IJC
International Journal of Cancer

Annexin A4-conferred platinum resistance is mediated by the copper transporter ATP7A

Shinya Matsuzaki[1,2], Takayuki Enomoto[3], Satoshi Serada[2], Kiyoshi Yoshino[1], Shushi Nagamori[4], Akiko Morimoto[1], Takuhei Yokoyama[1,2], Ayako Kim[2], Toshihiro Kimura[1], Yutaka Ueda[1], Masami Fujita[1], Minoru Fujimoto[2], Yoshikatsu Kanai[4], Tadashi Kimura[1] and Tetsuji Naka[2]

[1] Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka, Japan
[2] Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan
[3] Department of Obstetrics and Gynecology, Niigata University Graduate School of Medicine, Niigata, Japan
[4] Department of Pharmacology, Osaka University Graduate School of Medicine, Osaka, Japan

Although platinum drugs are often used for the chemotherapy of human cancers, platinum resistance is a major issue and may preclude their use in some cases. We recently reported that enhanced expression of Annexin A4 (Anx A4) increases chemoresistance to carboplatin through increased extracellular efflux of the drug. However, the precise mechanisms underlying that chemoresistance and the relationship of Anx A4 to platinum resistance *in vivo* remain unclear. In this report, the *in vitro* mechanism of platinum resistance induced by Anx A4 was investigated in endometrial carcinoma cells (HEC1 cells) with low expression of Anx A4. Forced expression of Anx A4 in HEC1 cells resulted in chemoresistance to platinum drugs. In addition, HEC1 control cells were compared with Anx A4-overexpressing HEC1 cells in xenografted mice. Significantly greater chemoresistance to cisplatin was observed *in vivo* in Anx A4-overexpressing xenografted mice. Immunofluorescence analysis revealed that exposure to platinum drugs induced relocation of Anx A4 from the cytoplasm to the cellular membrane, where it became colocalized with ATP7A, a copper transporter also well known as a mechanism of platinum efflux. ATP7A expression suppressed by small interfering RNA had no effect on HEC1 control cells in terms of chemosensitivity to platinum drugs. However, suppression of ATP7A in Anx A4-overexpressing platinum-resistant cells improved chemosensitivity to platinum drugs (but not to 5-fluorouracil) to a level comparable to that of control cells. These results indicate that enhanced expression of Anx A4 confers platinum resistance by promoting efflux of platinum drugs *via* ATP7A.

Platinum drugs, widely used for treating gynecological cancers, can improve survival rates dramatically, particularly in patients with ovarian and endometrial carcinomas.[1–6] Compared with platinum-sensitive tumors, prognosis is poorer for tumors that are (or become) platinum-resistant; for these tumors, other chemotherapeutic drugs also tend to be less effective. For example, an efficacy of 81% has been demonstrated for chemotherapy regimens that include platinum drugs for treatment of ovarian serous adenocarcinoma (SAC), the most common subtype of ovarian carcinoma; however, the efficacy of these regimens is only 18% for ovarian clear cell carcinomas (CCC), which are frequently resistant to multiple drugs.[7] Compared with advanced SAC, the clinical prognosis of patients with similarly advanced CCC is markedly worse largely because of the considerably higher rate of recurrence after CCC treatment.[7–11] Therefore, determining the mechanism underlying platinum resistance may aid in identification of therapeutic targets for platinum-resistant tumors such as CCC. Studies using proteomic screening approaches have previously demonstrated overexpression of Annexin A4 (Anx A4) protein in ovarian CCC, which is frequently a highly platinum-resistant tumor compared with SAC.[12] Similar findings have been reported in a study comparing SAC and CCC using a genomic screening approach.[13] Anx A4, a previously understudied member of the Annexin protein family, binds to phospholipids in a Ca^{2+}-dependent manner, self-associates on phospholipid

Key words: Annexin A4, ATP7A, platinum resistance, platinum transporter, copper transporter
Abbreviations: 5-FU: 5-fluorouracil; Anx A4: Annexin A4; CCC: clear cell carcinoma; D-MEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; PBS: phosphate-buffered saline; SAC: serous adenocarcinoma; siRNA: small interfering RNA
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Japanese Ministry of Education, Science, Culture and Sports; **Grant number:** 22791560; **Grant sponsors:** Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, Ministry of Health, Labour and Welfare of Japan
DOI: 10.1002/ijc.28526
History: Received 4 Dec 2012; Accepted 26 Sep 2013; Online 8 Oct 2013
Correspondence to: Dr. Tetsuji Naka, Laboratory for Immune Signal, National Institute of Biomedical Innovation, 7-6-8 Saito-asagi, Ibaraki, Osaka 567-0085, Japan, Tel.: +81-72-641-9843, Fax: +81-72-641-9837, E-mail: tnaka@nibio.go.jp

What's new?

Although platinum-based drugs are often used in chemotherapy, resistance to these drugs is frequently a problem. The protein Annexin A4 (Anx A4) is known to be involved in platinum efflux in ovarian tumours; however, its precise mechanism of action has been unclear. In this study, the authors demonstrated that the strong platinum-resistance in Anx A4-overexpressing cells involves the transporter protein ATP7A, both in vitro and in vivo. This suggests that Anx A4 may be a highly useful therapeutic target in Anx A4-expressing carcinomas.

membrane surfaces and causes membrane aggregation.[12,14–17] Enhanced expression of Anx A4 has recently shown to increase tumor chemoresistance to carboplatin (a key drug for treating gynecological cancers) *via* increased extracellular efflux of the drug.[12] Another study showed that Anx A4 suppresses NF-κB transcriptional activity, which is significantly upregulated early after etoposide treatment. Anx A4 translocates to the nucleus together with p50 and imparts greater resistance to apoptotic stimulation by etoposide treatment.[18] Anx A4 may also be associated with drug resistance in other types of tumors; enhanced expression of Anx A4 has been reported in colon, renal, lung and pancreatic cancers.[19–23] However, the details of Anx A4-mediated extracellular efflux of platinum drugs remain unclear.

HEC1 is an endometrial carcinoma cell line with low Anx A4 expression levels. In our study, Anx A4-overexpressing derivative HEC1 cell lines were established and their chemosensitivity toward platinum drugs was analyzed both *in vitro* and *in vivo*. Anx A4-conferred platinum chemoresistance was shown to be mediated by the copper transporter ATP7A.[24–28]

Material and Methods

Cell lines

The human endometrial carcinoma cell lines HEC1, HEC1A, HEC6, HEC88nu, HEC108, HEC116 and HEC251; SNGII and SNGM cells, the human ovarian SAC cell line OVSAHO and the ovarian CCC cell lines OVISE and OVTOKO were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan); A2780 cells from the human ovarian SAC cell line were obtained from the European Collection of Animal Cell Culture (Salisbury, Scotland). The identity of each cell line was confirmed by DNA fingerprinting *via* short tandem repeat profiling, as described previously.[29] HEC1, HEC1A, HEC6, HEC88nu, HEC108, HEC116 and HEC251 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1% penicillin–streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C under a humidified atmosphere of 5% CO_2. SNGII and SNGM cells were maintained in Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin–streptomycin. OVSAHO, A2780, OVISE and OVTOKO cells were maintained in Roswell Park Memorial Institute 1640 medium (Wako Pure Chemical Industries) supplemented with 10% FBS and 1% penicillin–streptomycin.

Generation of Anx A4 stably transfected cell lines

To generate cell lines that stably expressed Anx A4, HEC1 cells were transfected with the pcDNA3.1–Anx A4 expression plasmid, as described previously.[12] Transfected cells were selected with 600 μg/ml of Geneticin (Invitrogen). Clones were maintained in 250 μg/ml of Geneticin for stability of expression. Four stable Anx A4-expressing cell lines were established and designated HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77. A control cell line of HEC1 was also established and stably transfected with an empty vector. This cell line was designated as HEC1-CV.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% protease-inhibitor cocktail (Nacalai Tesque) and 1% phosphatase-inhibitor cocktail (Nacalai Tesque)]. After centrifugation (13,200 rpm, 4°C, 15 min), soluble proteins in the supernatant were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously.[12] Additional information can be found in Supporting Information Material and Methods.

Measurement of IC_{50} values after treatment with cisplatin or carboplatin

Cells were suspended in D-MEM medium supplemented with 10% FBS and were seeded in 96-well plates (2,000 cells per well) (Costar; Corning, Corning, NY) for 24 hr. They were then exposed to various concentrations of carboplatin (0–500 μM), cisplatin (0–100 μM) or 5-fluorouracil (5-FU) (0–50 μM) for 72 hr. Cell proliferation was evaluated using the WST-8 assay (Cell Counting Kit-SF; Nacalai Tesque) after treatment at the time points indicated by the manufacturer. The absorption of WST-8 was measured at a wavelength of 450 nm (reference wavelength: 630 nm) using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA). Absorbance values for treated cells indicative of proliferation rates were expressed as percentages relative to results for untreated controls, and the drug concentrations resulting in a 50% inhibition of cell growth (IC_{50} values) were calculated.

Small interfering RNA transfection

Two commercial small interfering RNAs (siRNAs) against ATP7A and a nonspecific control siRNA were obtained from

Qiagen (Venlo, The Netherlands) and designated ATP7A siRNA4 and ATP7A siRNA6, respectively. For gene silencing, a specific sense strand 5′-GCAGCUUGUAGUAUUGAA ATT-3′ was used for ATP7A siRNA4, and an antisense strand 5′-UUUCAAUACUACAAGCUGCTA-3′ was also used. For ATP7A siRNA6, a specific sense strand 5′-GCGUA GCUCCAGAGGUUUATT-3′ was used, and an antisense strand 5′-UAAACCUCUGGAGCUACGCAG-3′ was also used. Cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Selective silencing of ATP7A was confirmed by Western blot analysis.

In vivo model of cisplatin resistance

All animal experiments were conducted in accordance with the Institutional Ethical Guidelines for Animal Experimentation of our National Institute of Biomedical Innovation (Osaka, Japan). Four-week-old, female Institute of Cancer Research (ICR) nu/nu mice were obtained from Charles River Japan (Yokohama, Japan). For subcutaneous xenograft experiments, 2.5×10^6 HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were suspended in 100 μl of 1/1 (v/v) phosphate-buffered saline (PBS)/Matrigel (Becton Dickinson, Bedford, MA) and injected subcutaneously into the flanks of the ICR nu/nu mice ($n = 5$ per group). One week after xenograft establishment, tumors measured ~100 mm^3. Mice were then randomly divided into two groups and administered cisplatin (3 mg/kg) or PBS i.p. twice weekly for 4 weeks. Tumor volumes were determined twice weekly by measuring length (L), width (W) and depth (D). Tumor volume was calculated using the formula: tumor volume (mm^3) = $W \times L \times D$. At 56 days after tumor implantation, tumors were removed and weighed.

Quantification of intracellular platinum accumulation

Cisplatin accumulation in cells was analyzed according to a previously established method, with certain minor modifications. In brief, 6×10^6 cells (HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells) were seeded into two 150-mm tissue culture dishes and incubated for 24 hr. The cells were then exposed to 1 mM cisplatin for 60 min at 37°C and then washed twice with PBS. After 3 hr of incubation in cisplatin-free D-MEM medium (supplemented with 10% FBS), whole extracts were prepared and the concentration of intracellular platinum was determined using an Agilent 7500ce inductively coupled plasma mass spectrometer (ICP-MS; Agilent, Santa Clara, CA). The absolute concentration of platinum in each sample was determined from a calibration curve prepared with a platinum standard solution.

Preparation of crude membrane fractions

To investigate the localization of Anx A4, crude membrane fractions (CMFs) of cells treated in various ways were prepared. Cells were divided into three groups: those that received no treatment, those pretreated with 10 μM cisplatin for 4 hr and those pretreated with 50 μM carboplatin for 4 hr. CMF were prepared as described elsewhere,[30] with modifications. Prepared proteins were investigated using Western blot analysis. Additional information can be found in Supporting Information Material and Methods.

Biotinylation of HEC1 cell membrane surface proteins after cisplatin or carboplatin exposure

To investigate the localization of ATP7A after exposure to platinum drugs, treated or mock-treated HEC1 cells were surface-biotinylated and the presence of ATP7A was investigated by Western blot analysis. Additional information can be found in Supporting Information Material and Methods.

Immunofluorescence for ATP7A and Anx A4

Immunofluorescence staining was performed 2 days after cells had been seeded on cover slips. Before staining, cells in the treatment groups were pretreated with 10 μM cisplatin or 50 μM carboplatin for 4 hr. Cells were then analyzed for localization of Anx A4 and ATP7A. Additional information can be found in Supporting Information Material and Methods.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's analysis to evaluate the significance of differences. In all analyses, $p < 0.05$ was considered to be statistically significant.

Results

Expression of Anx A4 in endometrial carcinoma cell lines

To investigate Anx A4 expression in nine common endometrial carcinoma cell lines, Western blot analyses were performed. Expression of Anx A4 was strongest in SNGM cells compared with the other eight cell lines (Fig. 1*a*). Thus, enhanced expression of Anx A4 was confirmed in this endometrial carcinoma cell line.

