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Potential Role of ASC, a Proapoptotic Protein, for Determining the Cisplatin Susceptibility of Lung Cancer Cells

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Primary lung cancer is the most frequent cause of cancer-related deaths worldwide. Cisplatin has been used as a key drug in the treatment for patients with lung cancer; however, most of the patients failed to respond to cisplatin within several months, and the mechanisms underlying the cisplatin resistance have not been fully elucidated. Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) is a key adaptor protein in the formation of inflammasomes. ASC is also involved in apoptotic signaling. Importantly, ASC expression is decreased in lung cancer and various cancers, but its precise function in tumor progression remains unknown. To explore the hitherto unknown role of ASC in lung cancer, we initially searched for lung cancer cell lines with higher expression levels of ASC using Cancer Cell Line Encyclopedia (CCLE) database, thereby identifying the A549 human non-small cell lung cancer cell line. Accordingly, with retroviral shRNA, the expression of ASC was forced to decrease in A549 cells. Stable ASC-knockdown cells, thus established, showed the increased activities of proliferation, motility, and invasion, compared with control cells. Importantly, ASC-knockdown cells also became resistant to cisplatin, but not to other anti-cancer agents, 5-fluorouracil and paclitaxel. Bcl-2 and phospho-Src levels were increased in ASC-knockdown cells. A Bcl-2 inhibitor, ABT-199, induced an apoptotic response in ASC-knockdown cells, and dasatinib, a Src inhibitor, blocked cell invasiveness. Thus, ASC may be involved in tumor suppression and cell death via Bcl-2 and pSrc. Targeting Bcl-2 and Src in ASC-downregulated populations of lung cancer may improve treatment outcome.

Keywords: ASC; Bcl-2; cisplatin resistance; lung cancer; Src
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Introduction

Lung cancer is the most common form of malignancy and the leading cause of cancer deaths worldwide (CDC WONDER 2014). Surgical treatment for lung cancer is possible at an early stage. However, as tumors are commonly diagnosed at advanced or inoperable stages, anticancer agents occupy a pivotal position in therapy as well. Several molecular targeted therapies have been developed, but are limited to cases of driver gene alteration, such as epidermal growth factor receptor (EGFR) (Soda et al. 2007; Reungwetwattana et al. 2012). On the other hand, treatment with classic anticancer drugs is generally used for patients with v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (*KRAS*) mutations or without the above gene variations since no effective strategies have been established to date.

Cisplatin (*cis*-Diamminedichloroplatinum (II); CDDP)

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is one of the most effective and widely used anticancer drugs for advanced or unresectable lung cancers (Baggstrom et al. 2007; Aupérin et al. 2010). Moreover, post-surgical CDDP-based adjuvant chemotherapy is recommended to improve survival in patients with completely resected non-small cell lung cancer (Arriagada et al. 2004; Pignon et al. 2008). Although the drug is able to prolong overall survival in some cases, the clinical effects of CDDP are temporary and the majority of patients acquire chemoresistance (Herbst et al. 2008). Severe hematologic toxicity and nephrotoxicity have also greatly limited its application (D'Addario et al. 2005). Thus, a large number of CDDP-treated patients are destined for treatment failure and tumor recurrence. Secondary agents that can induce apoptosis of CDDP-resistant lung cancer cells are therefore of therapeutic interest.

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), also referred to as PYCARD, is a 22-kDa pro-apoptotic protein containing an N-terminal pyrin domain (PYD) and a C-terminal caspase recruitment domain (CARD) (Masumoto et al. 1999). The protein was found by our group to induce apoptosis in association with forming an insoluble aggregate "speck" near the nuclear periphery (Masumoto et al. 1999; Taniguchi and Sagara 2007). ASC has since become well known as a key adaptor protein in inflammasome formation that plays a crucial role in caspase-1 activation and the secretion of interleukin (IL)-1β and IL-18 in innate immune cells (Martinon et al. 2002). We and other groups have also identified the relationships between methylation-mediated silencing of ASC expression and prognosis in lung cancer (Machida et al. 2006; Zhang et al. 2006), and other malignancies (Conway et al. 2000; Guan et al. 2003; Das et al. 2006; Kordi Tamandani et al. 2009). Moreover, ASC protein expression was found to be generally downregulated in metastatic sites compared with primary tumors in melanoma (Liu et al. 2013). These studies imply that ASC expression affects cancer progression, but the underlying molecular functions of ASC remain unclear.

In this study, we aimed to explore the hitherto unknown roles of ASC in lung cancer using the A549 human lung cancer cell line. Understanding the role of ASC in cancer cells is an important step towards identifying novel therapeutic targets.

Materials and Methods

Reagents

5-Fluorouracil (5-FU), paclitaxel (PTX), and CDDP were purchased from Sigma-Aldrich (St. Louis, USA). A caspase-9 inhibitor (z-LEHD-fmk) and anti-human Fas antibody (clone CH-11) were obtained from Medical & Biological Laboratories (Nagoya, Japan). ABT-199 and dasatinib were purchased from Santa Cruz Biotechnology (Dallas, USA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, USA).

Cell culture

The A549 human lung cancer cell line was obtained from Riken BioResource Center (Tsukuba, Japan). BEAS-2B, an immortalized human normal bronchial epithelial cell line, was procured from ATCC (Manassas, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaile, France), penicillin, and streptomycin (Wako, Osaka, Japan) in a humidified atmosphere with 5% CO_2 at 37°C. For the serum starvation experiments, cells were cultured in DMEM supplemented with 0.5% FBS.

ASC knockdown cells

To generate stable ASC knockdown A549 cells, packaging cells were transfected with *ASC* short hairpin RNA (shRNA) or control shRNA in pSINsi-hU6 retroviral vector (Takara Bio, Shiga, Japan). The target sequence for human ASC was 5′-GGTTTCGAGTTG TCTTGAT-3′. For infection with retroviral vectors, retroviral packaging cells cultured in 100-mm dishes were incubated with 6 μg of recombinant retroviral vector and 18 μg of Genejuice transfection reagent (Merck Millipore, Darmstadt, Germany) for 8 hours. Thereafter, the cells were cultured in fresh DMEM with 10% FBS for 48 hours, during which time the supernatants containing the retroviral particles were harvested at 24 and 48 hours post-transfection. The supernatants were concentrated by centrifugation at 6,000 *g* for 16 hours, filtered through a 0.45-μm filter, and used to infect target cells. Twelve-well plates were coated with 50 μg/mL of RetroNectin (Takara Bio) as recommended by the manufacturer. One day later, 2 mL of concentrated virus supernatant were added to each well, and plates were centrifuged at 3,370 *g* for 4 hours at 32°C. The virus supernatants were removed and replaced by A549 cells (2×10^4 cells per well) in DMEM high glucose with 10% FBS, and the cells were incubated at 37°C for 48 hours. Infected cells were selected in fresh DMEM containing 950 μg/mL G418. G418-resistant cell pools were readily established within 10 days. The transfection efficiency of ASC knockdown cells was confirmed by real-time PCR and immunoblotting.

