

Figure 2. Periostin is upregulated upon histamine stimulation in cultured wild-type (WT) fibroblasts. WT fibroblasts were stimulated with histamine at the indicated concentrations for 2 hours (a, b) or 24 hours (c). Periostin mRNA expression was determined by reverse transcriptase–PCR (RT-PCR) analysis (a) and real-time PCR analysis (b). Periostin protein expression was evaluated by western blotting analysis (c). Three independent experiments were performed, and representative blots and quantitative analysis of signal density on blots from three independent experiments are shown (using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control). Values in b and c are shown as mean \pm SD for three independent experiments. *** P <0.001; NS, no significance, compared with control (0 μM histamine) by one-way analysis of variance (ANOVA) followed by Dunnett’s test.

but not by either H2R or H4R antagonists (Figure 3a and b), suggesting that histamine upregulates periostin expression through H1R activation *in vitro*.

Next, we determined whether periostin was induced by histamine via H1R *in vivo*. Histamine release was triggered through mast cell degranulation using the compound 48/80 in WT and H1R-deficient ($H1R^{-/-}$) mice. After treatment with compound 48/80 for three consecutive days, skin at the injected site was sampled. Periostin expression in WT and $H1R^{-/-}$ mouse skin was compared by western blotting analyses (Figure 3c). In WT mice, periostin expression markedly increased after compound 48/80 treatment, although no such increase was observed in $H1R^{-/-}$ mice (Figure 3c). These results suggest that H1R mediates histamine-induced periostin upregulation.

H1R activation upregulates periostin expression via the ERK1/2 pathway

Next, to investigate the signal transduction pathway involved after H1R activation by histamine in dermal fibroblasts, we used a commercial human phosphorylated kinase array kit to profile the phosphorylated kinases in normal human dermal fibroblasts (Figure 4a). Subsequently, phosphorylation of analogous kinases was confirmed in murine dermal fibroblasts by western blot analysis (Figure 4b). Compared with nontreated dermal fibroblasts, enhanced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and the downstream factor cAMP response element-binding protein (CREB) was observed after 10 minutes and 30 minutes of histamine stimulation (Figure 4a and b).

Furthermore, we found that histamine-induced phosphorylation of ERK1/2 and CREB was blocked not only with U0126 (a selective ERK1/2 kinase inhibitor) but also with an H1R antagonist (Figure 4c and d).

These observations demonstrated that histamine activates the ERK1/2 signal transduction pathway via H1R in dermal fibroblasts.

In addition, to verify the involvement of ERK1/2 activation in histamine-induced upregulation of periostin, western blotting analysis was performed (Figure 4c and d). Both U0126 and H1R antagonists decreased the expression of periostin, as well as suppressed the phosphorylation of CREB (Figure 4c and d). These results indicated that H1R-mediated signaling upregulated periostin expression via the ERK1/2 pathway.

H1R-mediated upregulation of periostin is essential for histamine-induced collagen production

To investigate the involvement of periostin in histamine-induced collagen production, primary dermal fibroblasts from WT and periostin-deficient ($PN^{-/-}$) mice were stimulated with histamine (100 μM) for 48 hours. The induction of collagen was abolished in $PN^{-/-}$ fibroblasts at both the mRNA (Figure 5a) and protein (Figure 5b and c) levels. Histamine-treated $PN^{-/-}$ fibroblasts did not exhibit increases in mRNA or protein expression of type I collagen (Col1; Figure 5a–c).

As described above, periostin was induced by histamine via the H1R pathway. To further clarify whether H1R was associated with histamine-induced collagen production, H1R antagonist was added to WT fibroblasts before histamine stimulation. After 48 hours of histamine stimulation, collagen production was evaluated as determined by quantitative real-time reverse transcriptase–PCR, Sircol Collagen Assay, and western blotting analyses (Figure 6). As expected, histamine-induced collagen synthesis was blocked by an H1R antagonist (Figure 6a–c). Furthermore, this inhibitory effect was rescued by the addition of recombinant mouse periostin (rmPeriostin; Figure 6a–c).

In addition, this mechanism was confirmed in cultured primary human dermal fibroblasts derived from healthy donor skin biopsies (Supplementary Figure S1 online).

Finally, we addressed the question of how strong the effect of histamine on tissue remodeling was in AD. Compared with normal skin and AD nonlesioned skin, increased expression of

periostin was observed in both acute AD lesioned skin and skin tissues with positive *Dermatophagoides farinae* (Dorf) scratch tests (Supplementary Figure S2 online). Our results

suggest that histamine may contribute to the initiation of tissue remodeling during the acute phase of AD.

DISCUSSION

Here, we report that histamine increases the expression of periostin in dermal fibroblasts. Moreover, periostin increases *de novo* synthesis of Col1 via an ERK1/2-mediated pathway.