Anx A4 and platinum resistance in HEC1 cell lines

From control HEC1 cells (low Anx A4 expression levels), four stable lines of Anx A4-overexpressing cells (HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells) and one line of empty vector transfected cells (HEC1-CV cells) were established. Overexpression of Anx A4 was confirmed using Western blot analysis and was compared with CCC cell lines (OVTOKO and OVISE) used as positive controls (Fig. 1*b*). Significantly higher IC_{50} values for cisplatin were observed in HEC1-A25 (32.1 μM, $p < 0.01$), HEC1-A43 (23.8 μM, $p < 0.01$), HEC1-A63 (34.9 μM, $p < 0.01$) and HEC1-A77 cells (17.3 μM, $p < 0.01$) compared with HEC1 (9.8 μM) and HEC1-CV cells (8.4 μM) (Fig. 1*c*). Similarly, IC_{50} values for carboplatin were significantly increased in HEC1-A25 (194.6 μM, $p < 0.01$), HEC1-A43 (153.3 μM, $p < 0.01$), HEC1-A63 (371.5 μM, $p < 0.01$) and HEC1-A77 cells (158.1 μM, $p < 0.01$) compared with HEC1 (59.1 μM) and HEC1-CV cells (60.9 μM) (Fig. 1*c*). Thus, Anx A4 overexpression conferred platinum resistance in HEC1 cell lines.

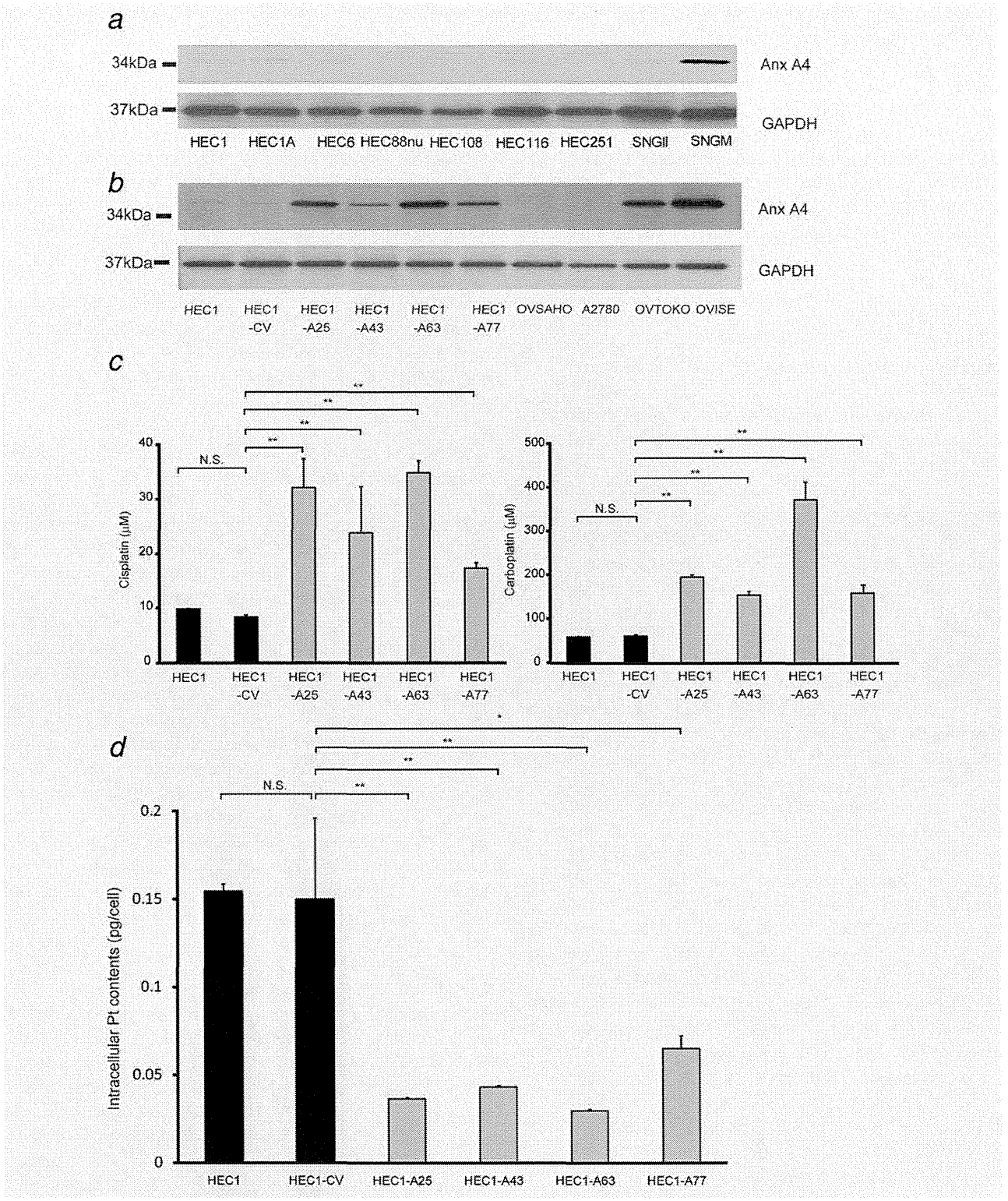

Figure 1. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vitro*. (*a*) Western blot analysis of nine endometrial carcinoma cell lines. Anx A4 was expressed in one cell line. (*b*) Establishment of an Anx A4-stably-expressing HEC1 cell line by transfection with the pcDNA3.1-Anx A4 expression plasmid into a HEC1 cell line with low Anx A4 expression levels. Enforced expression of Anx A4 was confirmed by Western blot analysis. (*c*) The IC_{50} sensitivity to cisplatin or carboplatin was investigated in HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells. (*d*) Intracellular platinum accumulation was investigated after treatment with 1 mM cisplatin for 60 min and further incubation with cisplatin-free medium for 180 min and was determined by ICP-MS analysis.

Intracellular platinum accumulation in Anx A4-overexpressing cells

To elucidate the mechanism underlying platinum resistance induced by Anx A4, intracellular platinum accumulation of HEC1, HEC1-CV, HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells after cisplatin exposure was analyzed. Significantly less platinum had accumulated in HEC1-A25, HEC1-A43, HEC1-A63 and HEC1-A77 cells compared with HEC1 and HEC1-CV cells (0.036 pg/cell, $p < 0.01$; 0.04 pg/cell, $p < 0.01$; 0.03 pg/cell, $p < 0.01$; 0.065 pg/cell, $p < 0.05$ and 0.154 and 0.150 pg/cell, respectively) (Fig. 1*d*). Thus, intracellular platinum accumulation was decreased in Anx A4-overexpressing cells.

Anx A4-overexpressing cells and cisplatin in xenograft models

To determine the involvement of Anx A4 in platinum resistance *in vivo*, HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were subcutaneously injected into nude mice. After the tumor xenograft had been established, cisplatin or PBS was given twice a week for 1 month. On Day 56, average tumor volumes were $11,496 \pm 950$ mm^3 in PBS-treated HEC1-CV control mice and $3,554 \pm 872$ mm^3 in cisplatin-treated HEC1-CV controls. A significant antitumor effect of cisplatin was therefore observed in HEC1-CV-xenografted mice compared with the PBS-treated group. The parent HEC1 and HEC1-CV xenografts responded similarly to cisplatin (Fig. 2*a*; $p < 0.01$).

In HEC1-A63-xenografted mice, the average tumor volume on Day 56 was $8,245 \pm 160$ mm^3 in the PBS-treated group and only slightly less ($7,078 \pm 257$ mm^3) in the cisplatin-treated group (Fig. 2*a*; $p = 0.42$). A similar response to cisplatin was observed in the HEC1-A63 and HEC1-A77 xenografts. On Day 56, no significant differences in tumor weight were found in HEC1-A63-xenografted mice between the PBS treatment (4.66 ± 0.42 g) and the cisplatin treatment groups (4.43 ± 0.16 g) (Fig. 2*b*). Similar results were observed in HEC1-A77 xenograft models. In contrast, a significant decrease in tumor weight was observed in HEC1-CV-xenografted mice between the PBS mock treatment (5.95 ± 1.16 g) and the cisplatin treatment groups (3.20 ± 0.76 g; $p < 0.05$) (Fig. 2*b*). Similar results were observed for the HEC1 and HEC1-CV xenografts. No significant differences in tumor weight in the PBS treatment group were observed among HEC1-CV-xenografted (5.95 ± 1.16 g), HEC1-xenografted (7.48 ± 0.34 g), HEC1-A63-xenografted (4.66 ± 0.42 g) and HEC1-A77-xenografted mice (4.82 ± 1.08 g) (Fig. 2*b*). These results indicated that overexpression of Anx A4 in HEC1 endometrial carcinoma cell lines conferred significant platinum resistance to the cells as tumors growing *in vivo*.

Translocation of Anx A4 and ATP7A after platinum exposure

In our study, platinum transporters were the focus of an investigation of the molecular mechanisms of chemoresistance induced by Anx A4. In previous research, intracellular platinum levels were decreased after enhanced expression of Anx A4, and ATP7A and ATP7B are well known as efflux transporters of platinum drugs.[27,28,31] However, the relationship of Anx A4 with ATP7A and ATP7B has not been previously examined. The results of our study demonstrated no change in expression of ATP7A at the protein levels owing to enforced overexpression of Anx A4 (Fig. 3*a*) and no ATP7B expression in HEC1 cells (data not shown). Therefore, the effects of Anx A4 expression on ATP7B in these cells were not investigated.

Because Anx A4 is normally localized to the cytoplasm, we theorized that exposure to platinum drugs may induce translocation of Anx A4 to the cellular membrane, resulting in an increase in chemoresistance owing to the influence of ATP7A. To investigate the possibility of induced translocation of Anx A4 and ATP7A by platinum drugs, CMFs were prepared. By Western blot analysis, Anx A4 expression in CMF of HEC1 and HEC1-CV cells before and after treatment with cisplatin or carboplatin was barely detectable because of its low endogenous expression in these cells (Fig. 3*b*). In contrast, Anx A4 expression was increased in CMF of HEC1-A63 cells and HEC1-A77 cells treated with cisplatin and carboplatin compared with untreated cells (Fig. 3*b*). Biotinylation-based cell surface membrane protein enrichment revealed a marked increase in biotinylation of ATP7A after exposure to cisplatin or carboplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (Fig. 3*c*). In the biotinylated samples, no Anx A4 expression was detected on the cell surface, although it had been previously detected in the cell CMF (data not shown). These results suggested that exposure to cisplatin or carboplatin induced massive translocation of Anx A4 to CMF, including the inner surface of the cell membrane (inaccessible to biotinylation). Before exposure of the cell to cisplatin or carboplatin, ATP7A was not expressed in biotinylated samples but after exposure, strong ATP7A expression was detected. These results suggested that exposure to cisplatin or carboplatin induced massive translocation of ATP7A to the outer surface of the cell (accessible to biotinylation).

Anx A4 and ATP7A localization

By immunofluorescence analysis, Anx A4 was localized in the perinuclear and cytoplasmic regions of untreated cells, whereas ATP7A was localized mainly in the perinuclear and cytoplasmic regions and slightly less in the cellular membrane in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (Figs. 4*a*–4*d*). After 4-hr exposure to cisplatin or carboplatin, Anx A4 and ATP7A were found to be colocalized to the cellular membrane in HEC1-A63 cells (Fig. 4*c*). Similar findings were observed in HEC1-A77 cells (Fig. 4*d*). Because of the low expression of Anx A4 in HEC1 and HEC1-CV cells, no Anx A4 was detected in the cellular membranes in these cells (Figs. 4*a* and 4*b*). Thus, the results of the immunofluorescence analysis were in accordance with those of both Western blot analysis of CMF preparations and biotinylation