ASC-overexpressing cells

To establish ASC-overexpressing cells, A549 cells were transfected with pMXs-IRES-rat CD2 vector carrying ASC cDNA by retroviral infection (Kitamura et al. 2003). The empty vector was used as a control. The retroviral infection method was same as the preparation of ASC-knockdown cells. BD IMag™Cell Separation System (Streptavidin Particles Plus-DM; BD Biosciences, San Jose, USA) were used for selecting transfected *ASC* positive cells according to the manufacturer's instruction. The transfection efficiency was confirmed, based on the rate of rat CD2-positive cells by flow cytometry.

Wound healing assay

ASC-knockdown and control cells were cultured on 60 mm plates and wounds were made by scratching with a sterile 200 μL pipette tip. Images of the wounds were taken at 0 and 36 hours after scratching, and the wounded areas were measured as a percentage of the total wounded area by image J software (http://rsb.info.nih.gov/ij/).

Cytotoxicity assay

ASC-knockdown and control cells were seeded into 96-well

plates at 5,000 cells/well. Following overnight incubation, serial dilutions of anticancer drugs were added to the cells and incubated for another 24 hours. Cell viability was determined by the MTS assay. Absorbance was measured in triplicate for each sample using a multiplate reader at the wavelength rate of 490 nm/650 nm. IC50 values were determined using the following formula: IC50 = 10^(LOG[A/B]*[50−C]/[D−C]+LOG[B]) from a sigmoidal dose-response curve, where A was the concentration higher than IC50, B was the concentration lower than IC50, C was the inhibition rate at point B, and D was the inhibition rate at point A.

Flow cytometry

ASC-knockdown and control cells were cultivated with respective reagents in each experiment and then analyzed using FACSCanto II and FACSDiva (BD Bioscience, Franklin Lakes, USA) systems along with Kaluza software (Beckman Coulter, Brea, USA).

Quantitative real-time PCR

RNA was purified by treatment with RNAiso Plus (Takara Bio) according to the standard protocol at indicated time points. RNA (500 ng) was reverse-transcribed using the Prime Script RT Master Mix (Takara Bio). Relative quantification of mRNA expression was performed in duplicate using SYBR premix Ex Taq II (Takara Bio) in a TP850 Thermal Cycler Dice Real Time System Single (Takara Bio). Data were normalized to β-actin levels. The sequences of the primer sets used are shown in Table 1.

Western blotting analysis

Cells were seeded at 1×10^6 cells into 100 mm plates. The medium was replaced with fresh medium on the following day and cells were treated with 24 μM CDDP for 24 hours. The pellet was homogenized in cell lysis buffer (20 mM HEPES-OH pH7.9, 0.5% NP-40, 15% glycerol, 300 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 1 unit of complete protease inhibitor cocktail EDTA-free [Roche, Pleasanton, USA]) and incubated at 4°C with gentle rocking. Protein samples were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% skim milk in TBS for 1 hour at room temperature. The following primary antibodies were used: anti-human ASC antibody was generated as previously described (Masumoto et al. 1999), while those for caspase-9, Bim, BID (pro-apoptotic molecules), Bcl-2, Bcl-xL (anti-apoptotic molecules), ERK, phospho-ERK (Tyr202/Tyr204), p38, phospho-p38 (Tyr180/Tyr182), JNK, phospho-JNK (Tyr183/Tyr185), NF-κB p65, phospho-NF-κB p65, Src, and phospho-Src (Tyr416) were all purchased from Cell Signaling Technologies (Beverly, USA). β-actin and GAPDH were obtained from Santa Cruz Biotechnology. Bax was purchased from Thermo (Waltham, USA). The secondary, HRP-conjugated antibodies used were anti-mouse, rabbit, and goat IgG (Dako, Glostrup, Denmark). Detection was performed using the Immobilon Western Chemiluminescent HRP substrate (Merck Millipore). All data were processed and analyzed using Printgraph and CS Analyzer software (ATTO, Tokyo, Japan).

Reactive oxygen species (ROS) measurements

ASC-knockdown and control cells were seeded into 12-well plates at 6×10^4 cells per well. Following overnight incubation, cells were treated with 24 μM CDDP for 24 hours. Cells were then incubated with 5 μM CellROX® Deep Red Reagent (Life technology, Carlsbad, USA) for 30 min at 37°C. Fluorescence was measured using flow cytometry.

Table 1. Primer sequences used.

Gene		Sequence
β-actin	Forward	5'-GGACTTCGAGCAAGAGATGG-3'
	Reverse	5'-GTGGATGCCACAGGACTCCAT-3'
GAPDH	Forward	5'-CACCACCAACTGCTTAGCACC-3'
	Reverse	5'-CAGTCTTCTGGGTGGCAGTGATG-3'
ASC	Forward	5'-CTCCTCAGTCGGCAGCCAAG-3'
	Reverse	5'-ACAGAGCATCCAGCAGCCAC-3'
NRF2	Forward	5'-CGGTCCACAGCTCATCATGATGG-3'
	Reverse	5'-CGCTGACTGAAGTCAAATACTTCTCGAC-3'
PPARGC1B	Forward	5'-TGACACGCAGGGTGGAGG-3'
	Reverse	5'-GAGTTCTCTGGGCACCACTG-3'
ABCB1	Forward	5'-CCATGCTCAGACAGGATGTGA-3'
	Reverse	5'-ATCATTGGCGAGCCTGGTAG-3'
ERCC1	Forward	5'-CTCCTGGCACCTTCCCTTTC-3'
	Reverse	5'-TAAGGGCTTGGCCACTCCAG-3'

Primers used for quantitative PCR measurement of gene expression.

Cell invasion assay

Transparent PET membranes (8-μm pore size; BD Falcon, Bedford, USA) were coated with 60 μg Matrigel (BD Biosciences). Aliquots of 1×10^5 cells in serum-free medium with or without dasatinib were transferred into the upper chambers of 24-well plates. The lower chambers were supplemented with 10% FBS DMEM and the cells were allowed to invade for 45 hours at 37°C. The filter was later removed and fixed for 10 min in 100% MetOH, followed by staining with 0.2% crystal violet. Cells located on the basal side were observed under a light microscope and counted in three randomly selected fields.