It is widely recognized that mast cells contribute to the healing of skin wounds (Hebda *et al.*, 1993; Artuc *et al.*, 1999; Trautmann *et al.*, 2000; Gailit *et al.*, 2001; Noli and Miolo, 2001). Impaired wound closure in mast cell-deficient mice indicates that mast cells have a crucial role in the wound repair process (Weller *et al.*, 2006). An increased number of mast cells in fibrotic tissues such as scleroderma, keloid, or healing wounds has been identified (Hawkins *et al.*, 1985; Atkins and Clark, 1987), although it is still unclear whether mast cells are fibrogenic. In many instances, chemical mediators, such as histamine, which is derived from degranulated mast cells or basophils, have been implicated as a cause of inflammation and tissue remodeling in AD (Davies and Greaves, 1980; Nishioka *et al.*, 1987; Wahlgren, 1999; Murota and Katayama, 2009). In support of these findings, H1R antagonist has been shown to inhibit the synthesis of Col1 by dermal fibroblasts (Murota *et al.*, 2008). Interestingly, histamine H1R antagonists but not H2R antagonists reduced wound closure in experimentally induced skin wounds in mice (Weller *et al.*, 2006). Therefore, histamine is believed to have an important role in the wound-healing process. Indeed, disruption of histamine in histidine decarboxylase knockout mice resulted in delayed cutaneous wound healing, and this phenotype was rescued by exogenous histamine administration (Numata *et al.*, 2006). It remains unclear how histamine promotes wound healing. Some reports indicated that histamine induces fibroblast proliferation after a long period of coculturing (Russel *et al.*, 1977; Topol *et al.*, 1981). In our study, increased expression of Col1 mRNA was observed after 48 hours of co-incubation with histamine. Thus, histamine-mediated tissue remodeling may require the expression of periostin as a second messenger in order to elicit tissue remodeling.