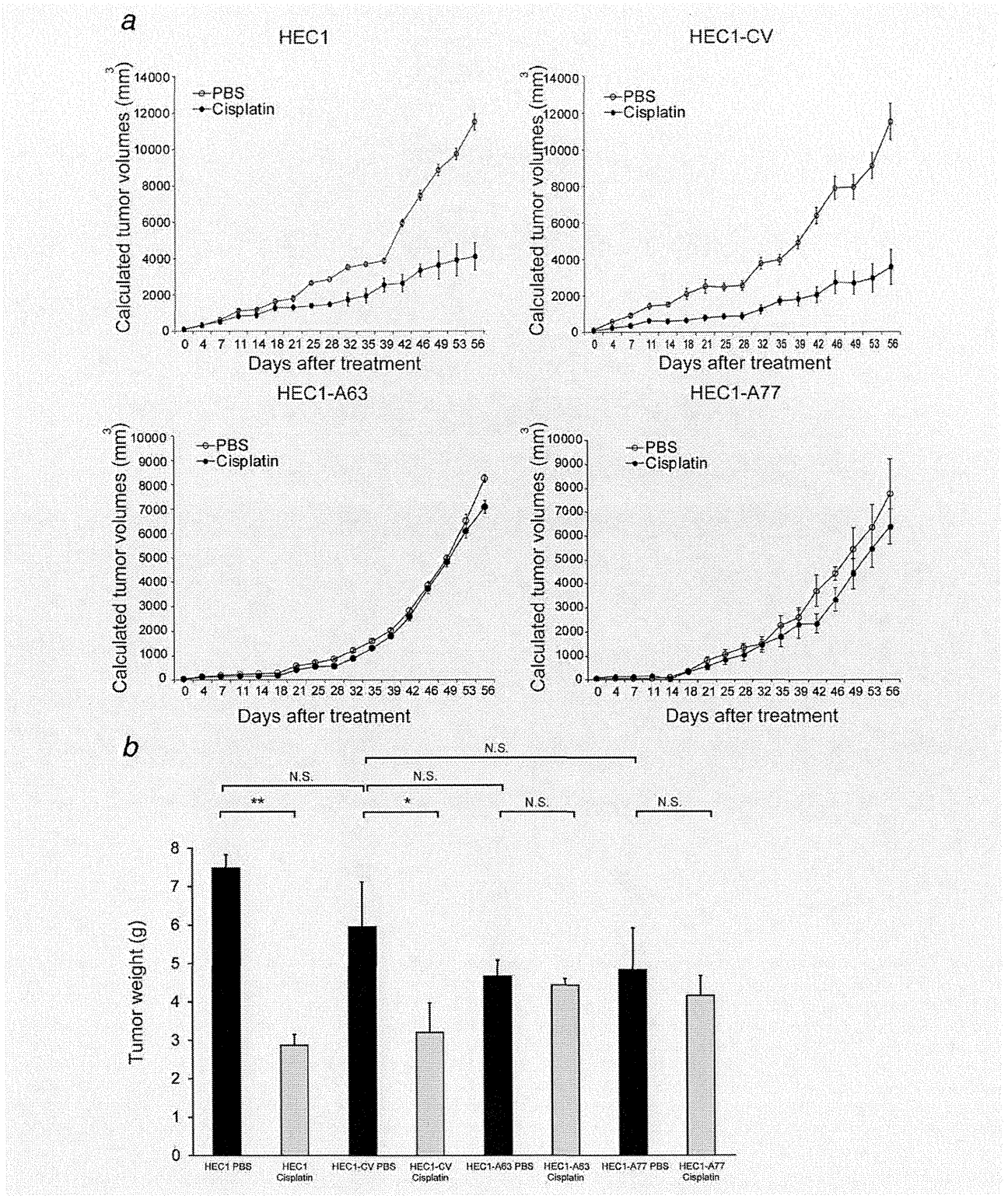

Figure 2. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vivo*. Analysis of Anx A4 as a platinum-resistant protein *in vivo*. (*a*) To determine the resistance of Anx A4-stably-expressing HEC1 cells to platinum *in vivo*, parent HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were subcutaneously injected into nude mice ($n = 5$ per group). After tumor xenografts were established, cisplatin (3 mg/kg) or PBS was administered i.p. twice weekly for 1 month. Figure shows the average (points) for five animals ± SD (bars). (*b*) Fifty-six days after implantation, tumors were removed and weighed. Values shown are the means (±SD) of five mice. NS: not significant (*$p <$ 0.05; **$p < 0.01$; one-way ANOVA, followed by Dunnett's analysis).

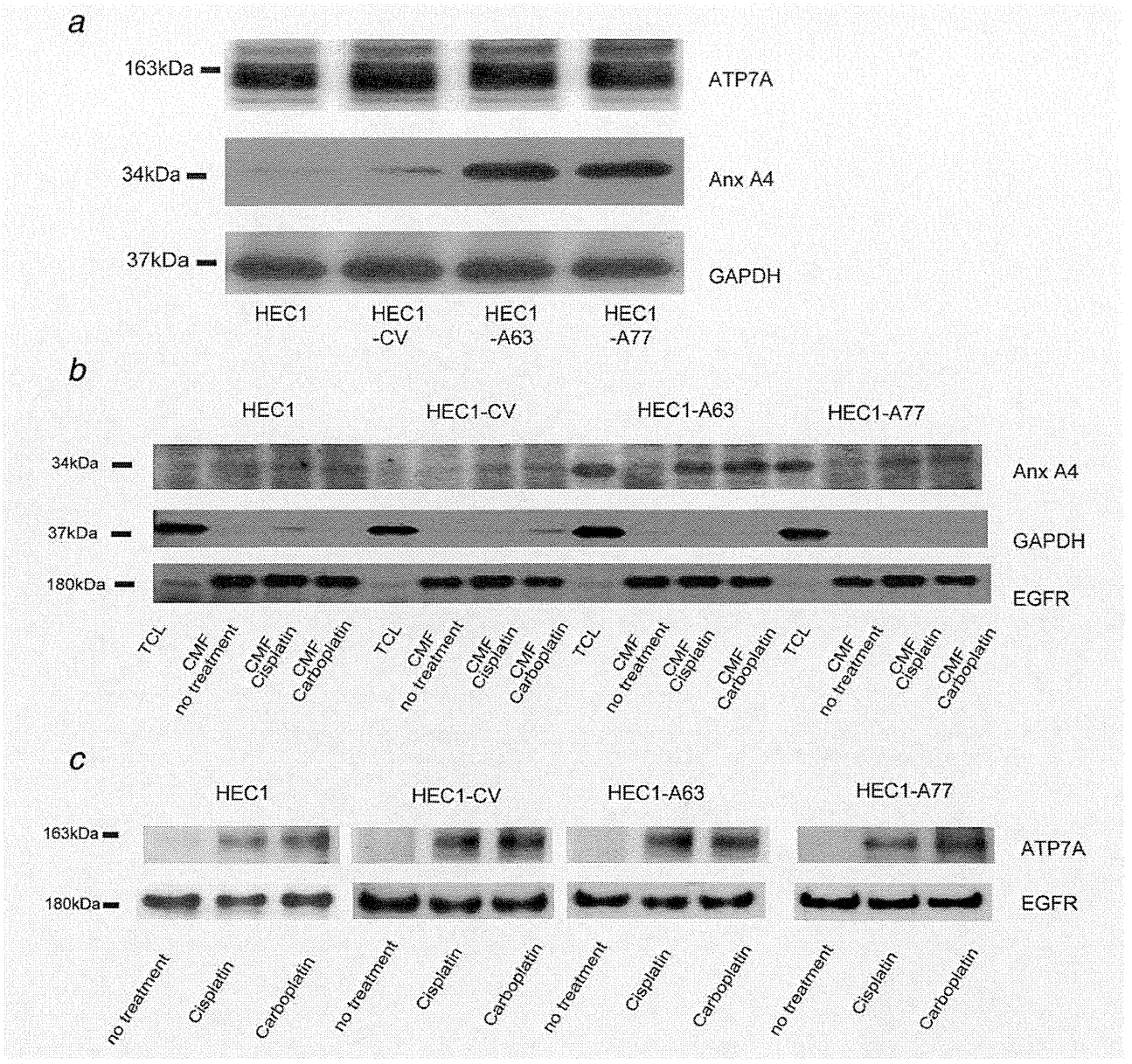

Figure 3. Localization of Anx A4 and ATP7A was investigated using Western blot analysis. The localization of Anx A4 and ATP7A was investigated using two techniques: orthogonal crude membrane fractions and biotinylation of cell surface proteins. (*a*) No significant change in expression levels of ATP7A was observed in HEC1, HEC1-CV, HEC1-A63 or HEC1-A77 cells. (*b*) In both HEC1-A63 and HEC1-A77 cells (but not in HEC1 and HEC1-CV cells), the drug-induced translocation of Anx A4 into the crude membrane fraction was shown by Western blot analysis after exposure to 10 μM cisplatin or 50 μM carboplatin for 4 hr. TCL: total cell lysate. Epidermal growth factor receptor was used as the control for cell surface protein labeling. (*c*) In HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells, translocation of ATP7A to the cell surface was shown by Western blot analysis. Cells were treated with 25 μM cisplatin or 150 μM carboplatin for 4 hr, and cell surface proteins were biotinylated with 500 μM sulfo-NHS-SS-biotin. Biotinylated surface proteins were enriched with UltraLink Immobilized Neutroavidin (Thermo Fisher Scientific, Waltham, MA) and analyzed by Western blot analysis using anti-ATP7A. Levels of epidermal growth factor receptor, a surface protein, are shown as loading controls.

assays (Figs. 3*b* and 3*c*). Anx A4 and ATP7A were localized in the cytoplasm before cisplatin or carboplatin exposure; Anx A4 and ATP7A were then translocated to the cellular membrane after cisplatin or carboplatin exposure. Thus, Anx A4 and ATP7A are colocalized to the cellular membrane in platinum-treated HEC1-A63 and HEC1-A77 cells but not in HEC1 and HEC1-CV cells.

Effect of ATP7A expression on resistance to platinum drugs

The mechanism of platinum resistance conferred by Anx A4 overexpression was explored further by suppression of ATP7A expression using siRNA. The suppression of ATP7A was confirmed using Western blot analysis (Fig. 5*a*). Anx A4 expression was unchanged by silencing ATP7A (Fig. 5*a*). The

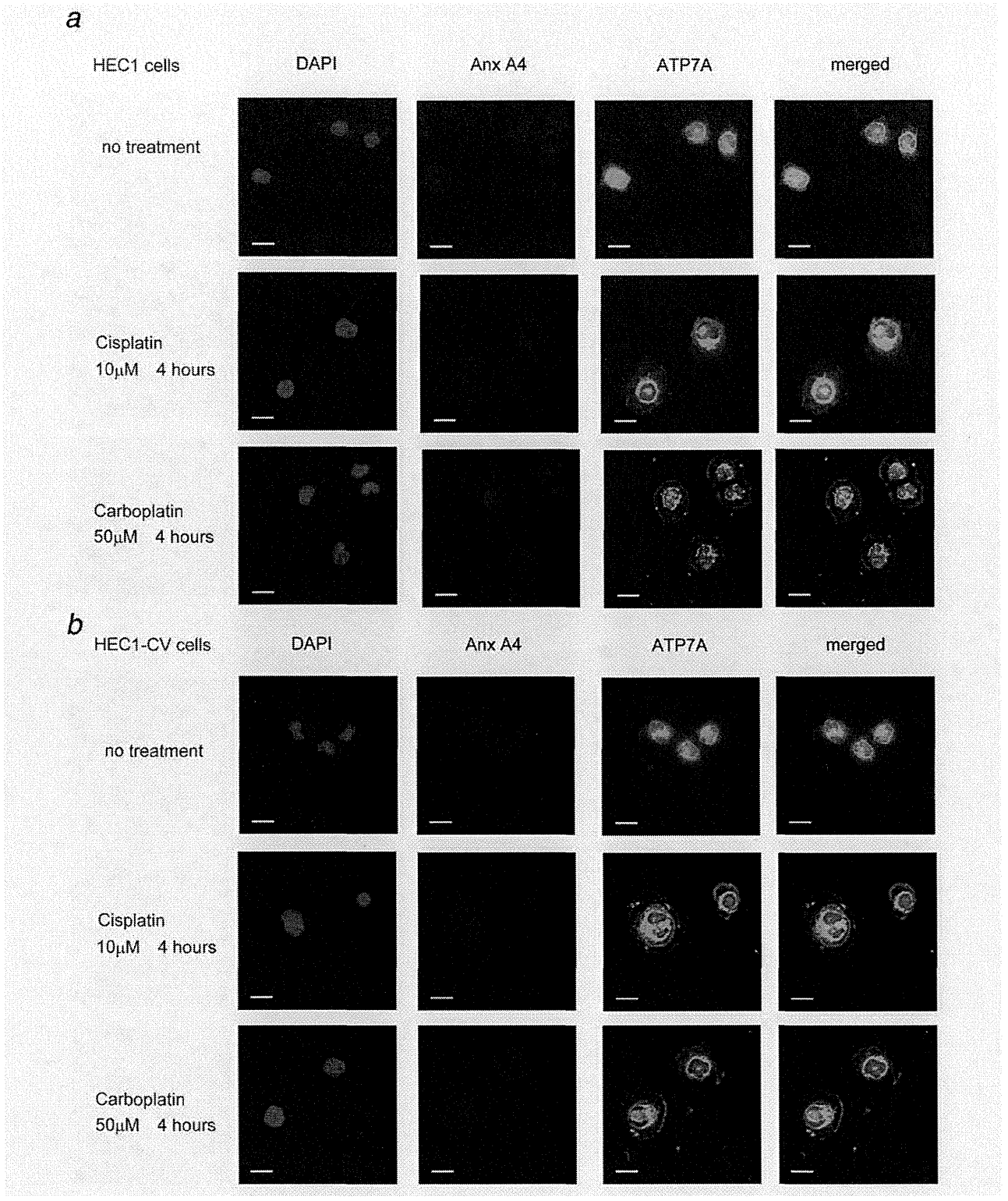

Figure 4. Immunofluorescence staining for ATP7A and Anx A4. HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were divided into three groups: the no treatment, cisplatin exposure and carboplatin exposure groups. (*a*) HEC1 cells, (*b*) HEC1-CV cells, (*c*) HEC1-A63 cells and (*d*) HEC1-A77 cells. Cells were incubated with anti-Anx A4 antibody (red) or anti-ATP7A antibody (green). Nuclei were stained with DAPI (blue). In the no treatment group for each cell, Anx A4 was localized in perinuclear and cytoplasmic regions and ATP7A was strongly localized in perinuclear regions. In HEC1 and HEC1-CV cells, after exposure to cisplatin or carboplatin, ATP7A was relocalized in the cellular membrane, although some ATP7A remained in the cytoplasm; however, no change in location of Anx A4 was observed. In HEC1-A63 and HEC1-A77 cells, Anx A4 and ATP7A were newly colocalized in the cellular membrane as well as remaining in the cytoplasm. In a comparison of HEC1 and HEC1-CV cells with HEC1-A63 and HEC1-A77 cells, expression of Anx A4 in HEC1-A63 and HEC1-A77 cells was stronger in the cytoplasm and cellular membrane. Scale bar = 30 μm.