Statistical analysis

Significance was determined by the unpaired two-tailed Student's *t*-test or one-way ANOVA. Results are expressed as the mean ± standard error. A P value of < 0.05 was accepted as statistically significant.

Results

Knockdown of ASC expression enhances malignant properties of lung cancer cells

To elucidate the effects of ASC on malignant properties, we first investigated the expression of endogenous ASC (PYCARD) mRNA in our experimental cell line according to the Cancer Cell Line Encyclopedia (CCLE) expression data set (http://www.broadinstitute.org/ccle). The expression of ASC in the A549 cell line is relatively high among human lung cancer cell lines (15^{th} of 186 cell lines) (Fig. 1a). As A549 has been extensively used in lung cancer experiments worldwide, its comparability with other reports also made it a suitable cell line. The expression of endogenous ASC was compared between A549 and BEAS-2B, a normal bronchial epithelial cell line. A549 cells produced less ASC expression than did BEAS-2B (Fig. 1b). To examine the potential role of ASC in human lung cancer, A549 cells were infected with retroviruses delivering shRNA targeting ASC to produce stable ASC knockdown cells (Fig. 1c). This knockdown of endogenous ASC expression promoted tumor growth, with a significant difference in tumor cell number between ASC knockdown and control cells at 10 days after cell inoculation (Fig. 1d), while we did not observe any significant difference in those phenotypes between ASC overexpression and control cells (data not shown). The number and size of colonies formed after sparsely plating the cells into culture dishes were increased in ASC knockdown cells compared with controls (Fig. 1e). We observed that ASC knockdown cells also exhibited increased cancer cell migration ability as shown by wound healing assays (Fig. 1f). These findings suggest that the suppression of ASC may induce more aggressive tumor phenotypes in this *in vitro* model.

ASC-depletion renders A549 cells resistant to CDDP

Since ASC was initially discovered as a proapoptotic protein (Masumoto et al. 1999), we examined whether it could modulate the anticancer effects of 5-FU, PTX, and CDDP, all widely used drugs for lung cancer treatment. While ASC knockdown A549 cells became insensitive to CDDP as compared with controls, this was not the case for 5-FU or PTX (Fig. 2a). The IC50 value of CDDP killing of sh-ASC and sh-control A549 cells was 33.6 μM and 17.7 μM, respectively. The number of apoptotic ASC knockdown cells was lower than that of controls with CDDP, confirming that the knockdown cells had become resistant to CDDP (Fig. 2b). By contrast, when we overexpressed ASC in A549 cells, neither the cell proliferation nor the apoptotic response to CDDP was significantly altered (data not shown).

We next directed our attention to how ASC was involved in the mechanism of the CDDP-induced apoptotic pathway. Decrease in the mitochondrial membrane potential ($\Delta\Psi$m) is generally recognized as a critical step in the apoptotic pathway (Green and Reed 1998). ASC knockdown inhibited the reduction of $\Delta\Psi$m during CDDP treatment (Fig. 2c), consequently decreasing the cleavage of caspase-9 to its active form (Fig. 2d). In support of this, addition of a selective caspase-9 inhibitor, z-LEHD-fmk (20 μM), to the cells rescued the CDDP-induced apoptosis in control cells (Fig. 2e). On the other hand, the apoptosis in ASC knockdown cells was not able to inhibit by using caspase-9 inhibitor. These findings collectively demonstrated that ASC knockdown cells were rendered less susceptible to CDDP cytotoxicity and indicated that ASC influenced more upper site of CDDP-induced apoptotic pathway, not in the site of releasing of cytochrome *c* and activation of caspase-9.

Anti-apoptotic Bcl-2 is involved in ASC-mediated CDDP resistance

It has also been reported that when NLR family pyrin domain containing 3 (NLRP3), NOD like receptor, one of the key molecule of inflammasome, and its adaptor, ASC, are stimulated with inflammatory or apoptotic signaling, ASC becomes redistributed to the perinuclear space or mitochondria (Ohtsuka et al. 2004; Zhou et al. 2011), respectively. Therefore, to clarify the mechanisms underlying ASC depletion and a decrease in CDDP-induced apoptosis, we examined Bcl-2 family proteins in the mitochondrial membrane (Joza et al. 2001). A major function of the Bcl-2 family in regulating caspase activation is the alteration of mitochondrial membrane permeability to control the release of caspase-activating cytochrome *c*. As shown in Fig. 3a, there were no significant differences between ASC knockdown cells and control cells for the expression of the apoptotic proteins Bax, Bim, or Bcl-xL under CDDP treatment. Although the inactive form of the pro-apoptotic protein BID was elevated in ASC knockdown cells, its cleaved/active form was not detectable. The anti-apoptotic protein Bcl-2 was more highly expressed in ASC knockdown cells than in controls both before and after treatment with CDDP. Taken together, these data suggested that Bcl-2 functioned as a key regulator of the mitochondrial apoptotic pathway in the treatment of ASC knockdown cells with CDDP.