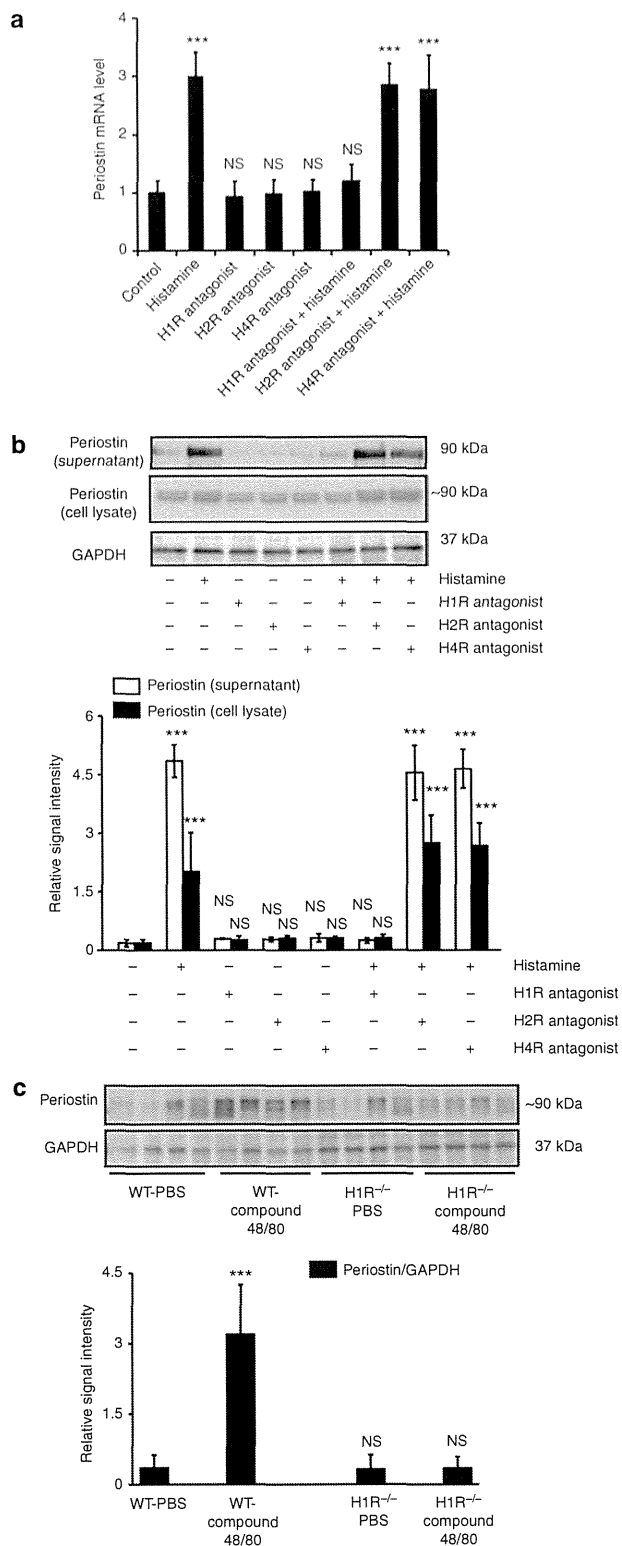


Figure 3. Histamine upregulates periostin expression via histamine receptor 1 (H1R) *in vitro* and *in vivo*. *In vitro*, wild-type (WT) fibroblasts were either treated with histamine antagonists (H1R, H2R, or H4R; 100 μ M) or left untreated for 2 hours, and then cells were stimulated with histamine (100 μ M) for an additional 2 hours (a) or 24 hours (b). Periostin expression was examined by quantitative real-time reverse transcriptase-PCR (qRT-PCR) (a) and western blotting analysis (b). *In vivo*, WT and H1R^{-/-} mice were treated with mast cell stimulator compound 48/80 for 3 days by subcutaneous injection, and periostin protein expression in the injected site skin was evaluated by western blotting analysis ($n=4$ mice per group); representative blots and quantitative analysis of signal density on blots from four mice of each group are shown (using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control). (c) Values in a and b are shown as mean \pm SD for three independent experiments. Values in c are shown as mean \pm SD for blot signals from four mice. *** $P<0.001$; NS, no significance, compared with control (0 μ M histamine in a and b; WT-phosphate-buffered saline in c) by one-way analysis of variance (ANOVA) followed by Dunnett's test.

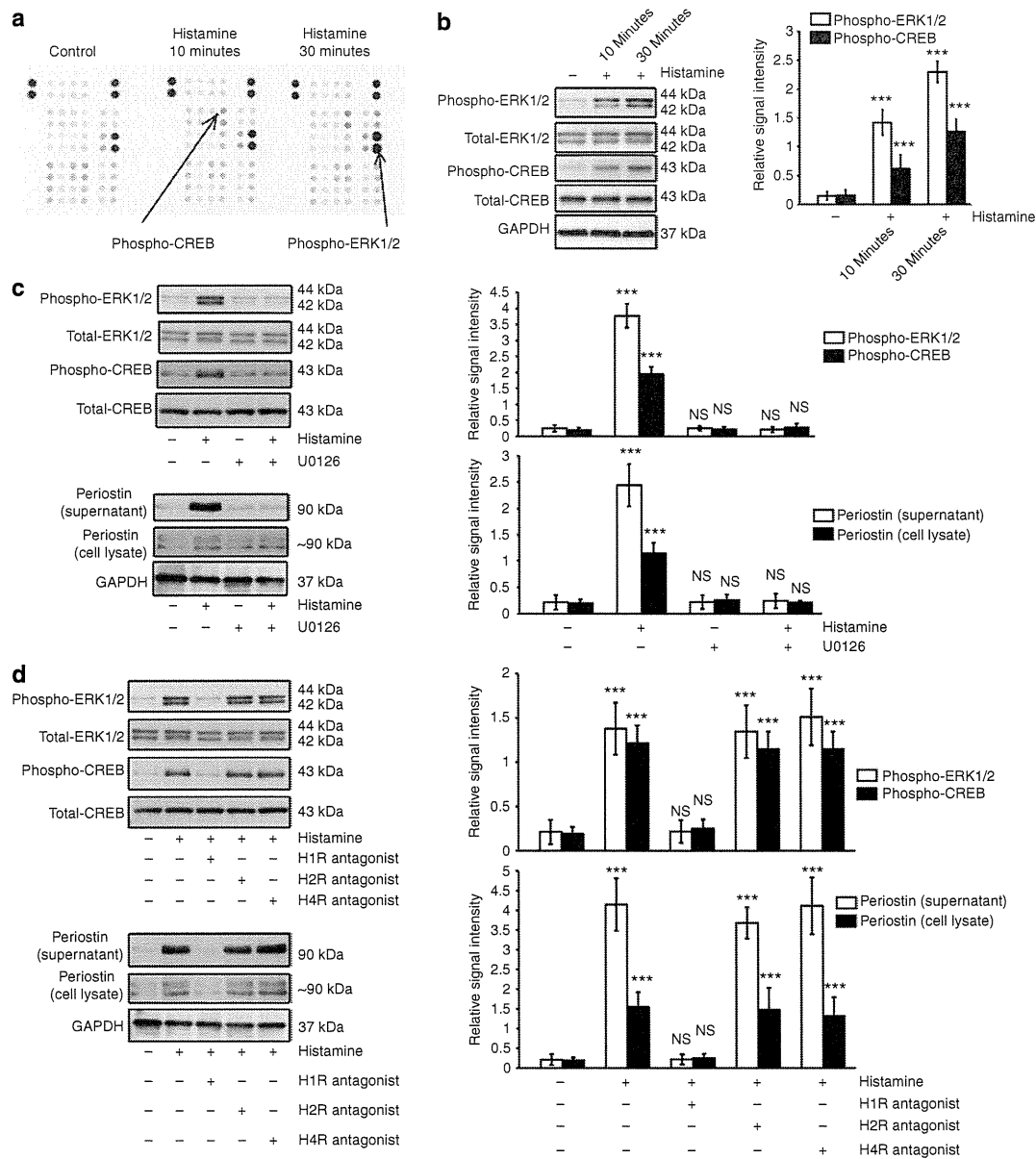
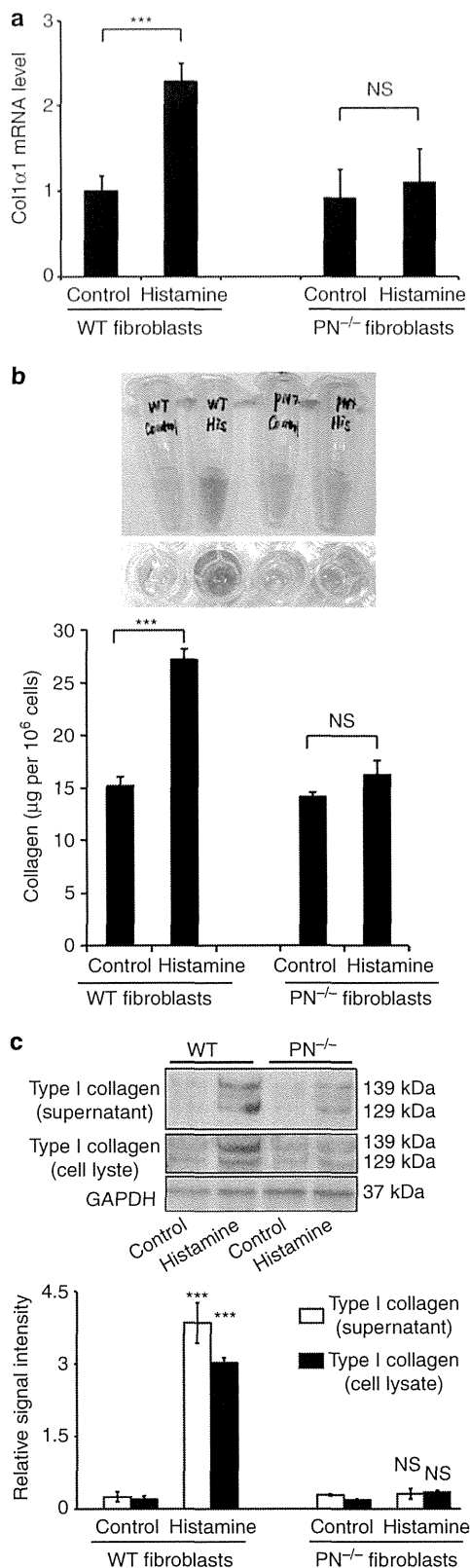