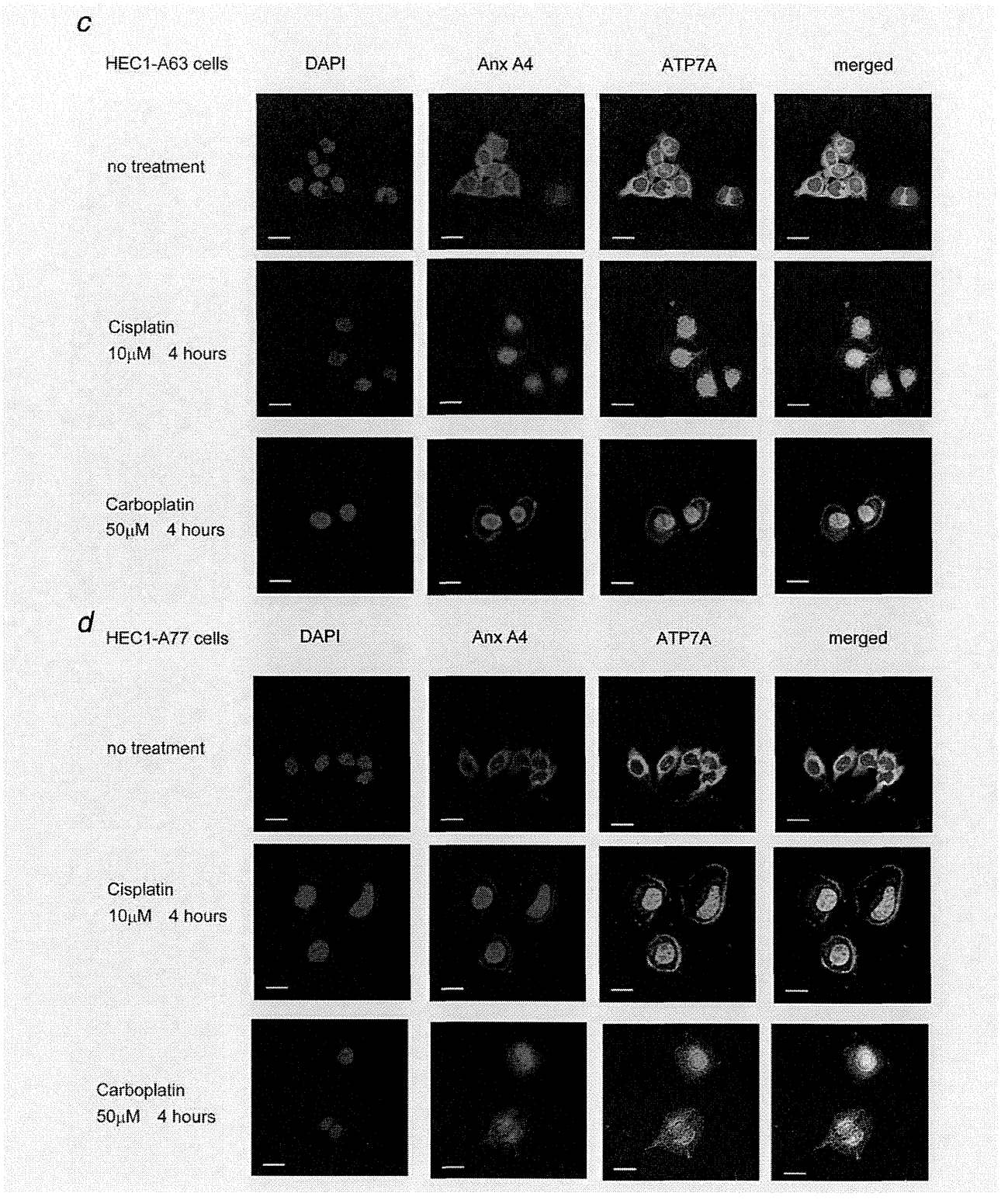

Figure 4. (Continued)

control and commercial siRNAs against ATP7A were transfected and the IC_{50} values of cisplatin and carboplatin were determined for each cell line. The IC_{50} value for cisplatin was significantly lower for the two kinds of ATP7A-silenced HEC1-A63 cells (ATP7A siRNA4, $IC_{50} = 11.0$ μM, $p < 0.01$; ATP7A siRNA6, $IC_{50} = 11.2$ μM, $p < 0.01$) compared with

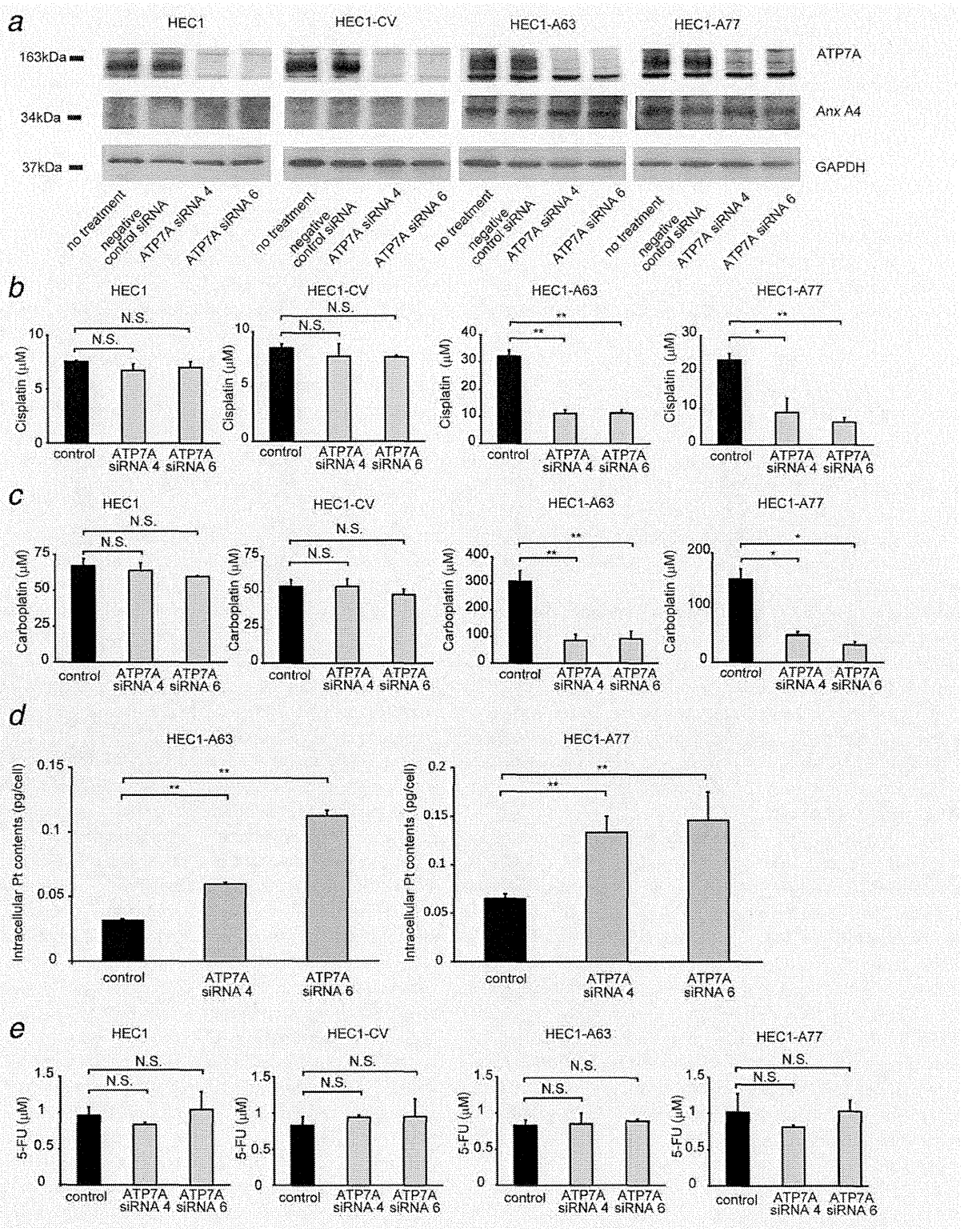

Figure 5. Knockdown of ATP7A expression improves platinum chemosensitivity in Anx A4-overexpressing cells. (*a*) Knockdown expression of ATP7A by siRNA in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells by Western blot analysis. (*b*) IC_{50} values are shown for cisplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells transfected with negative control siRNA and two types of siRNA targeting ATP7A. A significant decrease in IC_{50} value for cisplatin was observed for the two types of ATP7A-silenced HEC1-A63 and HEC1-A77 cells but not for the HEC1 and HEC1-CV cells. (*c*) IC_{50} values are shown for carboplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells transfected with negative control siRNA and two kinds of siRNA targeting ATP7A. A significant decrease in IC_{50} value for carboplatin was observed for the two types of ATP7A-silenced HEC1-A63 and HEC1-A77 cells but not for the HEC1 and HEC1-CV cells. (*d*) Intracellular platinum content after treatment with 1 mM cisplatin for 60 min and further incubation with cisplatin-free medium for 180 min in D-MEM medium in HEC1-A63 cells and HEC1-A77 cells transfected with negative control siRNA and ATP7A-targeting siRNA, as determined by ICP-MS analysis. Significantly higher intracellular platinum accumulation was observed in HEC1-A63 cells and HEC1-A77 ATP7A-silencing cells than in control siRNA-transfected HEC1-A63 cells and HEC1-A77 cells. (*e*) No significant differences in IC_{50} values for 5-FU were noted between HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells. Similar results were observed in ATP7A-silenced cell lines for HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells (*$p < 0.05$; **$p < 0.01$; one-way ANOVA followed by Dunnett's analysis).

the HEC1-A63 control siRNA-transfected cells ($IC_{50} = 32.2$ μM) (Fig. 5*b*).

In addition to cisplatin, improved chemosensitivity associated with ATP7A silencing was observed with carboplatin. Significantly lower IC_{50} values for carboplatin were observed in both types of ATP7A-silenced HEC1-A63 cells (siRNA4, $IC_{50} = 85.9$ μM, $p < 0.01$; siRNA6, $IC_{50} = 92.8$ μM, $p < 0.01$) compared with the HEC1-A63 control siRNA-transfected cells ($IC_{50} = 300.7$ μM) (Fig. 5*c*). Similar results were found for HEC1-A77 ATP7A-silenced cells, where a significantly lower IC_{50} value for cisplatin was observed (siRNA4, $IC_{50} = 8.9$ μM, $p < 0.05$; siRNA6, $IC_{50} = 6.2$ μM, $p < 0.01$) compared with that for HEC1-A77 control siRNA-transfected cells ($IC_{50} = 23.3$ μM). IC_{50} values for carboplatin were also significantly lower for the two kinds of ATP7A-silenced HEC1-A77 cells (siRNA4, $IC_{50} = 49.8$ μM, $p < 0.05$; siRNA6, $IC_{50} = 31.9$ μM, $p < 0.05$) compared with the HEC1-A77 control siRNA-transfected cells ($IC_{50} = 152.1$ μM, $p < 0.01$) (Fig. 5*c*). In contrast, siRNA treatments targeting ATP7A were ineffective in HEC1 and HEC1-CV cells treated with cisplatin or carboplatin (Figs. 5*b* and 5*c*). Intracellular platinum accumulation after cisplatin exposure was significantly increased in HEC1-A63 cells treated with ATP7A siRNA (0.060 pg/cell, $p < 0.01$ to 0.113 pg/cell, $p < 0.01$) compared with control siRNA-transfected cells (0.030 pg/cell) (Fig. 5*d*). Similarly, a significant increase in intracellular platinum accumulation was observed in HEC1-A77 cells treated with ATP7A siRNA (0.133 pg/cell, $p < 0.01$ to 0.146 pg/cell, $p < 0.01$) compared with control siRNA-transfected cells (0.065 pg/cell) (Fig. 5*d*).

To investigate the relationship between resistance to drugs other than platinum drugs and Anx A4 or ATP7A expression, IC_{50} values for 5-FU were determined for each cell line. No significant change in IC_{50} values for 5-FU was observed in HEC1 ($IC_{50} = 0.96$ μM), HEC1-CV ($IC_{50} = 1.00$ μM), HEC1-A63 ($IC_{50} = 0.83$ μM) or HEC1-A77 cells ($IC50 = 1.01$ μM) (Fig. 5*e*). Similar results were observed in the ATP7A-silenced cell lines for HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells as well as in ATP7A-silenced cell lines (Fig. 5*e*). These results suggested that platinum resistance induced by enforced expression of Anx A4 was mainly dependent on the platinum transporter ATP7A and that expression of Anx A4 and ATP7A was unrelated to resistance to 5-FU.

Discussion

In our study, overexpression of Anx A4 in HEC1 cells decreased cell sensitivity to platinum drugs *in vitro*. Increased drug efflux was the mechanism underlying this change. In addition, an association between Anx A4 and platinum resistance was demonstrated for the first time *in vivo*. The mechanism of Anx A4-induced drug efflux may prove to be a promising therapeutic target because blockage of that mechanism may improve the prognosis of patients with Anx A4-associated platinum-resistant tumors.