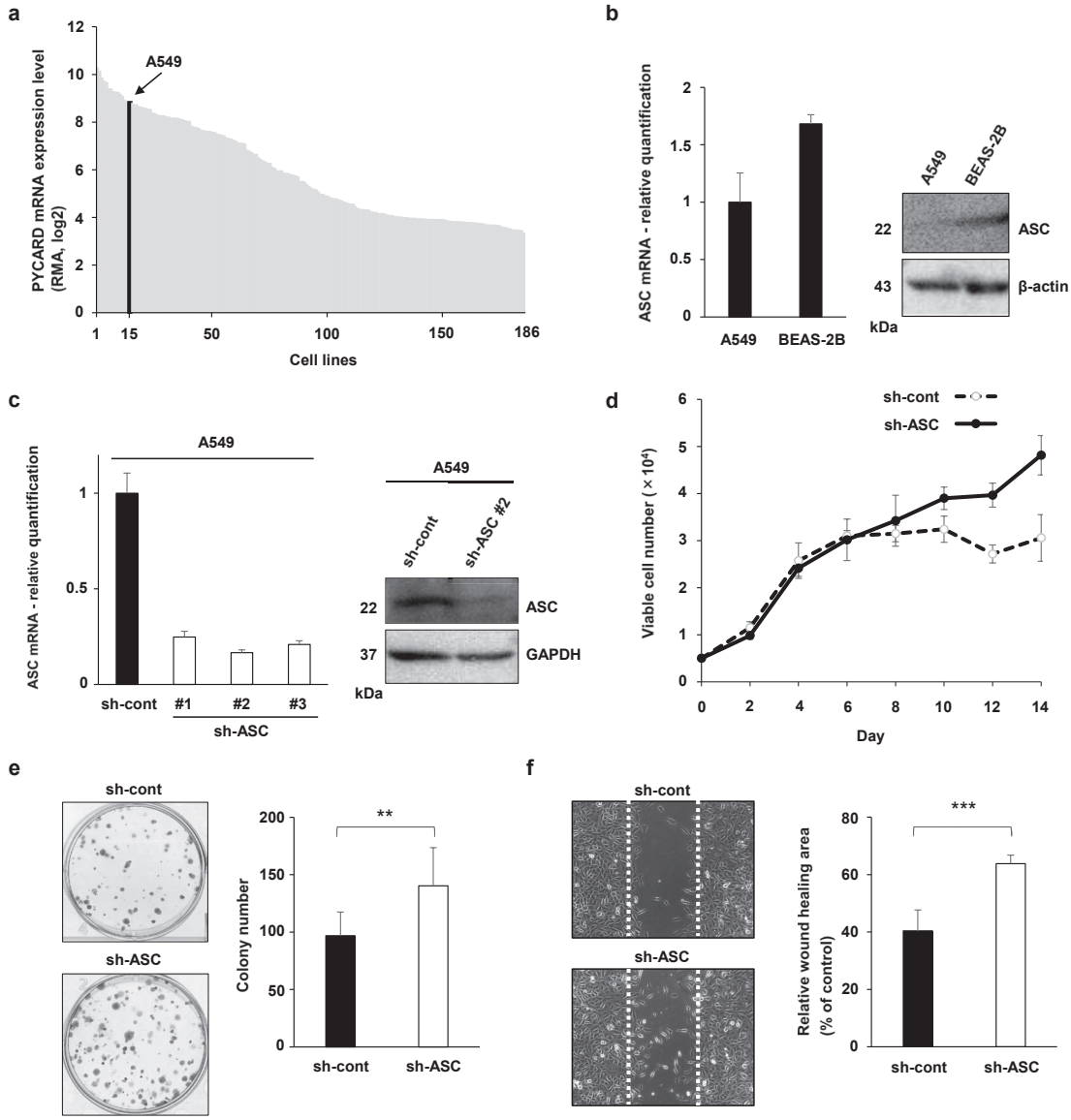

Fig. 1. Knockdown of ASC expression enhances malignant properties of lung cancer cells.

(a) Gene expression levels of PYCARD (29108_at probe), which codes for ASC, across 186 human non-small cell lung cancer cell lines (RMA; Robust Multi-array Average, log2). Data are from the RMA normalized CCLE expression data set (http://www.broadinstitute.org/ccle). A549 is shown as a black vertical line (15th of 186 cell lines).

(b) The expression levels of endogenous ASC in A549 and BEAS-2B cell lines were examined by real-time PCR and immunoblotting. *β*-actin was used as a loading control.

(c) A549 cells were transfected with *ASC* shRNA or control shRNA. Three clones were independently isolated and showed comparable phenotypic tendencies throughout all experiments. The knockdown efficiency of A549 cells was confirmed by real-time PCR. The expression levels of relative *ASC* mRNA were normalized by those of *β-actin*. Protein expression levels of ASC in *ASC* knockdown (sh-ASC) and control cells (sh-cont) were examined by immunoblotting. GAPDH was used as a loading control.

(d) Growth curves of *ASC* stable knockdown and control A549 cells. Cells were cultured under serum starvation in 12-well plates (5×10^4 cells per well). Every 2 days of culture, the medium was replaced with fresh medium. To measure cell number, cells were mixed with trypan blue and the numbers of living, dead, and total cells were counted. Triplicate measurements were done to determine the average number of viable cells.

(e) ASC knockdown and control A549 cells were sparsely seeded in 6-well plates. Cells were grown for 9-11 days. To visualize colonies, cells were fixed in 70% ethanol and stained with 0.4% crystal violet solution. Colonies were counted manually. Error bars represent results from triplicate wells for each experimental treatment (n = 3). $^{**}P < 0.01$.

(f) Representative images of wound healing assays for *ASC* knockdown and control cells at 36 hours after cellular scratch under serum starvation. Summary bar graphs of the percentage closure at 36 hours. $^{***}P < 0.001$.