Figure 4. Histamine receptor 1 (H1R) activation upregulates periostin expression via the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. (a) The phosphorylation state was detected by R&D Systems Proteome Profiler Phospho-Kinase Array in normal human dermal fibroblasts, which were either untreated or treated with histamine for 10 minutes or 30 minutes. The activated kinases are indicated by arrows. (b) Phosphorylated ERK1/2 and phosphorylated cAMP response element-binding protein (CREB) were evaluated by western blotting analyses in murine wild-type (WT) fibroblasts following histamine stimulation for 10 minutes and 30 minutes. (c) WT fibroblasts with or without ERK1/2 inhibitor (U0126, 20 μ M) preincubation were stimulated with histamine (30 minutes, upper panel; 24 hours, lower panel). Phosphorylated ERK1/2, phosphorylated CREB, and periostin protein expression was examined by western blotting analyses. (d) WT fibroblasts cultured in the presence or absence of preincubation with histamine receptor antagonists (H1R, H2R, or H4R; 100 nM) were stimulated with histamine (30 minutes, upper panel; 24 hours, lower panel). Phosphorylated ERK1/2, phosphorylated CREB, and periostin protein expression was examined by western blotting analyses. Three independent experiments were performed, and representative blots and quantitative analysis of signal density on blots from three independent experiments are shown (using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control). *** P <0.001; NS, no significance, compared with control (0 μ M histamine) by one-way analysis of variance (ANOVA) followed by Dunnett's test.

Periostin, a recently characterized matricellular protein, has been reported to have crucial roles in tooth and periodontium development (Horiuchi *et al.*, 1999), cancer proliferation and invasion (Siriwardena *et al.*, 2006; Baril *et al.*, 2007; Kudo *et al.*, 2012), cardiac healing after acute myocardial infarction

(Shimazaki *et al.*, 2008), idiopathic interstitial pneumonia (Okamoto *et al.*, 2011), and bone marrow fibrosis (Oku *et al.*, 2008). Furthermore, periostin is highly expressed in connective tissue and at the remodeling tissue site after injury or inflammation. This protein is secreted from fibroblasts via



transforming growth factor beta stimulation (Horiuchi *et al.*, 1999). Periostin was shown to accelerate cardiac healing after acute myocardial infarction (Dorn, 2007; Oka *et al.*, 2007; Shimazaki *et al.*, 2008) and during full-thickness cutaneous wound repair (Nishiyama *et al.*, 2011; Elliott *et al.*, 2012; Otsuka *et al.*, 2012) by modulating fibroblast differentiation.