Anx A4 itself is not a drug transporter, but it does bind to phospholipids in a Ca^{2+}-dependent manner and self-associates onto phospholipid membrane surfaces, causing membrane aggregation.[12,14–17] Thus, we assumed an indirect mediating effect of Anx A4 on drug efflux through an association between an unidentified drug transporter and Anx A4. Recently, MRP2 (an ABC ATPase-like multidrug-resistant protein) and ATP7A and ATP7B (two P-type Cu-transporting ATPases) were identified as platinum efflux transporters strongly associated with platinum resistance.[32,33] In an analysis of clinical gynecological samples, expression of MRP2 failed to predict tumor response to chemotherapy and did not correlate with overall survival.[34–36] In contrast, poor survival rates were associated with overexpression of ATP7A in patients with ovarian cancer.[27] Similarly, a correlation was found between ATP7B overexpression in endometrial carcinomas and an unfavorable clinical outcome in patients treated with cisplatin-based chemotherapy.[37] Therefore, we focused on the platinum transporters ATP7A and ATP7B and investigated their relationships with expression of Anx A4. In normal, unchallenged cells, ATP7A and ATP7B are localized in the Golgi apparatus and are involved in copper homeostasis, using ATP hydrolysis to transport copper ions across cellular membranes. They function in both the export of excess copper and its delivery to copper-dependent enzymes. ATP7A and ATP7B are also known to be efflux transporters of platinum drugs.[8,27,28,31,38,39] In one study, only a slight increase in expression of transfected ATP7A was seen in a human ovarian cancer cell line; however, that small increase was sufficient to confer significant resistance to cisplatin or carboplatin.[40] In a similar study in another human cisplatin-resistant ovarian cancer cell line, silencing of ATP7B by siRNA transfection resulted in a 2.5-fold decrease in cisplatin IC_{50} levels and a significant increase in DNA-platinum adduct formation.[41] Preparing CMF of treated cells facilitated the localization of Anx A4 expression in cells before and after exposure to platinum drugs. The abundance of AnxA4 in the membrane fraction along with the translocation to the membrane was increased. Using the orthogonal method of cell surface protein labeling to monitor proteins appearing on the cell surface, biotinylated ATP7A was increased after cisplatin or carboplatin exposure both in HEC1 and HEC1-CV cells (cells expressing low levels of Anx A4) and HEC1-A63 and HEC1-A77 cells (cells overexpressing Anx A4). Taken together, these results suggest that platinum drug exposure causes relocalization of Anx A4 expression to the membrane fraction and relocalization of ATP7A transporters (to a minimum) to the external surface of the cellular membrane. Unfortunately, no similar analysis of ATP7B was possible because it is not expressed in HEC1 cells (data not shown). However, in cells that express both ATP7A and ATP7B proteins, other immunofluorescence studies have shown similar changes in localization of both proteins after cisplatin exposure.[42] After cisplatin or carboplatin exposure in HEC1-A63 and HEC1-A77 Anx

A4-overexpressing cells, immunofluorescence showed that Anx A4 expression was relocated from the perinuclear and cytoplasmic Golgi regions to the cellular membrane. This relocalization was not observed in HEC1 and HEC1-CV cells, in which overexpression of Anx A4 does not occur.

ATP7A also relocates from the perinuclear and cytoplasmic regions to the cellular membrane after cisplatin or carboplatin exposure. However, this occurs both in HEC1 and HEC1-CV cells (cells expressing low levels of Anx A4) and HEC1-A63 and HEC1-A77 cells (cells overexpressing Anx A4). Although no direct interaction between ATP7A and Anx A4 was detected by coimmunoprecipitation analysis (data not shown), immunofluorescence analysis showed colocalization of ATP7A and Anx A4 at least within the cellular membrane in Anx A4-overexpressing cells. These results suggested that Anx A4 is not required for ATP7A translocation and that ATP7A translocation is unrelated to expression of Anx A4.

Translocation of Anx A4 to plasma membranes is reportedly mediated by an increase in intracellular free Ca^{2+}, which is increased by exposure to platinum drugs.[43,44] In addition to the translocation of ATP7A and Anx A4 to the plasma membrane, our results also showed translocation of ATP7A to the nucleus in HEC1 and HEC1-CV cells. Translocation to the nucleus and colocalization of both ATP7A and Anx A4 were also observed in HEC1-A63 and HEC1-A77 cells after exposure to cisplatin or carboplatin in the immunofluorescence staining analysis in our study (Fig. 4). Anx A4 translocates to the nucleus after etoposide treatment and suppresses NF-κB transcriptional activity, which induces expression of Bax, a proapoptotic Bcl-2 family protein.[18] In addition, a correlation has been reported between nuclear staining of Anx A4 and poor survival in patients with ovarian cancer.[45] However, the role of ATP7A in the nucleus and its relationship with NF-κB transcriptional activity has not been investigated. Further investigation is needed to elucidate the role of nuclear colocalization of Anx A4 and ATP7A in platinum resistance.

In our study, translational silencing of ATP7A in HEC1 and HEC1-CV (Anx A4-nonexpressing cells) and HEC1-A63 and HEC1-A77 cells (Anx A4-overexpressing cells) was performed. Western blot analysis demonstrated no detectable changes in protein expression of Anx A4 when ATP7A was silenced in any of these four cell lines.

In HEC1 and control HEC1-CV cells (low Anx A4 expression levels), IC_{50} values for cisplatin or carboplatin cells after the knockdown of ATP7A expression caused no improvement in the sensitivity of these cells to cisplatin or carboplatin. Similar results were observed in a previous study in which no improvement in sensitivity to cisplatin resulted from silencing of ATP7A in platinum-resistant or -sensitive ovarian cancer cell lines.[41] However, Mangala *et al.* reported improved sensitivity to cisplatin in both platinum-resistant ovarian cancer cells and parental cells expressing ATP7B as a result of silencing of ATP7B expression.[41] An important discovery related to ATP7A was communicated in our study: in cells overexpressing both Anx A4 and ATP7A, silencing of ATP7A significantly improved sensitivity to cisplatin and carboplatin, thus restoring them to sensitivity levels comparable to those of HEC1 and HEC1-CV cells. These results were supported by a quantitative analysis of the accumulation of intracellular platinum, demonstrating that siRNA silencing of ATP7A in Anx A4-overexpressing HEC1-A63 and HEC1-A77 cells resulted in greater intracellular platinum accumulation than HEC1-A63 and HEC1-A77 cells transfected with a control siRNA. On the other hand, the analysis of IC_{50} values for 5-FU showed no relationship between overexpression of Anx A4 and resistance to 5-FU. In addition, no improvement in sensitivity to 5-FU was observed as a result of ATP7A silencing. These results suggested a specific relationship of Anx A4 with ATP7A and resistance to platinum drugs but with to nonplatinum drugs such as 5-FU. Differences in efficacy and improvement in drug sensitivity of ATP7A silencing were observed between cell lines (HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells). These variations may be related to the colocalization of Anx A4 and ATP7A in the cellular membrane after cisplatin or carboplatin exposure. Colocalization of Anx A4 and ATP7A after exposure to platinum drugs was specific to changes in Anx A4-overexpressing cells, which are probably related to drug efflux. These results suggest that in conjunction with higher Anx A4 expression levels, ATP7A had a positive effect on efflux of platinum drugs, resulting in significantly increased platinum resistance. Because overexpression of Anx A4 had no effect on ATP7A expression and because no direct interaction between ATP7A and Anx A4 was detected in the coimmunoprecipitation analysis, Anx A4 seems to promote ATP7A activity in a manner which is currently unexplained.

In addition to the effects of Anx A4 on drug resistance in ovarian cancer, similar findings have been reported for other overexpressed members of the Annexin family such as Annexin A3 (Anx A3).[46,47] Intracellular platinum concentrations of cisplatin and levels of platinum DNA binding in that study were significantly lower in Anx A3-overexpressing cells than in control cells, suggesting a more general involvement of the Annexin family in platinum resistance.[46] From the results of these related reports and those of our study, we conclude that the Annexin family may potentially enhance the activity of numerous drug transporters. Identifying these enhancement mechanisms may be extremely useful for developing additional therapeutic targets for drug-resistant tumors.

In summary, our study demonstrated that enhanced expression of Anx A4 induces chemoresistance by promoting platinum drug efflux *via* ATP7A. These findings suggested that Anx A4 is a potential therapeutic target for chemosensitization, particularly in tumors with higher expression of both Anx A4 and ATP7A. Thus, our study provides a clear example of applied genotoxicology. However, platinum resistance induced by overexpression of Anx A4 may occur as a

result of multiple processes, including regulation of apoptosis and efflux of platinum drugs. Thus, other unknown chemoresistant mechanisms may be induced by overexpression of Anx A4. Because overexpression of Anx A4 has been reported in several other types of clinically important cancers, such as rectal, renal, lung and pancreatic cancer,[19–23] targeting Anx A4 may lead to the development of an effective therapy for overcoming chemoresistance in more types of cancer.

Acknowledgements

The authors thank Y. Kanazawa and S. Sugiyama for their secretarial assistance, M. Urase for technical assistance and Dr. G.S. Buzard for helpful editing.

References

1. Omura G, Blessing JA, Ehrlich CE, et al. A randomized trial of cyclophosphamide and doxorubicin with or without cisplatin in advanced ovarian carcinoma. *A Gynecologic Oncology Group Study. Cancer* 1986;57:1725–30.
2. Thigpen T, Vance R, Puneky L, et al. Chemotherapy in advanced ovarian carcinoma: current standards of care based on randomized trials. *Gynecol Oncol* 1994;55:S97–S107.
3. Vaughan S, Coward JI, Bast RC, Jr, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer* 2011;11:719–25.
4. Fleming GF, Brunetto VL, Cella D, et al. Phase III trial of doxorubicin plus cisplatin with or without paclitaxel plus filgrastim in advanced endometrial carcinoma: a Gynecologic Oncology Group Study. *J Clin Oncol* 2004;22:2159–66.
5. Hoskins PJ, Swenerton KD, Pike JA, et al. Paclitaxel and carboplatin, alone or with irradiation, in advanced or recurrent endometrial cancer: a phase II study. *J Clin Oncol* 2001;19:4048–53.
6. Obel JC, Friberg G, Fleming GF. Chemotherapy in endometrial cancer. *Clin Adv Hematol Oncol* 2006;4:459–68.
7. Enomoto T, Kuragaki C, Yamasaki M, et al. Is clear cell carcinoma and mucinous carcinoma of the ovary sensitive to combination chemotherapy with paclitaxel and carboplatin? *Proc Am Soc Clin Oncol* 2003;22:(abstr 1797).
8. Nakayama K, Kanzaki A, Terada K, et al. Prognostic value of the Cu-transporting ATPase in ovarian carcinoma patients receiving cisplatin-based chemotherapy. *Clin Cancer Res* 2004;10:2804–11.
9. Pectasides D, Fountzilas G, Aravantinos G, et al. Advanced stage clear-cell epithelial ovarian cancer: the Hellenic Cooperative Oncology Group experience. *Gynecol Oncol* 2006;102:285–91.
10. Goff BA, Sainz de la Cuesta R, Muntz HG, et al. Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. *Gynecol Oncol* 1996;60:412–17.
11. Sugiyama T, Kamura T, Kigawa J, et al. Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. *Cancer* 2000;88:2584–9.
12. Kim A, Enomoto T, Serada S, et al. Enhanced expression of Annexin A4 in clear cell carcinoma of the ovary and its association with chemoresistance to carboplatin. *Int J Cancer* 2009;125:2316–22.
13. Miao Y, Cai B, Liu L, et al. Annexin IV is differentially expressed in clear cell carcinoma of the ovary. *Int J Gynecol Cancer* 2009;19:1545–9.
14. Gerke V, Moss SE. Annexins: from structure to function. *Physiol Rev* 2002;82:331–71.
15. Kaetzel MA, Hazarika P, Dedman JR. Differential tissue expression of three 35-kDa annexin calcium-dependent phospholipid-binding proteins. *J Biol Chem* 1989;264:14463–70.
16. Kaetzel MA, Mo YD, Mealy TR, et al. Phosphorylation mutants elucidate the mechanism of annexin IV-mediated membrane aggregation. *Biochemistry* 2001;40:4192–9.
17. Kim A, Serada S, Enomoto T, et al. Targeting annexin A4 to counteract chemoresistance in clear cell carcinoma of the ovary. *Expert Opin Ther Targets* 2010;14:963–71.
18. Jeon YJ, Kim DH, Jung H, et al. Annexin A4 interacts with the NF-kappaB p50 subunit and modulates NF-kappaB transcriptional activity in a Ca2+-dependent manner. *Cell Mol Life Sci* 2010;67:2271–81.
19. Alfonso P, Canamero M, Fernandez-Carbonie F, et al. Proteome analysis of membrane fractions in colorectal carcinomas by using 2D-DIGE saturation labeling. *J Proteome Res* 2008;7:4247–55.
20. Duncan R, Carpenter B, Main LC, et al. Characterisation and protein expression profiling of annexins in colorectal cancer. *Br J Cancer* 2008;98:426–33.
21. Sitek B, Luttges J, Marcus K, et al. Application of fluorescence difference gel electrophoresis saturation labelling for the analysis of microdissected precursor lesions of pancreatic ductal adenocarcinoma. *Proteomics* 2005;5:2665–79.
22. Zimmermann U, Balabanov S, Giebel J, et al. Increased expression and altered location of annexin IV in renal clear cell carcinoma: a possible role in tumour dissemination. *Cancer Lett* 2004;209:111–18.
23. Wei R, Zhang Y, Shen L, et al. Comparative proteomic and radiobiological analyses in human lung adenocarcinoma cells. *Mol Cell Biochem* 2012;359:151–9.
24. Furukawa T, Komatsu M, Ikeda R, et al. Copper transport systems are involved in multidrug resistance and drug transport. *Curr Med Chem* 2008;15:3268–78.
25. Gourdon P, Liu XY, Skjorringe T, et al. Crystal structure of a copper-transporting PIB-type ATPase. *Nature* 2011;475:59–64.
26. Owatari S, Akune S, Komatsu M, et al. Copper-transporting P-type ATPase, ATP7A, confers multidrug resistance and its expression is related to resistance to SN-38 in clinical colon cancer. *Cancer Res* 2007;67:4860–8.
27. Samimi G, Varki NM, Wilczynski S, et al. Increase in expression of the copper transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients. *Clin Cancer Res* 2003;9:5853–9.
28. Safaei R, Holzer AK, Katano K, et al. The role of copper transporters in the development of resistance to Pt drugs. *J Inorg Biochem* 2004;98:1607–13.
29. Iwahori K, Serada S, Fujimoto M, et al. SOCS-1 gene delivery cooperates with cisplatin plus pemetrexed to exhibit preclinical antitumor activity against malignant pleural mesothelioma. *Int J Cancer* 2013;132:459–71.
30. Khunweeraphong N, Nagamori S, Wiriyasermkul P, et al. Establishment of stable cell lines with high expression of heterodimers of human 4F2hc and human amino acid transporter LAT1 or LAT2 and delineation of their differential interaction with (alpha)-alkyl moieties. *J Pharmacol Sci* 2012;119:368–80.
31. Rabik CA, Maryon EB, Kasza K, et al. Role of copper transporters in resistance to platinating agents. *Cancer Chemother Pharmacol* 2009;64:133–42.
32. Galluzzi L, Senovilla L, Vitale I, et al. Molecular mechanisms of cisplatin resistance. *Oncogene* 2012;31:1869–83.
33. Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* 2007;7:573–84.
34. Arts HJ, Katsaros D, de Vries EG, et al. Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. *Clin Cancer Res* 1999;5:2798–805.
35. Guminski AD, Balleine RL, Chiew YE, et al. MRP2 (ABCC2) and cisplatin sensitivity in hepatocytes and human ovarian carcinoma. *Gynecol Oncol* 2006;100:239–46.
36. Materna V, Pleger J, Hoffmann U, et al. RNA expression of MDR1/P-glycoprotein, DNA-topoisomerase I, and MRP2 in ovarian carcinoma patients: correlation with chemotherapeutic response. *Gynecol Oncol* 2004;94:152–60.
37. Aida T, Takebayashi Y, Shimizu T, et al. Expression of copper-transporting P-type adenosine triphosphatase (ATP7B) as a prognostic factor in human endometrial carcinoma. *Gynecol Oncol* 2005;97:41–5.
38. Katano K, Kondo A, Safaei R, et al. Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. *Cancer Res* 2002;62:6559–65.
39. Kuo MT, Chen HH, Song IS, et al. The roles of copper transporters in cisplatin resistance. *Cancer Metastasis Rev* 2007;26:71–83.
40. Samimi G, Safaei R, Katano K, et al. Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells. *Clin Cancer Res* 2004;10:4661–9.
41. Mangala LS, Zuzel V, Schmandt R, et al. Therapeutic targeting of ATP7B in ovarian carcinoma. *Clin Cancer Res* 2009;15:3770–80.
42. Kalayda GV, Wagner CH, Buss I, et al. Altered localisation of the copper efflux transporters ATP7A and ATP7B associated with cisplatin

