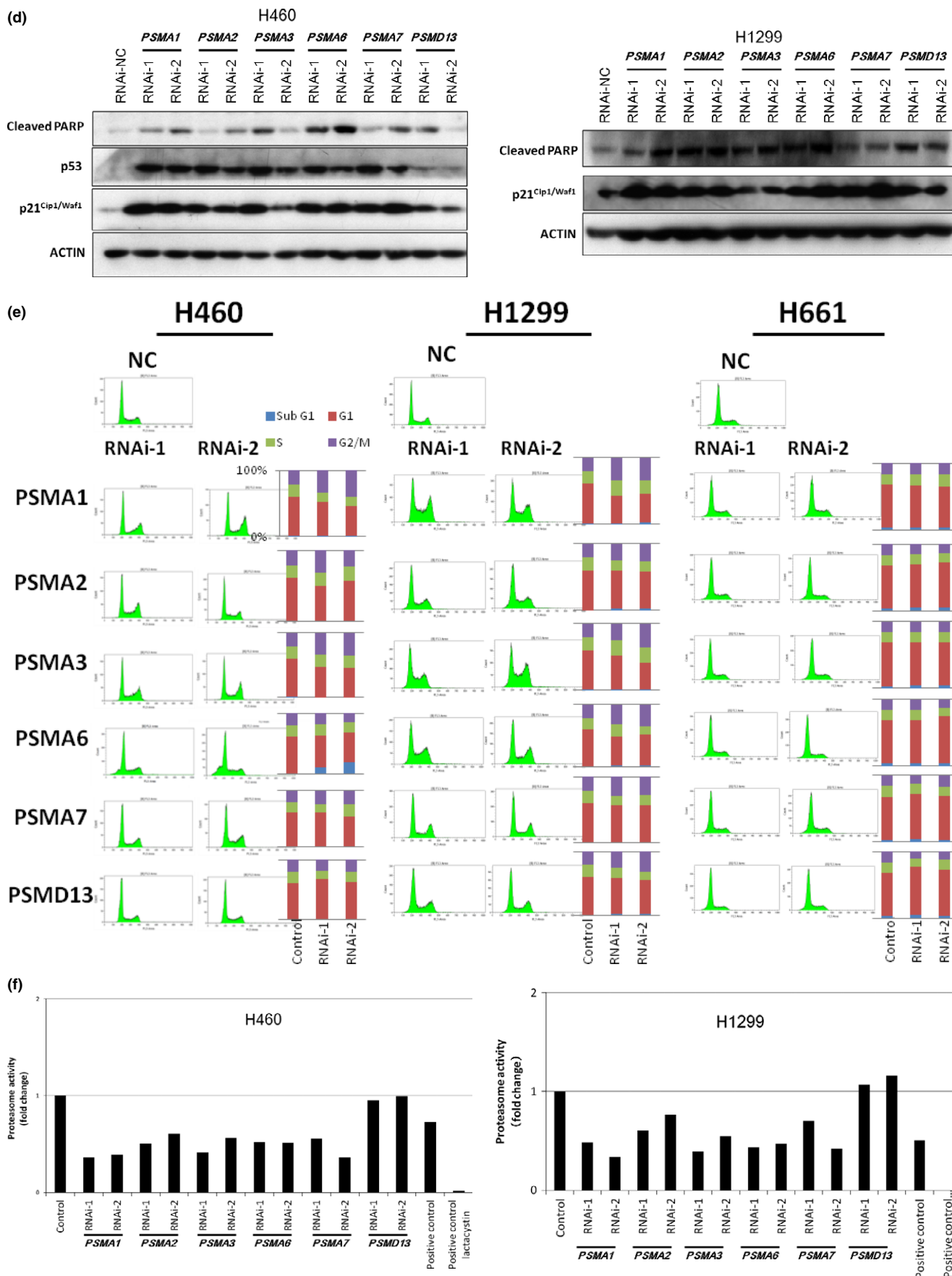
(d)

(e)

(f)