Periostin has also been reported to be induced by other factors, including bone morphogenetic proteins, vascular endothelial growth factor, connective tissue growth factor, vitamin K, IL-3, IL-4, IL-6, and IL-13 (Asano *et al.*, 2005; Takayama *et al.*, 2006; Iekushi *et al.*, 2007; Blanchard *et al.*, 2008; Coutu *et al.*, 2008; Banerjee *et al.*, 2009; Norris *et al.*, 2009). Recently, the increased expression of periostin was confirmed in various allergic diseases such as bronchial asthma (Takayama *et al.*, 2006), AD (Masuoka *et al.*, 2012), and eosinophilic chronic rhinosinusitis (Hur *et al.*, 2012). As an IL-4- and IL-13-inducible protein, periostin is associated with tissue remodeling in bronchial asthma (Takayama *et al.*, 2006), allergic eosinophilic esophagitis (Blanchard *et al.*, 2008), AD (Masuoka *et al.*, 2012), and allergic rhinitis (Hur *et al.*, 2012). In the present study, histamine was found to directly induce periostin expression, whereas the expression levels of transforming growth factor beta, IL-4, and IL-13 were not altered by histamine stimulation (data not shown). Thus, we postulate that periostin is involved in the initiation of tissue remodeling in chronic allergic diseases.

AD is known to develop tissue remodeling, which is characterized by epidermal thickening, hyperkeratosis and fibrosis of the papillary dermis, increased fibroblast proliferation, and collagen accumulation, and these features are caused by nonspecific stimuli, constant scratching, and rubbing (Lee *et al.*, 2009). Tissue remodeling and repair are thought to be the underlying causes of chronic allergic inflammation, such as in asthmatic diseases and AD (Leung, 1995). Recently, increased expressions of periostin and the inducers of periostin (IL-4, IL-13, and transforming growth factor beta) were identified in a screening of AD-associated genes in genome-wide association studies and quantitative mRNA expression analysis in lesion tissues (Hoffjan and Epplen, 2005; Wood *et al.*, 2009a, 2009b). Furthermore, in the present study, elevated expression of periostin was found in lesional skin of patients with AD. These results suggest that periostin may be involved in AD and in asthma.

Figure 5. Periostin is essential in histamine-induced collagen production *in vitro*. Primary dermal fibroblasts from wild type (WT) and periostin-deficient (PN^{-/-}) mice were stimulated with histamine (100 μM) or phosphate-buffered saline (PBS) (control) for 48 hours. The collagen type-I alpha 1 (Col1α1) mRNA level was examined by quantitative real-time reverse transcriptase-PCR (qRT-PCR) (a), and collagen protein expression was evaluated by Sircol assay (b) and western blotting analysis; representative blots and quantitative analysis of signal density on blots from three independent experiments are shown (using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control) (c). Values are shown as mean ± SD for three independent experiments. ***P < 0.001; NS, no significance, compared with paired control (WT fibroblasts control or PN^{-/-}-fibroblasts control) by Student's *t*-test.

In WT and $PN^{-/-}$ mice, a mite extract-induced AD model was established and analyzed. In contrast to WT mice, $PN^{-/-}$ mice showed amelioration of epidermal hyperplasia and

inflammatory cell infiltration (Masuoka *et al.*, 2012). Moreover, periostin directly induces production of thymic stromal lymphoprotein in keratinocytes (Masuoka *et al.*, 2012). Thus, periostin was suggested to have a critical role in the amplification and chronicity of allergic skin inflammation.

The results of the present study demonstrate the role of periostin in histamine-mediated collagen production. We found that H1R-mediated phosphorylation of ERK1/2 had a crucial role in histamine-induced collagen production. These observations may open a new window of therapeutic opportunity against airway remodeling in asthma or dermal remodeling in AD, as histamine H1R antagonists are expected to ameliorate tissue remodeling.

As refractory chronic allergic symptoms are known to impair the quality of life, work productivity, and overall activity (Meltzer *et al.*, 1999; Thompson *et al.*, 2000; Kawashima *et al.*, 2002; Baiardini *et al.*, 2003; Spector *et al.*, 2007), we believe that these studies will provide a basis for exploring the fibrotic components of allergic diseases in skin and other tissues.

MATERIALS AND METHODS

Mice

WT mice (C57BL/6 strain) were purchased from CLEA Japan (Osaka, Japan). Periostin gene knockout ($PN^{-/-}$) mice (C57BL/6 strain) were generated as previously described (Shimazaki and Kudo, 2008). Histamine receptor 1 gene knockout ($H1R^{-/-}$) mice (C57BL/6 strain) were purchased from Oriental Bio Service (Kyoto, Japan). Mice were maintained in our pathogen-free animal facility. Animal care and experimentation were performed in accordance with the institutional guidelines of the National Institute of Biomedical Innovation, Osaka, Japan and Osaka University, Osaka, Japan. Six-week-old male mice were used in all experiments. All experiments used four mice per group.