resistance in human ovarian carcinoma cells. *BMC Cancer* 2008;8:175.
43. Al-Bahlani S, Fraser M, Wong AY, et al. P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. *Oncogene* 2011;30:4219–30.
44. Splettstoesser F, Florea AM, Busselberg D. IP(3) receptor antagonist, 2-APB, attenuates cisplatin induced Ca2+-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis. *Br J Pharmacol* 2007;151: 1176–86.
45. Choi CH, Sung CO, Kim HJ, et al. Overexpression of annexin A4 is associated with chemoresistance in papillary serous adenocarcinoma of the ovary. *Hum Pathol* 2013;44:1017–23.
46. Yan X, Yin J, Yao H, et al. Increased expression of annexin A3 is a mechanism of platinum resistance in ovarian cancer. *Cancer Res* 2010;70:1616–24.
47. Yin J, Yan X, Yao X, et al. Secretion of annexin A3 from ovarian cancer cells and its association with platinum resistance in ovarian cancer patients. *J Cell Mol Med* 2012;16:337–48.

Cancer Science
The official journal of the Japanese Cancer Association JCA

Molecular mechanism underlying the antiproliferative effect of suppressor of cytokine signaling-1 in non-small-cell lung cancer cells

Kazuki Shimada,[1,2] Satoshi Serada,[1] Minoru Fujimoto,[1] Shintaro Nomura,[3] Rie Nakatsuka,[4] Emi Harada,[1] Kota Iwahori,[1,2] Isao Tachibana,[2] Tsuyoshi Takahashi,[4] Atsushi Kumanogoh,[2] Tadamitsu Kishimoto[5] and Tetsuji Naka[1,6]

[1]Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka; [2]Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, Osaka; [3]Faculty of Animal Bioscience, Nagahama Institute of Bio-Science and Technology, Shiga; [4]Department of Surgery, Osaka University Graduate School of Medicine, Osaka; [5]Laboratory of Immune Regulation, Osaka University Graduate School of Frontier Biosciences, Osaka, Japan

(Received March 21, 2013/Revised July 28, 2013/Accepted August 12, 2013/Accepted manuscript online August 21, 2013/Article first published online September 19, 2013)

Lung cancer (LC) is the major cause of death by cancer and the number of LC patients is increasing worldwide. This study investigated the therapeutic potential of gene delivery using suppressor of cytokine signaling 1 (SOCS-1), an endogenous inhibitor of intracellular signaling pathways, for the treatment of LC. To examine the antitumor effect of SOCS-1 overexpression on non-small-cell lung cancer (NSCLC) cells, NSCLC cells (A549, LU65, and PC9) were infected with adenovirus-expressing SOCS-1 vector. The cell proliferation assay showed that A549 and LU65, but not PC9, were sensitive to *SOCS-1* gene-mediated suppression of cell growth. Although JAK inhibitor I could also inhibit proliferation of A549 and LU65 cells, *SOCS-1* gene delivery appeared to be more potent as SOCS-1 could suppress focal adhesion kinase and epidermal growth factor receptor, as well as the JAK/STAT3 signaling pathway. Enhanced phosphorylation of the p53 protein was detected by means of phospho-kinase array in SOCS-1 overexpressed A549 cells compared with control cells, whereas no phosphorylation of p53 was observed when JAK inhibitor I was used. Furthermore, treatment with adenoviral vector AdSOCS-1 *in vivo* significantly suppressed NSCLC proliferation in a xenograft model. These results suggest that the overexpression of *SOCS-1* gene is effective for antitumor therapy by suppressing the JAK/STAT, focal adhesion kinase, and epidermal growth factor receptor signaling pathways and enhancing p53-mediated antitumor activity in NSCLC. (*Cancer Sci* 2013; 104: 1483–1491)

Lung cancer is the leading cause of cancer death in Japan and is a growing health epidemic worldwide.[1] Moreover, therapies that can cure metastatic LC have not been yet established,[2,3] so there is an urgent need for the development of novel interventions to cure LC.

One of the potential therapeutic targets of NSCLC is STAT3. Constitutively activated STAT3 has been shown to promote tumor cell growth, survival, and tumor angiogenesis, and persistently activated STAT3 has been found in 50% of lung adenocarcinomas.[4] It is thought that STAT3 is activated by JAK, EGFR, or Src family kinases.[5] Among these TYKs, JAK family kinases play an important role in the phosphorylation of STAT3 in NSCLC.[6,7] Dysregulated activation of the JAK/STAT3 signaling pathway, the major downstream pathway of cytokines such as interleukin-6, has been detected in various cancers including NSCLC.[8] Moreover, it has been recently reported that ruxolitinib, which is a potent and selective JAK1 and JAK2 inhibitor, is associated with marked and durable clinical benefits for patients with myelofibrosis, suggesting that JAK kinases are promising therapeutic targets for cancer.[9]

Cytokine signaling pathways are tightly controlled by negative regulatory mechanisms under homeostatic conditions. Suppressors of cytokine signaling family proteins play a role in the negative regulation of cytokine responses by terminating the activation of the JAK/STAT and other signaling pathways.[10–12]

The SOCS family, characterized by a central src homology 2 domain and a conserved C-terminus SOCS box, is composed of eight structurally related proteins.[13] Of these, SOCS-1 is known as the most potent negative regulator of pro-inflammatory cytokine signaling.[14] It interacts with phosphotyrosine residues on proteins such as JAK kinases to interfere with the activation of STAT proteins or other signaling intermediates.[15,16] Also, SOCS-1 recruits the Elongin BC-containing E3 ubiquitin-ligase complex through the conserved SOCS box to promote the degradation of target proteins.[17] Studies of SOCS-1 deficient mice have indicated that SOCS-1 is essential for the inhibition of excessive immune responses and is also involved in the suppression of tumor development.[18,19]

Although it is still not clear whether SOCS shows therapeutic benefit for NSCLC, preclinical analyses of SOCS in therapies of several types of cancers have been carried out worldwide.[20] Previously reported studies by us showed that SOCS-1 or SOCS-3 were effective when used for therapies of malignant pleural mesothelioma or gastric cancer.[21–23] In addition, it has been reported that overexpression of the *SOCS-3* gene showed antitumor effects in NSCLC.[24–26]

In the study presented here, we used the NSCLC cell lines A549, LU65, and PC9 to investigate the possibility of the application of *SOCS-1* gene transduction to NSCLC therapies and the mechanisms of antitumor effects by SOCS-1.

Material and Methods

Cell lines. Both A549 and LU65 cell lines were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). The PC9 cell line was kindly provided by Prof. Nishio of Kinki University of Medicine, Department of Genome Biology (Osaka, Japan). Details are described in Data S1.

Reagents. PD153035 and JAK inhibitor I were purchased from EMD Millipore (Billerica, MA, USA) and Calbiochem (La Jolla, CA, USA), respectively.

[6]To whom correspondence should be addressed.
E-mail: tnaka@nibio.go.jp

doi: 10.1111/cas.12266

Preparation of adenoviruses. The replication-defective recombinant adenoviral vector expressing the mouse *SOCS-1* gene was provided by Dr. Hiroyuki Mizuguchi (Osaka University, Osaka, Japan), which was constructed with an improved *in vitro* ligation method, as described previously.[27,28] An adenoviral vector expressing the *LacZ* gene was constructed using a similar method and expression of these genes was regulated by means of a CMV promoter/enhancer and intron A. Details are described in Data S1.

Phospho-kinase array. Expression of phosphorylated proteins was detected with the Proteome Profiler Human Phospho-Kinase Array kit (R&D Systems, Minneapolis, MN, USA). Details are described in Data S1.

Cell viability assay. The NSCLC cell lines were plated in 96-well plates at a density of 1×10^3 cells per well and incubated in RPMI-1640 medium containing 10% FCS. Details are described in Data S1.

SDS-PAGE and Western blot analysis. Whole cell protein extracts were prepared from NSCLC cells and tumor tissue in RIPA buffer containing phosphatase inhibitor cocktail and a protease inhibitor cocktail (both from Nacalai Tesque, Kyoto, Japan and both at a concentration of 1×) followed by centrifugation (16 100*g*, 4°C, 15 min). Details are described in Data S1.

Small interfering RNA transfection. Commercial FAK siRNA was obtained from Qiagen (Hilden, Germany). Details are described in Data S1.

Mouse xenograft model. All animal experiments were carried out according to the institutional ethical guidelines for animal experimentation of the National Institute of Biomedical Innovation (Osaka, Japan). Details are described in Data S1.

Immunohistochemistry. Subcutaneously implanted tumors were harvested and paraffin embedded for immunohistochemical analysis using anti-SOCS-1 antibody (Abcam, Cambridge, MA, USA) and anti-Ki-67 antibody (Novocastra Laboratories, Newcastle, UK). A TUNEL assay (with DAPI nuclear counterstaining) for apoptosis was carried out using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions.

Statistical analysis. Data are shown the mean ± SD (unless stated otherwise) for the number of experiments indicated. Details are described in Data S1.

Results

***SOCS-1* gene delivery shows marked antiproliferative effects in A549 and LU65 cells.** We used A549, LU65, and PC9 cell lines, which have been used as NSCLC cell lines in many other experiments. Because the JAK/STAT3 pathway has recently received attention as a novel target for treatment of NSCLC, we investigated the expression levels of JAK family kinases and of STAT3, as well as tyrosine phosphorylation of STAT3 in NSCLC cells. The four known mammalian members of the JAK family are TYK2, JAK1, JAK2, and JAK3. These kinases are widely expressed in a variety of different cell types, with the exception of JAK3, which is selectively expressed in cells of hematopoietic origin.[29] The expression of each JAK was at a similar level in the three cell lines (Fig. 1a). Although phosphorylated STAT3 was detectable in all cell lines, it was markedly more elevated in A549 and LU65 (Fig. 1b).

We next investigated whether SOCS-1 could suppress proliferation of A549, LU65, and PC9. Following *SOCS-1* gene delivery, overexpression of SOCS-1 was detected in all cell lines by Western blot analysis (Fig. 1c), but overexpression of SOCS-1 had antiproliferative effects on A549 and LU65 but not on PC9 (Fig. 1d).