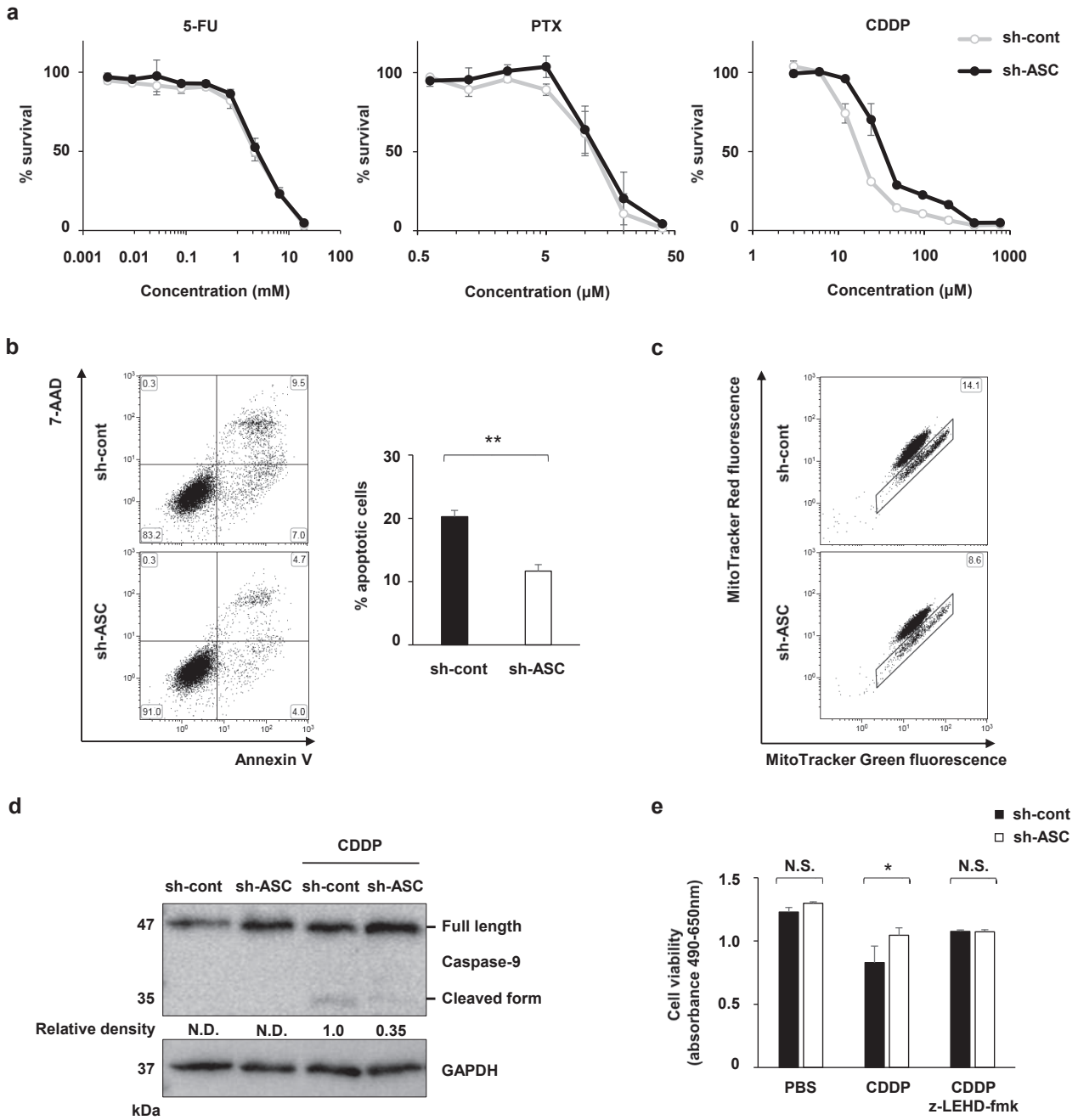

Fig. 2. ASC-depletion renders A549 cells resistant to CDDP.

(a) ASC knockdown and control A549 cells were seeded into 96-well plates at 5,000 cells/well. Serially diluted 5-FU, PTX, and CDDP solutions were administered to each well 24 hours after plating. Cells were further incubated under serum starvation for 24 hours and cell viability was determined by the MTS assay.

(b) Cells were treated with 24 μM CDDP for 24 hours under serum starvation. Total cells were collected and stained with Annexin V and 7-AAD and incubated for 15 min in the dark at 37°C. Flow cytometric analysis was performed to evaluate CDDP-induced apoptotic cells. $^{**}P < 0.01$.

(c) Cells were treated with 24 μM CDDP for 24 hours under serum starvation. To measure mitochondrial membrane potential, 50 nM each of MitoTracker green and red were added to the CDDP-treated cells, incubated for 30 min at 37°C, and analyzed by flow cytometry.

(d) Cells were treated with 24 μM CDDP for 24 hours under serum starvation. Cell extracts were electrophoresed and analyzed by immunoblotting with anti-caspase-9 antibody. The relative density of cleaved caspase-9/GAPDH was densitometrically determined. N.D.; not detectable.

(e) Cells were pretreated with or without z-LEHD-fmk for 1 hour prior to 24 μM CDDP addition. After 24 hours under serum starvation, cells were harvested and caspase-9 activity was evaluated as shown in (e). The caspase-9 inhibitor induced apoptosis in control cells, but not in ASC knockdown cells (n = 3). $^{*}P < 0.05$. N.S.; not significant.

Apart from the mitochondria-related pathway, several genes have been implicated in sensitivity to CDDP and are all involved in regulating the uptake of anticancer drugs or DNA repair. We examined the mRNA expression levels of such genes as *NRF2* (Wang et al. 2008), *PPARGC1β* (Yao et al. 2013), *ABCB1* (Cole et al. 1992), and *ERCC1* (Dabholkar et al. 1992) (Fig. 3b), but none exhibited significant expression differences between ASC knockdown and control cells.

Phosphorylated Src is upregulated in ASC-knockdown cells

In order to clarify the mechanism of malignant phenotype procurement by ASC knockdown, we determined the phosphorylation levels of several molecules affecting cellular malignant phenotype that were reportedly induced by ASC suppression, including MAPK, NF-κB (Masumoto et al. 2003; Hasegawa et al. 2009; Liu et al. 2013), and Src (Okada et al. 2016). Src was constitutively phosphorylated in ASC knockdown cells as compared with controls (Fig. 4a, b), implying that the malignant properties in ASC knockdown A549 cells was partially dependent on Src activation.

Intracellular ROS were previously reported to activate Src tyrosine kinase (Giannoni et al. 2005). ROS have been defined as physiological signals that promote cancer cell proliferation and migration and have also been observed upon Bcl-2 overexpression (Chen and Pervaiz 2007). Furthermore, another study demonstrated that the increased susceptibility in ASC-expressing cancer cells to genotoxic stress was partially dependent on mitochondrial ROS in colorectal cancer cells (Hong et al. 2013). To assess the influence of intracellular ROS in ASC knockdown cells, ROS production under CDDP treatment was examined by flow cytometry but revealed no difference in production between ASC knockdown and control cells (Fig. 4c).

Targeting Bcl-2 and pSrc abrogates malignant phenotypes in ASC-knockdown cells

Elevated Src levels promote tumor cell survival, angiogenesis, and invasive properties and contribute to correlations with stage advancement and metastatic potential (Slamon et al. 1989; Cartwright et al. 1990). Accordingly, we evaluated the effects of a Src inhibitor, dasatinib, alone and in combination with CDDP on cell viability. Dasatinib effectively inhibited cell proliferation of both ASC knockdown and control cells in combination with CDDP, but the agent failed to eliminate the difference in sensitivity to CDDP between cell types (Fig. 5a).

Matrigel invasion assays were performed with or without dasatinib to elucidate the involvement of Src in the malignant phenotype of invasiveness in ASC knockdown cells. As expected, the cells exhibited stronger invasiveness than controls, while dasatinib effectively attenuated the invasiveness of ASC knockdown cells (Fig. 5b). We could not confirm the permeation ability of combined CDDP and dasatinib, since they strongly induced cancer cell death, even at low doses. These findings indicated that an increase in phospho-Src appeared to be involved in enhancing invasion ability rather than cell proliferation in ASC knockdown cells.

In line with the hypothesis that Bcl-2 repressed the ASC-mediated apoptosis pathway, we evaluated the sensitivity of ASC knockdown and control cells to a Bcl-2 inhibitor, ABT-199, in cytotoxic assays. Both cell types displayed similar sensitivity to the agent (Fig. 5c). Overall, ABT-199 in combination with dasatinib elicited a stronger apoptotic response than did either drug in combination with CDDP (Fig. 5d).