Compound 48/80 treatment

Compound 48/80 (Sigma, St Louis, MO) was dissolved in phosphate-buffered saline at a concentration of 1 mg ml^{-1} and sterilized by filtration. With the use of a 27-gauge needle, $100 \mu\text{l}$ of compound 48/80 or phosphate-buffered saline was subcutaneously injected into the back side of mice each day for 3 days. One day after the final injection, the skin at the injected site was removed and solubilized at 4°C in lysis buffer (0.5% sodium deoxycholate, 1% Nonidet P40,

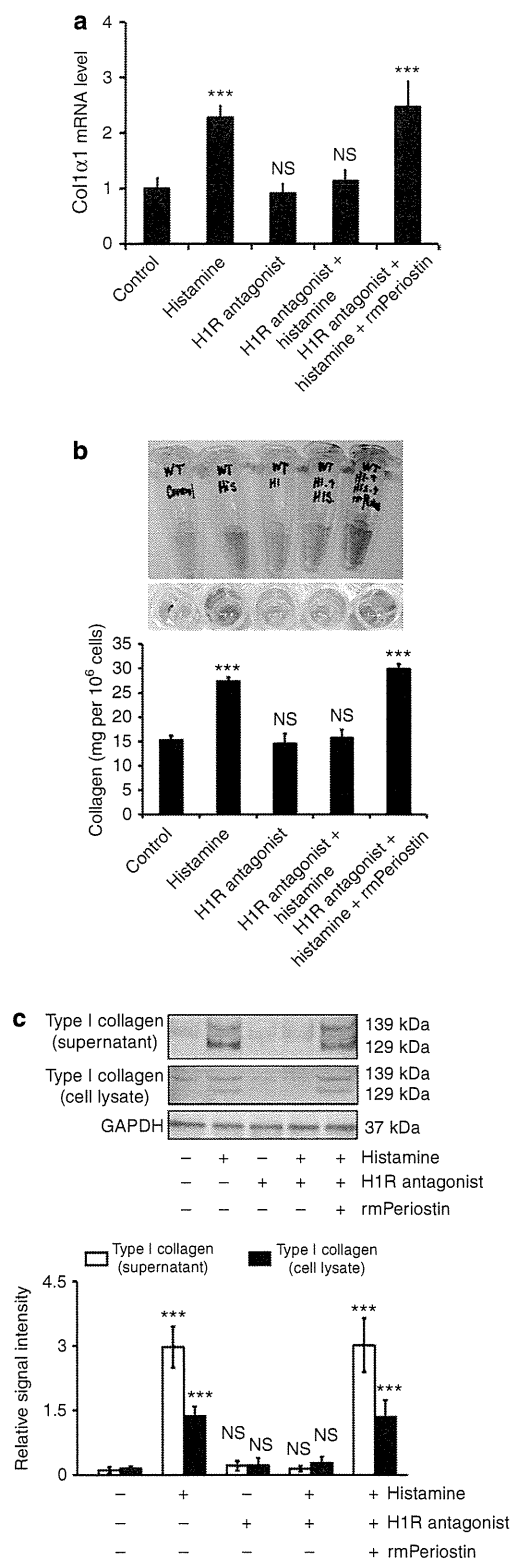


Figure 6. Histamine receptor 1 (H1R)-mediated upregulation of periostin is essential in histamine-induced collagen production. Wild-type (WT) fibroblasts cultured with or without H1R antagonist preincubations were stimulated with histamine ($100 \mu\text{M}$) alone or in the presence of recombinant mouse periostin (rmPeriostin, 100 ng/ml^{-1}). The collagen type-I alpha 1 (Col1α1) mRNA level was examined by quantitative real-time reverse transcriptase-PCR (qRT-PCR) (a), and collagen protein expression was evaluated by Sircol assay (b) and western blotting analysis; representative blots and quantitative analysis of signal density on blots from three independent experiments are shown (using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control) (c). Values are shown as mean \pm SD for three independent experiments. *** $P < 0.001$; NS, no significance compared with control ($0 \mu\text{M}$ histamine) according to one-way analysis of variance (ANOVA) followed by Dunnett's test.

0.1% sodium dodecyl sulfate, 100 $\mu\text{g ml}^{-1}$ phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail) for western blotting analysis.

Cell culture

Murine primary dermal fibroblasts from the skin of four newborn WT and four newborn $PN^{-/-}$ mice were isolated and cultured as previously described (Terao *et al.*, 2010). Human primary dermal fibroblasts were purchased from DS Pharma Biomedical (Osaka, Japan). After 24 hours of serum starvation, dermal fibroblasts at confluence were treated with 0.1 to 100 μM histamine (Sigma–Aldrich, Tokyo, Japan) or 100 ng ml^{-1} recombinant mouse periostin (rmPeriostin, R&D Systems, Minneapolis, MN) for the indicated periods of time before extraction of RNA and protein. Cells were used at passage three. In each experiment, the obtained fibroblasts were examined at the same time point and under the same culture conditions (e.g., cell density, passage, and days after plating). For inhibition experiments, fibroblasts were preincubated for 2 hours with specific histamine receptor antagonists (Pyrilamine maleate, Cimetidine, JNJ7777120, 100 μM , Sigma–Aldrich) or ERK1/2 inhibitor (U0126, 20 μM , Cell Signaling Technology, Beverly, MA) before the addition of histamine. We performed serial dilutions of each agent to identify the most effective concentrations to be used in the experiments, as determined by MTT assays and western blotting analyses.