Overexpression of SOCS-1 shows stronger antiproliferative effect than JAK inhibitor in NSCLC cells. Immunoblotting analysis showed that the expression levels of some of the JAK family kinases in all cell lines were reduced by overexpression of SOCS-1 (Fig. 2a). Next, phosphorylation levels of STAT3 (Tyr705), a downstream molecule of the JAK kinase family, were analyzed by Western blot analysis. Phosphorylation levels of STAT3 (Tyr705) were decreased in response to overexpression of the *SOCS-1* gene in all cell lines (Fig. 2b). These results agree with previously reported findings that SOCS-1 can act directly on JAK family kinases to suppress their kinase activity as well as to accelerate their degradation by the recruitment of an E3 ubiquitin ligase.[17]

Although the JAK/STAT3 pathway was downregulated by SOCS-1 in all cell lines, overexpression of the *SOCS-1* gene showed an antiproliferative effect only on A549 and LU65 cells. Accordingly, we next used JAK inhibitor I, a blocker of JAK1, JAK2, and JAK3, instead of AdSOCS-1 to examine the effects of JAK inhibition on all cell lines. Proliferation of A549 and LU65 cells was significantly suppressed by treatment with JAK inhibitor I, however, proliferation of PC9 was not significantly suppressed (Fig. 2c). Immunoblotting analysis showed that tyrosine phosphorylation of STAT3 was downregulated in all cell lines after introduction of JAK inhibitor I (Fig. 2d). These findings suggest that JAK-mediated signals are critical for the proliferation of A549 and LU65 cells, but are not needed for that of PC9 cells. In addition, because JAK1 and JAK2 are overlapping target molecules between SOCS-1 and JAK inhibitor I, the primary target molecules of SOCS-1 in NSCLC cells are likely to be JAK1 and/or JAK2. Therefore, we assumed that the antiproliferative effect of SOCS-1 was essentially determined by the JAK-dependence of NSCLC cells.

Immunoblotting analysis showed that introduction of both the *SOCS-1* gene (at 40 MOI) and of JAK inhibitor I (5 μM) could effectively suppress STAT3 phosphorylation (Fig. 2b,d). However, SOCS-1 was more effective than JAK inhibitor I for inhibiting proliferation of A549 and LU65 cells (Fig. 2e). Because SOCS-1 is known as an adaptor of several molecules other than JAK family kinases,[19] it was expected that AdSOCS-1 could also exert JAK-independent action in A549 and LU65. To investigate in detail the mechanisms of antitumor effects induced by overexpression of SOCS-1, we used A549 cells for further analyses.

Focal adhesion kinase is downregulated by overexpression of SOCS-1 in A549. Focal adhesion kinase is known as an adhesion molecule and previous investigations have indicated that FAK contributes to tumor cell proliferation, survival, and metastasis.[30] SOCS-1 was found to inhibit FAK-dependent signaling events by suppressing FAK-associated kinase activity and tyrosine phosphorylation of FAK,[31] and also by promoting polyubiquitination and degradation of FAK in a SOCS box-dependent manner.[17] For this reason, we next focused on the FAK pathway and examined the levels of FAK expression and of FAK tyrosine phosphorylation after overexpression of the *SOCS-1* gene.

Focal adhesion kinase expression was upregulated by introduction of the *LacZ* gene in A549 cells (Fig. 3a), probably reflecting the previously reported non-specific stimulation by adenovirus vectors.[32] Compared to the cells introduced by *LacZ*, FAK phosphorylation (Tyr397) was downregulated after the introduction of the *SOCS-1* gene in A549 (Fig. 3a). We also investigated whether JAK inhibitor I, instead of SOCS-1, can suppress activation of FAK in A549 cells, and found that JAK inhibitor could not (Fig. 3b).

We assumed that downregulation of FAK by SOCS-1 could contribute to the inhibition of A549 cell proliferation. Therefore, examined whether FAK siRNA indeed had an antiproliferative effect on A549, and found that proliferation of A549 cells was suppressed by FAK knockdown, indicating that these cells are dependent on FAK (Fig. 3c,d). We also investigated

doi: 10.1111/cas.12266

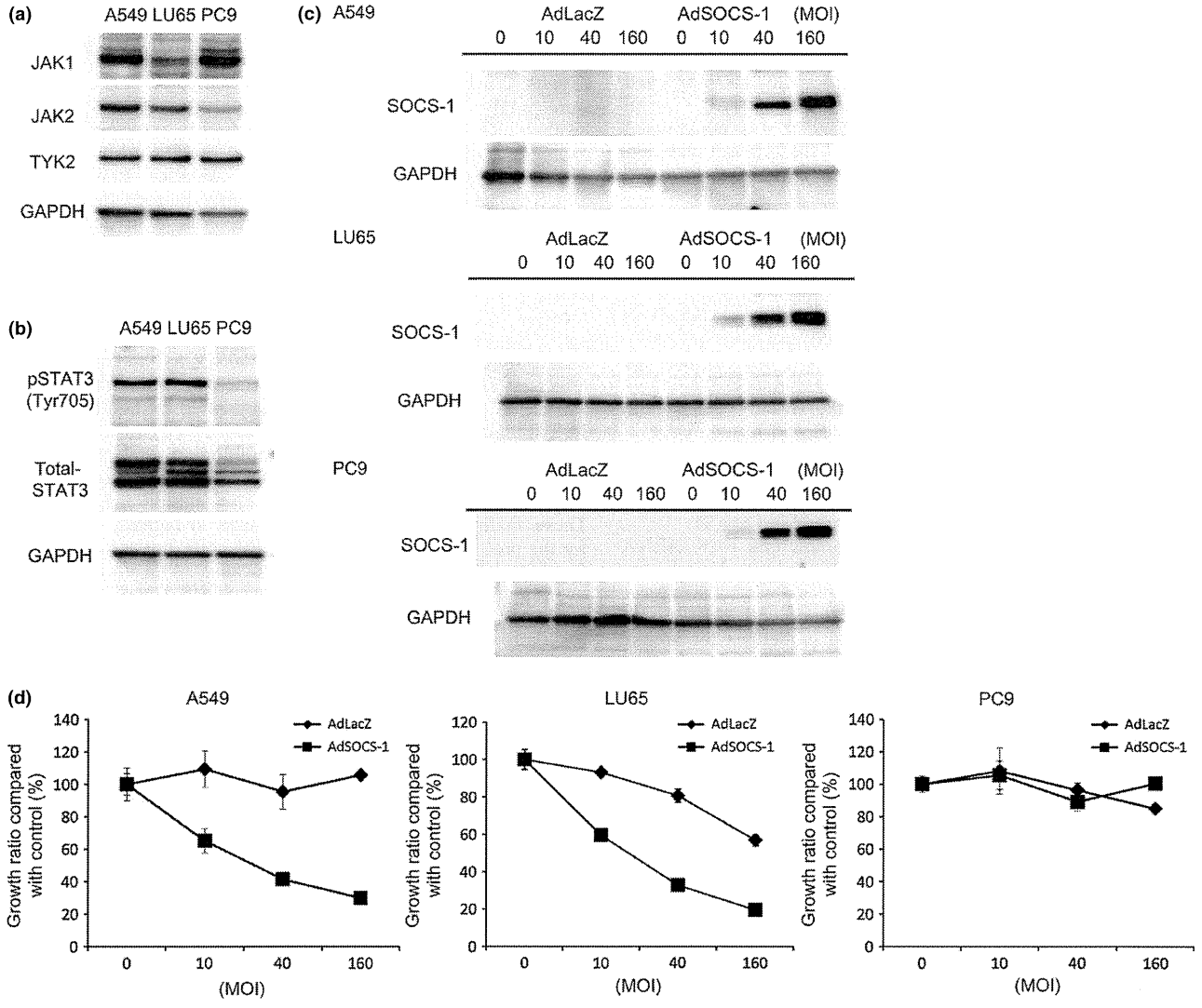

Fig. 1. Delivery of *SOCS-1* gene shows marked antiproliferative effects in A549 and LU65 lung cancer cell lines. (a) Expression of JAK family kinases in all cell lines determined by Western blotting. Cell lysates were immunoblotted with JAK1, JAK2, or tyrosine kinase 2 (TYK2) antibodies. (b) Comparative analysis of phosphorylation and expression of signal transducer and activator of transcription 3 (STAT3) determined by Western blotting. (c) Cell lysates were prepared 48 h after treatment with AdSOCS-1or AdLacZ adenoviral vectors at an MOI of 10–160, and immunoblotted with anti-SOCS-1 antibody. (d) Cells were infected with AdSOCS-1 or AdLacZ at an MOI of 10–160. Cell proliferation was determined by MTS assay 72 h after transfection. Growth ratio of AdLacZ or AdSOCS-1-infected cells was calculated by a percentage of that for non-treated cells. Each value represents the average ± SD of hexaplicate wells.

the combined effect of JAK inhibitor I and FAK siRNA on A549 cell proliferation. The results showed that the combined effect was stronger than that of JAK inhibitor I alone (Fig. 3e). These findings suggest that FAK has an important role in A549 cell proliferation independent of JAK and that SOCS-1-mediated FAK inhibition might contribute to the suppression of proliferation of A549 cells treated with AdSOCS-1.

Epidermal growth factor receptor is downregulated by overexpression of SOCS-1 in A549. Expression of SOCS-1 reportedly interacts with the cytoplasmic domain of EGFR and is likely to induce ubiquitination and degradation of ligand-bound EGFR.[33] This notion was confirmed by means of immunoblotting analysis in our study, in that EGFR expression was downregulated 48 h after introduction of the *SOCS-1* gene in A549 cells. The EGFR phosphorylation (Tyr1068) was also downregulated after overexpression of the *SOCS-1* gene had occurred in A549 cells (Fig. 4a). In contrast, JAK inhibitor I did not suppress EGFR activation in A549 cells (Fig. 4b).

We next examined the antiproliferative effects of the EGFR inhibitor PD153035 on A549 cells. Expression of EGFR in A549 cells was much lower than that in PC9 cells (data not shown), which harbor a deletion of an EGFR mutation.[34] Nevertheless, our findings show that proliferation of A549 cells with wild-type EGFR were also somewhat dependent on EGFR (Fig. 4c,d). We also investigated the combined effect of JAK inhibitor I and PD153035 on proliferation of A549 cells and found that they have an additive effect (Fig. 4e). This finding suggests that EGFR is also involved in A549 cell proliferation independent of JAK and that its downregulation by SOCS-1 may have an inhibitory effect on proliferation of A549 cells.

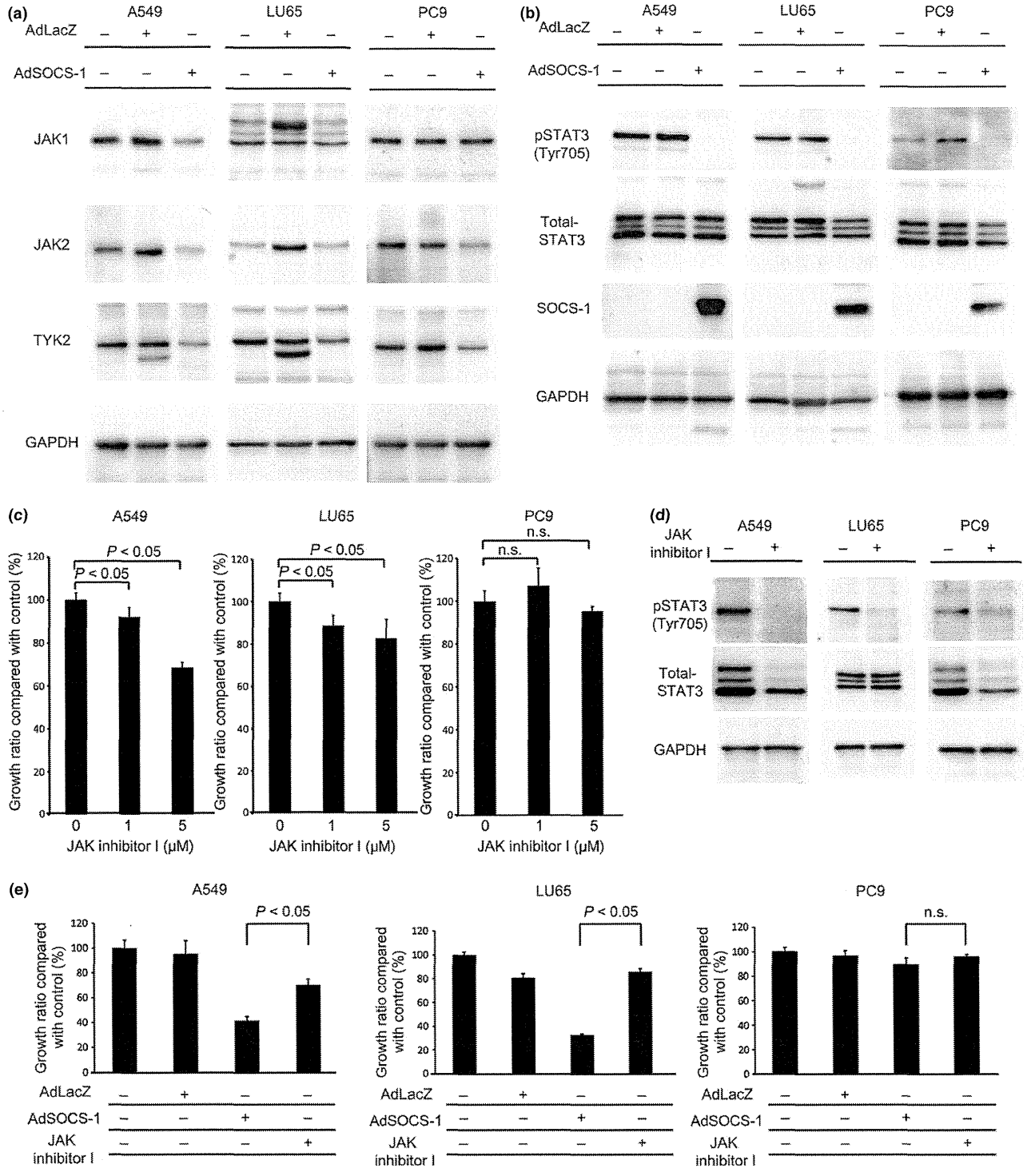

Fig. 2. Antiproliferative effect of suppressor of cytokine signaling-1 (SOCS-1) correlated with JAK dependence is stronger than JAK inhibitor. (a) Cell lysates were prepared 48 h after treatment with AdSOCS-1 or AdLacZ adenoviral vectors at an MOI of 40 and immunoblotted with JAK1, JAK2, or tyrosine kinase 2 (TYK2) antibodies. (b) SOCS-1 suppressed activation of signal transducer and activator of transcription 3 (STAT3). Cell lysates were prepared as described above and immunoblotted with anti-p-STAT3 (Tyr705), anti-STAT3, and anti-SOCS-1 antibodies. (c) Cells cultured in RPMI-1640 medium containing 10% FBS were exposed to 1–5 μM JAK inhibitor I. After incubation for 72 h, viable cells were counted with the MTS assay. (d) Cells were cultured in RPMI-1640 medium containing 0.5% FBS with 5 μM JAK inhibitor I. Cell lysates were immunoblotted with anti-p-STAT3 (Tyr705) and anti-STAT3 antibodies. (e) All cell lines cultured in RPMI-1640 medium containing 10% FBS were exposed to 5 μM JAK inhibitor I, or infected with AdLacZ or AdSOCS-1 at an MOI of 40. After incubation for 72 h, viable cells were counted with the MTS assay.