Fig. 4. Continued
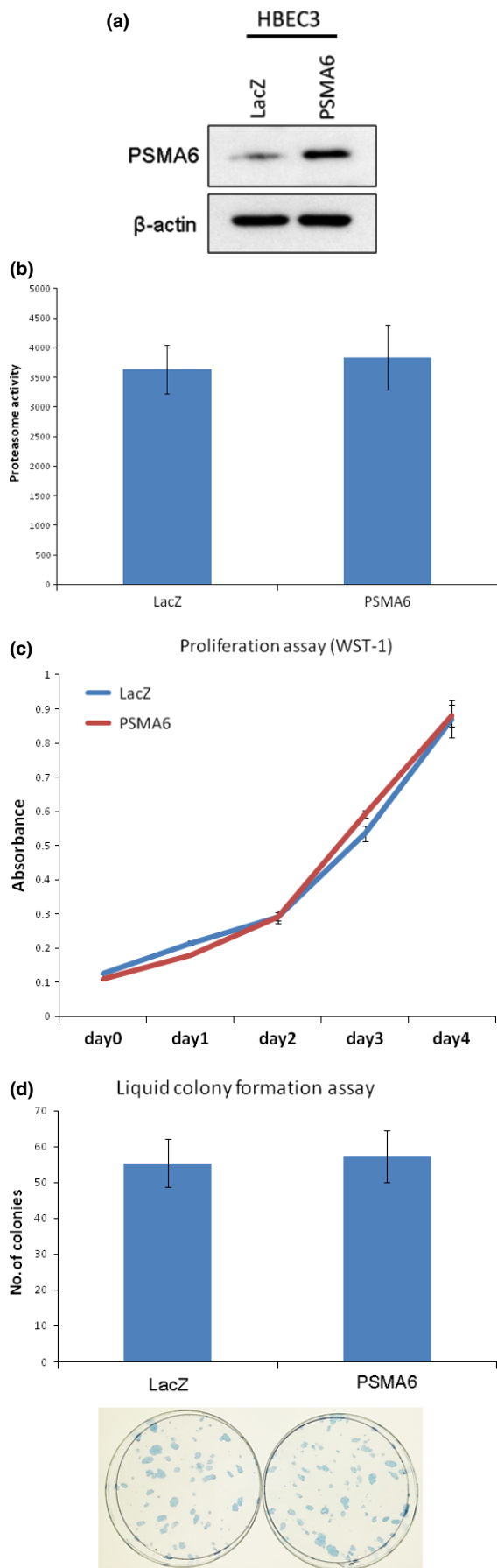
(a)

(b)

(c)

(d)

Fig. 5. *PSMA6* overexpression in HBEC3 does not affect proteasome activity or enhance their proliferation. (a) Western blot of PSMA6 in HBEC3 cells expressing *PSMA6* or *LacZ*. Proteasome activity (b), proliferation assessment of WST1 absorbance (c) and liquid colony formation assay (d) for HBEC3 cells expressing *PSMA6* or *LacZ*.

showed the potential of *PSMA6* as an oncogenic gene in lung cancer, suggesting that *PSMA6* is another target in this region that could contribute to the development of lung cancer treatment.

Elevated PSMA6 protein expression without gene amplification in lung cancer specimens suggested that other mechanisms cause PSMA6 upregulation. One possible cause is nuclear factor E2-related factor 2 (Nrf2), which has been shown to upregulate the expression of many proteasome subunits, including *PSMA6*, and proteasome activity.[38,39] There was no correlation between Nrf2 and *PSMA6* expression in our analysis of microarray data for lung cancer cell lines. Because Nrf2 is a nuclear factor, we cannot assertively exclude the possibility that it plays a role in *PSMA6* upregulation. However, considering the function of Nrf2 as a pan-upregulator of the majority of proteasome subunits, we suggest that Nrf2 does not primarily contribute to PSMA6 upregulation in lung cancer because we observed that only a few proteasome subunits were specifically upregulated in lung cancer.

In conclusion, we have identified *PSMA6* as a promising therapeutic target for lung cancer. Further studies focused on clarifying the mechanisms underlying *PSMA6* knockdown -induced growth inhibitory effects, with more expanded lung cancer cell lines, are required to warrant the performance of compound library screening to search for drugs that target *PSMA6*.

Disclosure Statement

Authors do not have any conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Kaplan–Meier plots of OS according to PSMA6 protein expression levels in 96 non-small cell lung cancer cases. The *P*-value was calculated by the log-rank test.

Fig. S2. Knockdown efficiency of *PSMA1*, *PSMA2*, *PSMA3*, *PSMA6*, *PSMA7*, or *PSMD13* in H460 and H1299. Quantitative real time PCR was done for H460 and H1299 transfected with *PSMA1*, *PSMA2*, *PSMA3*, *PSMA6*, *PSMA7*, *PSMD13* RNAi or control oligos.

Table S1. List of cell lines used for microarray expression analysis.

Table S2. List of cell lines used for gene copy analysis.

Table S3. Correlation between PSMA6 protein expression and clinicopathological characteristics.