Quantitative real-time and direct reverse transcriptase–PCR analysis of mRNA

Total RNA was isolated from fibroblasts using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. First, 100 ng of RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN). For quantitative real-time reverse transcriptase–PCR analysis, standard curves for periostin, collagen, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were generated from serial dilutions of positively expressing cDNA. Relative quantification of the PCR products was carried out using the ABI prism 7000 (Applied Biosystems, Darmstadt, Germany) and the comparative threshold cycle (C_T) method. The “fold-induction” was calculated as the ratio to values of cells that were not incubated with histamine or periostin. The primers used for real-time PCR were as follows: periostin, sense 5'-GAACGAATCATTACAGGTCC-3', antisense 5'-GGAGACCTCTTTTGCAAGA-3'; collagen type-I alpha 1 (Col1- α 1), sense 5'-GAGCCCTCGTTCCTACTC-3', antisense 5'-TGTTCCCTACTCAGCCGTCTGT-3'; and GAPDH, sense 5'-TGTCATCATACTTGGCAGGTTTCT-3', antisense 5'-CATGGCCTTCCGTGTTCTTA-3'. Each reaction was performed in triplicate. Variation within samples was less than 10%. Statistical analysis was performed with the Student's paired *t*-test.

Western blotting analyses

For preparation of protein samples, cell pellets and skin samples were extracted as described above, and 5 μg of extracted protein was used for western blotting analysis, as described previously (Terao *et al.*, 2010). The primary antibodies were used at the following dilutions: anti-type I collagen (Calbiochem, San Diego, CA) at 1:500, anti-periostin (R&D Systems, Minneapolis, MN) at 1:500, anti-phospho-ERK1/2 (Cell Signaling Technology) at 1:1,000, anti-total ERK1/2 (Cell Signaling Technology) at 1:1,000, anti-phospho-CREB (Cell Signaling Technology) at 1:1,000, anti-total CREB (Cell Signaling Technology)

at 1:1,000, and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500. Staining with the anti-GAPDH antibody was used as a loading control. Signal intensity of bands was quantified using the ImageJ densitometry software (<http://rsb.info.nih.gov/ij/index.html>) and normalized to GAPDH signal intensity.

Sircol collagen assay

The soluble collagen levels in culture supernatants were measured using a Sircol Collagen Assay (Biocolor, Belfast, UK). This assay measured total secreted collagen from cultured cells. Briefly, cells were cultured for 48 hours with or without treatment, and then supernatants were collected. One milliliter of Sirius red, an anionic dye that specifically reacts with the basic side chain groups of collagens, was added to 200 μl of the supernatant and incubated with gentle rotation for 30 minutes at room temperature. After centrifugation, the collagen-bound dye was resolubilized in 1 ml of 0.5 M NaOH, and the absorbance at 540 nm was measured.

Phosphorylated kinase array

Phosphorylated kinase was profiled with the Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems). The procedures were performed according to the manufacturer's protocol using 300 μg of protein lysate per array.

Statistical analysis

All experiments reported in this paper were repeated at least three times, yielding similar results, and data are presented as mean \pm SD. The Student's two-tailed *t*-test (Microsoft Excel software, Redmond, WA) was used for comparison between two groups. When analysis included more than two groups, one-way analysis of variance (ANOVA) followed by Dunnett's test was used. *P*-values less than 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Artuc M, Hermes B, Steckelings UM *et al.* (1999) Mast cells and their mediators in cutaneous wound healing—active participants or innocent bystanders? *Exp Dermatol* 8:1–16
- Asano M, Kubota S, Nakanishi T *et al.* (2005) Effect of connective tissue growth factor (CCN2/CTGF) on proliferation and differentiation of mouse periodontal ligament-derived cells. *Cell Commun Signal* 3:11
- Atkins FM, Clark RA (1987) Mast cells and fibrosis. *Arch Dermatol* 123:191–3
- Baiardini I, Giardini A, Pasquali M *et al.* (2003) Quality of life and patients' satisfaction in chronic urticaria and respiratory allergy. *Allergy* 58:621–3
- Banerjee I, Fuseler JW, Intwala AR *et al.* (2009) IL-6 loss causes ventricular dysfunction, fibrosis, reduced capillary density, and dramatically alters the cell populations of the developing and adult heart. *Am J Physiol Heart Circ Physiol* 296:H1694–704