doi: 10.1111/cas.12266

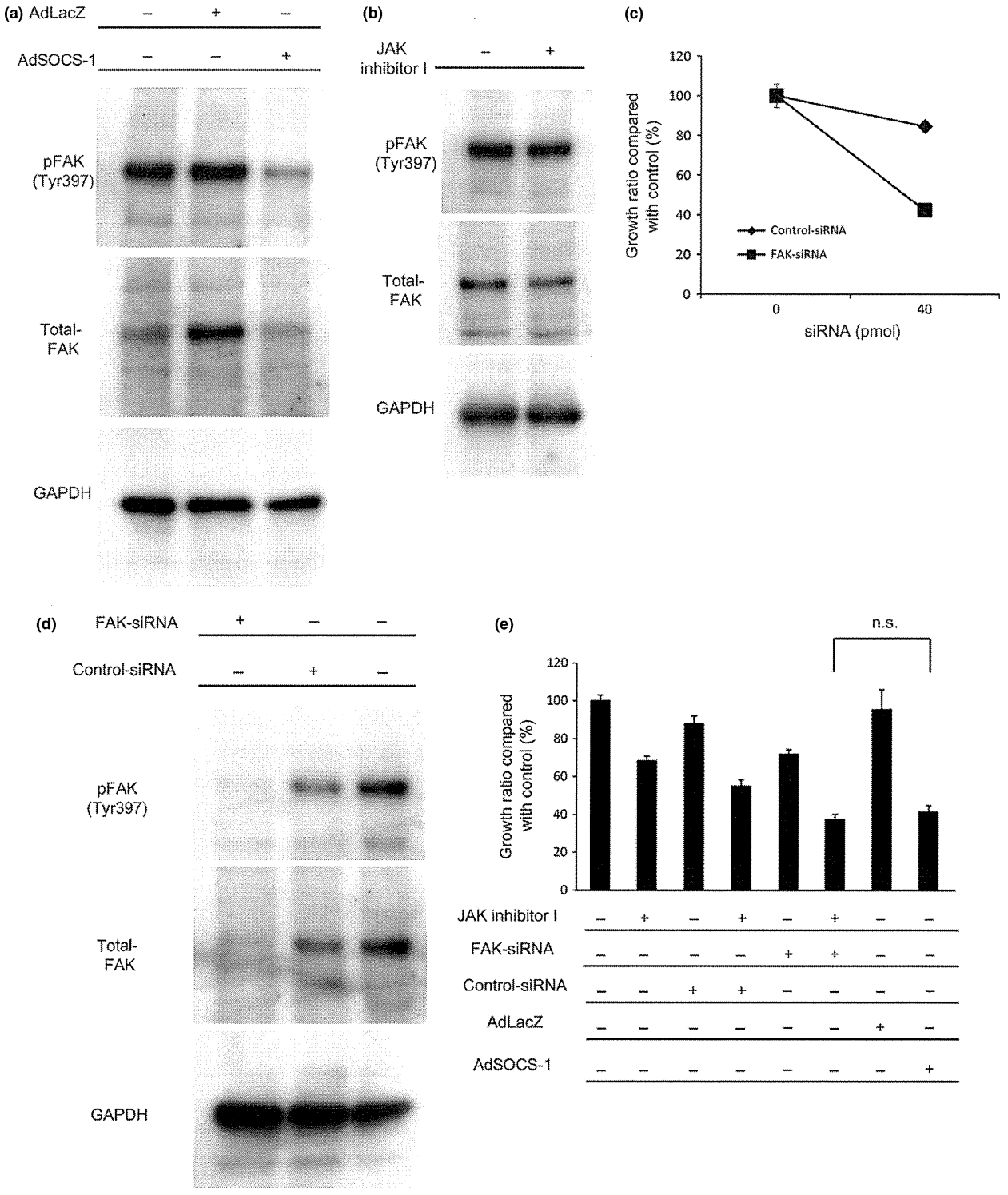

Fig. 3. Focal adhesion kinase (FAK) is downregulated by adenovirus vector containing suppressor of cytokine signaling-1 (AdSOCS-1) in A549 lung cancer cells. (a) Cell lysates were prepared 48 h after treatment with AdSOCS-1 or AdLacZ at an MOI of 40, and immunoblotted with p-FAK (Tyr397) and FAK antibodies. (b) Cell lysates were prepared 48 h after exposure to 5 μM JAK inhibitor I and immunoblotted with p-FAK (Tyr397) and FAK antibodies. (c) Cells were exposed to either FAK siRNA or non-specific siRNA as control. Cells were cultured in RPMI-1640 medium containing 10% FBS. After a 3-day culture, viable cells were counted with the MTS assay. (d) Protein extracts prepared at 48 h after treatment with FAK siRNA or non-specific siRNA were blotted with anti-p-FAK (Tyr397) and anti-FAK antibodies. (e) A549 cells cultured in RPMI-1640 medium containing 10% FBS were exposed to 5 μM JAK inhibitor I or 40 pmol FAK siRNA; 40 pmol non-specific siRNA was used as control. Infections of AdLacZ or AdSOCS-1 were carried out at an MOI of 40. Cell proliferation activity was assessed by MTS assay 72 h after treatment. n.s., no significant change detected by Tukey's post-hoc comparisons.

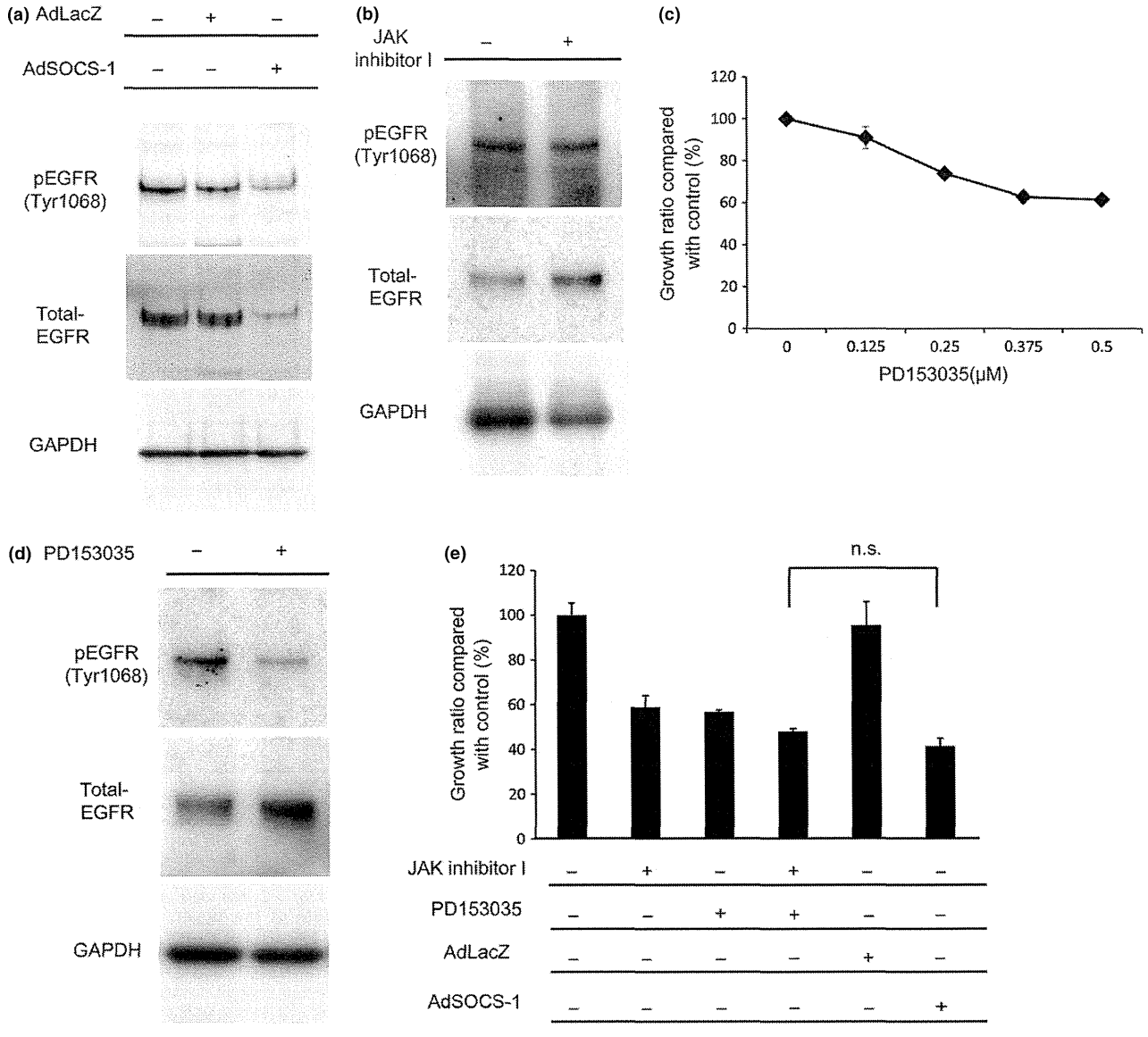

Fig. 4. Epidermal growth factor receptor (EGFR) is downregulated by adenovirus vector containing suppressor of cytokine signaling-1 (AdSOCS-1) in A549 lung cancer cells. (a) Cells were infected with AdLacZ or AdSOCS-1 at an MOI of 40 for 24 h. Cell lysates were immunoblotted with anti-p-EGFR (Tyr1068) and anti-EGFR antibodies. (b) Cell lysates were prepared 48 h after exposure to 5 μM JAK inhibitor I and immunoblotted with anti-p-EGFR (Tyr1068) and anti-EGFR antibodies. (c) Cells cultured in RPMI-1640 medium containing 10% FBS were exposed to 0.125–0.5 μM PD153035. Cell proliferation activity was assessed by MTS assay 72 h after exposure. (d) A549 cells were cultured in RPMI-1640 medium containing 0.5% FBS with 0.5 μM PD153035. Cell lysates were prepared 48 h after treatment and immunoblotted with anti-p-EGFR (Tyr1068) and anti-EGFR antibodies. (e) A549 cells cultured in RPMI-1640 medium containing 10% FBS were exposed to 5 μM JAK inhibitor I or 0.5 μM PD153035. Infection of AdLacZ or AdSOCS-1 was carried out at an MOI of 40. Cell proliferation activity was assessed by MTS assay 72 h after treatment. n.s., no significant change detected by Tukey's post-hoc comparisons.

Accordingly, JAK1, JAK2, FAK, and EGFR should be considered critical for the proliferation of A549 cells, so that simultaneous inhibition of these molecules by SOCS-1 may have a potent antiproliferative effect on A549.

Upregulation of p53 by AdSOCS-1 in A549 cells. We also used a phospho-kinase array to determine the expression profile of phosphorylated proteins in A549 in order to identify other target molecules for SOCS-1 in addition to JAK, FAK, and EGFR (Fig. 5a). Phosphorylation of p53 was upregulated in A549 cells after the introduction of the *SOCS-1* gene. This was confirmed by immunoblotting analysis, which showed that p53 phosphorylation was upregulated in A549 cells by overexpression of the *SOCS-1* gene (Fig. 5b). We also investigated whether JAK inhibitor I, instead of SOCS-1, could activate p53 in A549 cells, but found that it did not (Fig. 5b). Given the well-established antitumor effect of p53 phoshorylation,[35–38] SOCS-1-induced p53 activation may thus also contribute to the suppression of A549 cell proliferation.

Antitumor activity of *SOCS-1* in a lung cancer xenograft model. We also evaluated the therapeutic effect of AdSOCS-1 injection on the growth of NSCLC cells *in vivo*. We established a xenograft model of ICR nu/nu mice in which A549 cells were s.c. implanted. Injection of AdSOCS-1 vector

doi: 10.1111/cas.12266