Discussion

The present study sought to clarify the roles of ASC in lung cancer progression. A previous report showed that hypermethylation of ASC gene was detected in the sputum containing lung cancer-derived cells, which was correlated with the later stages of tumor progression and predictive of prognosis in individuals receiving resection for early-stage disease (Machida et al. 2006). Here, we showed that the downregulated ASC expression enhanced proliferative potential and invasiveness of A549-derived cells. We also observed that ASC-knockdown cells acquired chemoresistance to CDDP. These findings suggest that the downregulation of ASC confers malignant phenotypes on lung cancer cells.

In the present study, ASC was correlated with mitochondrial apoptotic signaling via caspase-9 in the A549-derived cell line. Other study reported that both caspase-8 and caspase-9 apoptotic pathways are involved in ASC-mediated cell death (Masumoto et al. 2003). While caspase-8-dependent apoptosis induces the direct activation of caspase-3, caspase-8 also cleaves BID, one of pro-apoptotic molecule functioning through mitochondria, to produce an active form (Hasegawa et al. 2007). According to our data, however, no such activated BID proteins were detected in ASC-knockdown cells under CDDP treatment, indicating that signals other than BID activation were involved in. It is generally recognized that the activated form of BID is generated upon proteolytic cleavage by caspase-8 in response to pro-inflammatory stimuli, such as the Fas ligand or tumor necrosis factor α (TNFα) signaling (Luo et al. 1998). The activation of caspases and induction of an apoptotic response by Fas-mediated death signaling or TNFα stimulation essentially require a sustained amount of ASC, such that an inhibition of ASC expression diminishes the apoptotic response (Ohtsuka et al. 2004). In fact, treatment of ASC-knockdown cells with an anti-Fas antibody (clone CH-11) or TNFα alone or in combination with CDDP failed to elicit a full apoptotic response (data not shown). Among other Bcl-2 family proteins, several studies have shown that ASC can interact with Bax and recruits it to the mitochondria (Ohtsuka et al. 2004); however, we did not witness any interactions between ASC and Bax by immunoprecipitation (data not shown), nor did ASC expres-

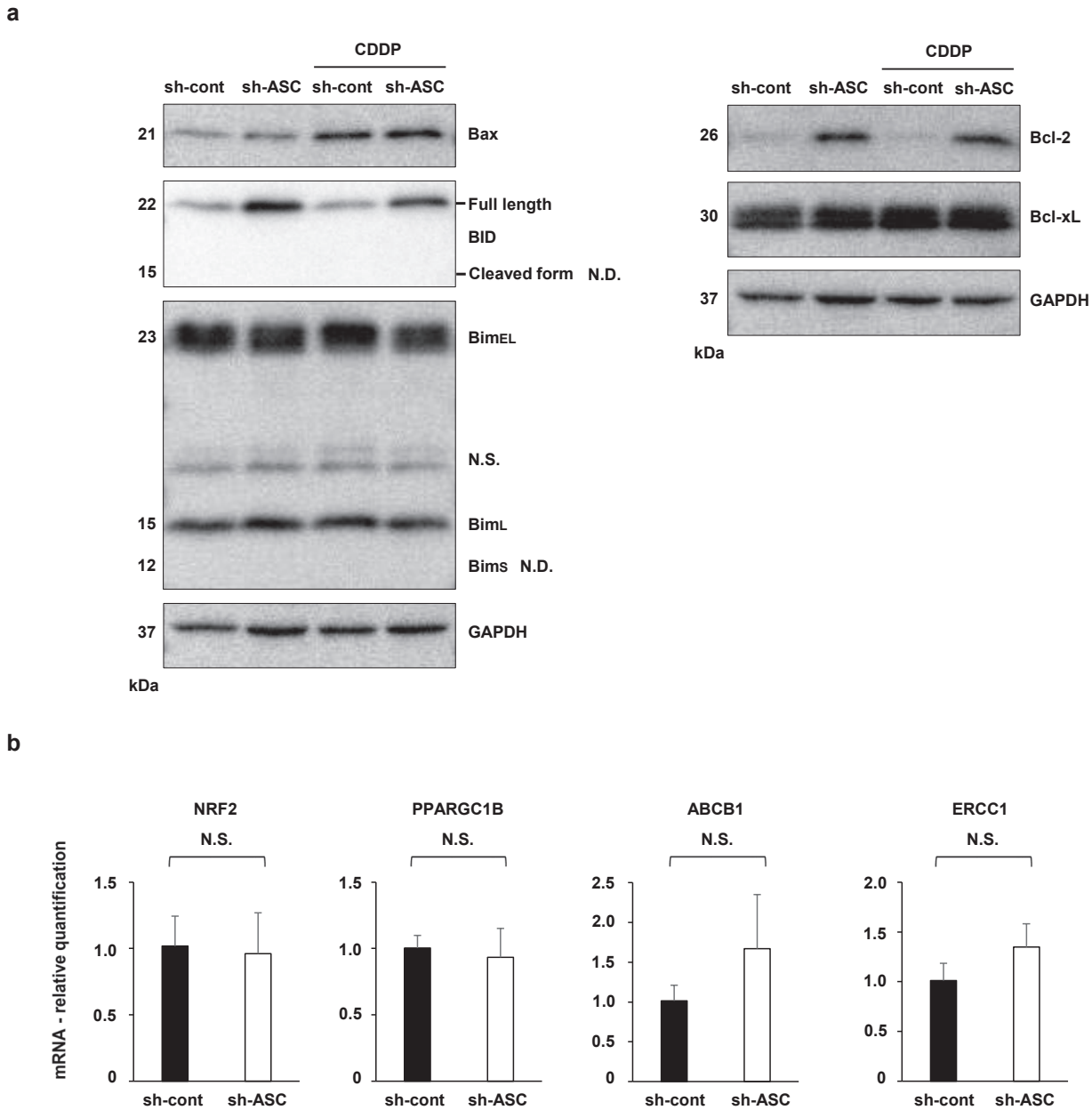

Fig. 3. Anti-apoptotic Bcl-2 is involved in ASC-mediated CDDP resistance.
(a) Pro- and anti-apoptotic proteins related to the mitochondrial apoptosis pathway were examined in ASC knockdown and control A549 cells by immunoblotting. Cells were treated with or without 24 μM CDDP under serum starvation for 24 hours and then harvested and separated by SDS/PAGE. GAPDH was used as a loading control. N.S., non-specific band; N.D., not detectable.
(b) The mRNA expression of genes reported to be involved in CDDP resistance was analyzed by real-time PCR. These data were observed under serum starvation. Each gene expression was normalized by β-actin expression (the value for the sh-control was set to 1).

sion level differences affect the expression of Bax (Fig. 3a), suggesting that CDDP-induced apoptosis in A549 cells was independent of Bax as well. Bax-related apoptosis signaling may depend on the cell type.

We observed that anti-apoptotic Bcl-2 was induced in ASC-knockdown cells. This protein is recognized as a key regulator of the mitochondrial apoptotic pathway. Bcl-2 controls cell survival by binding the BH3 domains of pro-apoptotic proteins and preventing permeabilization of the mitochondrial outer membrane. Overexpression of Bcl-2 was associated with CDDP resistance in several models (Han et al. 2003; Michaud et al. 2009). Our experiments clarified that the Bcl-2 inhibitor ABT-199 promoted CDDP-induced apoptosis in ASC-knockdown cells. ABT-199 is a potent and selective Bcl-2 inhibitor that is useful for malignancies in which Bcl-2 plays a central role, such as chronic lymphocytic leukemia and acute myelogenous leukemia, and does not cause thrombocytopenia (Souers et al. 2013).

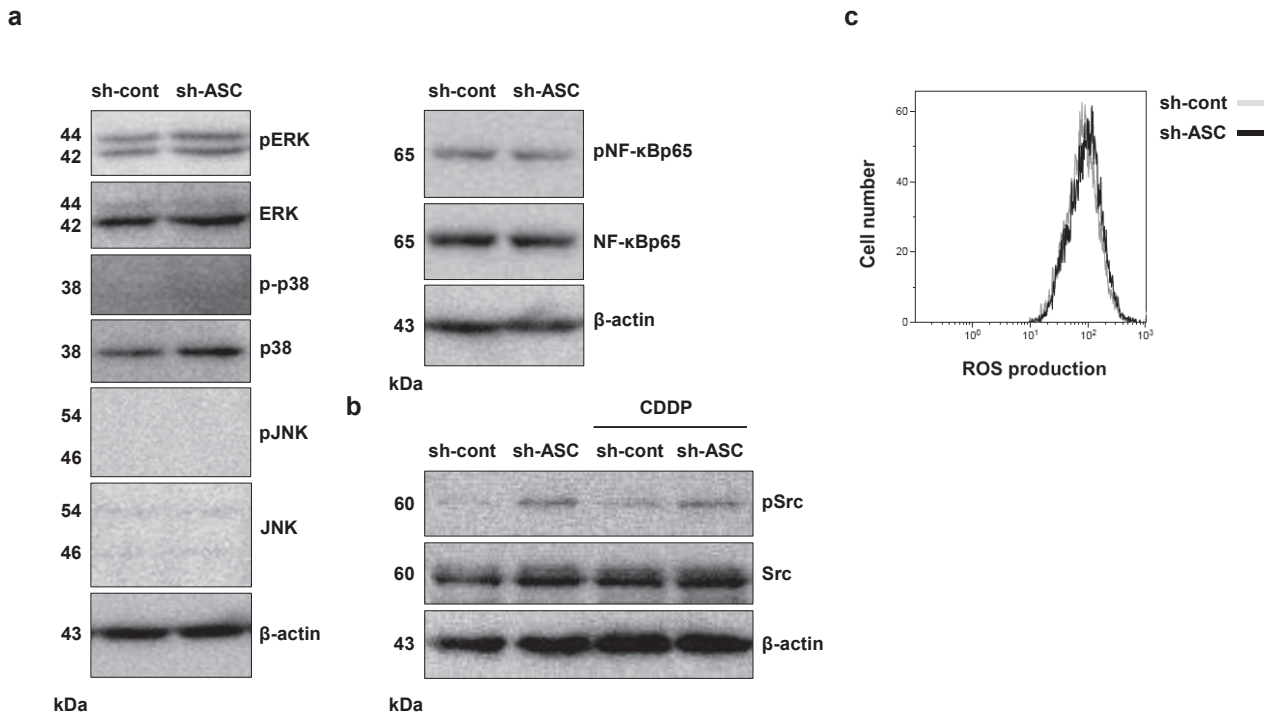

Fig. 4. Phosphorylated Src is upregulated in ASC-knockdown cells.
(a) Immunoblotting analysis of molecules related to carcinogenesis and cancer progression. Cell lysates cultured under serum starvation for 24 hours were separated by SDS/PAGE.
(b) Src expression and its phosphorylation in cells treated with 24 μM CDDP under serum starvation. β-actin was used as a loading control.
(c) Intracellular ROS measurements. Cells were treated with 24 μM CDDP under serum starvation for 24 hours and then incubated with 5 μM CellROX® Deep Red Reagent (Life technology, Carlsbad, USA) for 30 min at 37°C. ROS levels were analyzed by flow cytometry.

Therefore, it may be an alternative drug to CDDP, which also induces hematotoxicity as a major adverse effect.

Another characteristic of ASC-knockdown cells is the aberrantly enhanced phosphorylation of Src kinase. Src is involved in various cellular functions, including the regulation of cell proliferation, adhesion, migration, and invasion (Thomas and Brugge 1997; Guarino 2010), and can induce lung and bone metastasis (Rucci et al. 2006). Given that ASC abrogation was seen to increase phosphorylation levels of Src, we assessed intracellular ROS in ASC knockdown cells since Src activity is influenced by ROS. ROS levels, however, did not differ significantly between ASC knockdown and control cells. Interestingly, Src was constitutively activated in ASC-knockdown melanoma cells (Okada et al. 2016).

This study attempted to inhibit Src activity of A549-derived cells using dasatinib, an orally administered Bcr-Abl kinase inhibitor that has been approved for patients with chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia (Breccia et al. 2013). Dasatinib substantially abrogated the invasive properties of ASC-knockdown cells to those comparable to controls, suggesting that treatments containing Src inhibitors might increase the susceptibility of CDDP-resistant cells. Although more study is required to explain the precise regulatory mechanisms of Bcl-2 and Src signaling in connection with ASC suppression, it appears that cell proliferation is dependent on Bcl-2 and invasiveness is reliant on Src kinase in ASC-downregulated cells. Our data showed that cell proliferation and CDDP sensitivity in ASC knockdown cells did not recover to their respective levels in control cells when ABT-199 and dasatinib were used, indicating that other signaling pathways likely contributed to cell proliferation and the resistance mechanism to CDDP. We suspect that ASC affects cell proliferation and CDDP resistance at least partially through Bcl-2 and phosphorylated Src. Furthermore, we believe that ABT-199 and dasatinib are notable prospective treatment regimens for CDDP-resistant patients that may be effective in heterogeneously populated tumor cells, including those with downregulated ASC, because the drugs can inhibit the growth of both ASC-downregulated cells and ASC-positive cells.

Using ASC-knockdown cells, derived from A549 lung cancer cells, we provide the evidence that ASC expression is an important factor in determining susceptibility to CDDP chemotherapy in non-small cell lung cancer. However, additional studies are required to prove this biological function of ASC in overall non-small cell lung cancer.

In conclusion, our results demonstrate that ASC deple-

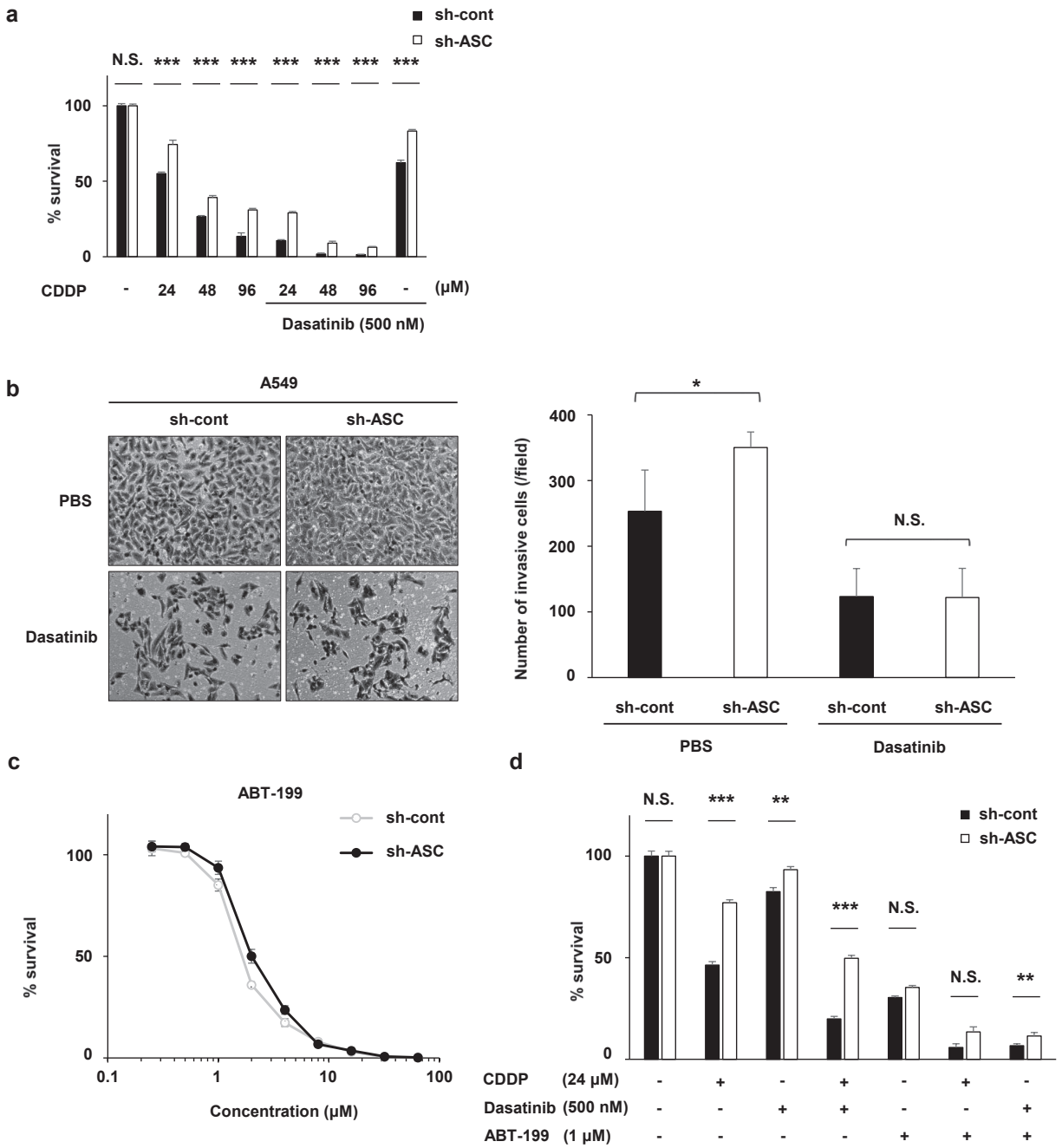

Fig. 5. Targeting Bcl-2 and pSrc abrogates malignant phenotypes in ASC- knockdown cells.

(a) Effect of a Src inhibitor in cell proliferation. ASC knockdown and control cells were seeded into 96-well plates. After 24 hours, serial dilutions of CDDP and 500 nM dasatinib were added to cells. Cells were then incubated under serum starvation for 24 hours and cell viability was evaluated by the MTS assay. ***$P < 0.001$. N.S.: not significant.

(b) ASC modulated cell invasiveness. Cells were seeded into transwells and treated for 48 hours with or without 100 nM dasatinib. Cell invasion assays were performed in the presence of matrigel transwell filters. Representative crystal violet staining images of invasive cells are shown. Summary bar graphs of numbers of invading cells. *$P < 0.05$. N.S., not significant.

(c) ASC knockdown and control cells were seeded into 96-well plates. After 24 hours under serum starvation, serial dilutions of ABT-199 was added. Cells were then incubated for 24 hours, and cell viability was evaluated by the MTS assay.

(d) ASC knockdown and control cells were seeded into 96-well plates. Following overnight incuvation under serum starvation, indicated combinations of agents were added to the cells (CDDP; 24 μM, dasatinib; 500 nM, ABT-199; 1 μM). Cells were then incubated for another 24 hours, and cell viability was evaluated by the MTS assay (n = 3). **$P <$ 0.001. ***$P < 0.001$. N.S., not significant.

tion in A549 cells enhances cell proliferation, motility, and invasiveness, and also show that ASC expression is an important factor in determining susceptibility to CDDP chemotherapy. Moreover, we provide evidence for the therapeutic potential of Bcl-2 and Src inhibitors as chemo-sensitizing agents in CDDP-resistant tumors. Particularly, combination therapy of dasatinib and ABT 199 for patients with tumors containing ASC-downregulated cancer cells may remedy the shortcomings of currently popular chemotherapies using CDDP that have limited curative impact and are usually accompanied with severe side effects.

Conflict of Interest

The authors declare no conflict of interest.

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