- Baril P, Gangeswaran R, Mahon PC *et al.* (2007) Periostin promotes invasiveness and resistance of pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k pathway. *Oncogene* 26:2082–94
- Blanchard C, Mingler MK, McBride M *et al.* (2008) Periostin facilitates eosinophil tissue infiltration in allergic lung and esophageal responses. *Mucosal Immunol* 1:289–96
- Cohen IK, Beaven MA, Horáková Z *et al.* (1972) Histamine and collagen synthesis in keloid and hypertrophic scar. *Surg Forum* 23:509–10
- Coutu DL, Wu JH, Monette A *et al.* (2008) Periostin, a member of a novel family of vitamin K-dependent proteins, is expressed by mesenchymal stromal cells. *J Biol Chem* 283:17991–8001
- Davies MG, Greaves MW (1980) Sensory responses of human skin to synthetic histamine analogues and histamine. *Br J Clin Pharmacol* 9:461–5
- Dorn GW (2007) Periostin and myocardial repair, regeneration, and recovery. *N Engl J Med* 357:1552–4
- Elliott CG, Wang J, Guo X *et al.* (2012) Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound repair. *J Cell Sci* 125:121–32
- Gailit J, Marchese MJ, Kew RR *et al.* (2001) The differentiation and function of myofibroblasts is regulated by mast cell mediators. *J Invest Dermatol* 117:1113–9
- Hawkins RA, Claman HN, Clark RA *et al.* (1985) Increased dermal mast cell populations in progressive systemic sclerosis: a link in chronic fibrosis? *Ann Intern Med* 102:182–6
- Hebda PA, Collins MA, Tharp MD (1993) Mast cell and myofibroblast in wound healing. *Dermatol Clin* 11:685–96
- Hoffjan S, Epplen JT (2005) The genetics of atopic dermatitis: recent findings and future options. *J Mol Med (Berl)* 83:682–92
- Horiuchi K, Amizuka N, Takeshita S *et al.* (1999) Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. *J Bone Miner Res* 14:1239–49
- Hur DG, Khalmuratova R, Ahn SK *et al.* (2012) Roles of periostin in symptom manifestation and airway remodeling in a murine model of allergic rhinitis. *Allergy Asthma Immunol Res* 4:222–30
- Iekushi K, Taniyama Y, Azuma J *et al.* (2007) Novel mechanisms of valsartan on the treatment of acute myocardial infarction through inhibition of the antiadhesion molecule periostin. *Hypertension* 49:1409–14
- Kawashima M, Harada S, Tango T (2002) Review of fexofenadine in the treatment of chronic idiopathic urticaria. *Int J Dermatol* 41:701–6
- Kudo Y, Iizuka S, Yoshida M *et al.* (2012) Periostin directly and indirectly promotes tumor lymphangiogenesis of head and neck cancer. *PLoS One* 7:e44488
- Lee JH, Chen SY, Yu CH *et al.* (2009) Noninvasive *in vitro* and *in vivo* assessment of epidermal hyperkeratosis and dermal fibrosis in atopic dermatitis. *J Biomed Opt* 14:014008
- Leung DY (1995) Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. *J Allergy Clin Immunol* 96:302–18
- Masuoka M, Shiraishi H, Ohta S *et al.* (2012) Periostin promotes chronic allergic inflammation in response to Th2 cytokines. *J Clin Invest* 122:2590–600
- Meltzer EO, Casale TB, Nathan RA *et al.* (1999) Once-daily fexofenadine HCl improves quality of life and reduces work and activity impairment in patients with seasonal allergic rhinitis. *Ann Allergy Asthma Immunol* 83:311–7
- Murakami T, Yoshioka M, Yumoto R *et al.* (1998) Topical delivery of keloid therapeutic drug, tranilast, by combined use of oleic acid and propylene glycol as a penetration enhancer: evaluation by skin microdialysis in rats. *J Pharm Pharmacol* 50:49–54
- Murota H, Bae S, Hamasaki Y *et al.* (2008) Emedastine difumarate inhibits histamine-induced collagen synthesis in dermal fibroblasts. *J Invest Allergol Clin Immunol* 18:245–52
- Murota H, Katayama I (2009) Emedastine difumarate: a review of its potential ameliorating effect for tissue remodeling in allergic diseases. *Expert Opin Pharmacother* 10:1859–67
- Nishioka K, Katayama I, Doi T (1987) Histamine release by scratching inflamed skin. *J Dermatol* 14:284–5
- Nishiyama T, Kii I, Kashima TG *et al.* (2011) Delayed re-epithelialization in periostin-deficient mice during cutaneous wound healing. *PLoS One* 6:e18410
- Noli C, Miolo A (2001) The mast cell in wound healing. *Vet Dermatol* 12:303–13
- Norris RA, Moreno-Rodriguez R, Hoffman S *et al.* (2009) The many facets of the matricellular protein periostin during cardiac development, remodeling, and pathophysiology. *J Cell Commun Signal* 3:275–86
- Numata Y, Terui T, Okuyama R *et al.* (2006) The accelerating effect of histamine on the cutaneous wound-healing process through the action of basic fibroblast growth factor. *J Invest Dermatol* 126:1403–9
- Oka T, Xu J, Kaiser RA *et al.* (2007) Genetic manipulation of periostin expression reveals a role in cardiac hypertrophy and ventricular remodeling. *Circ Res* 101:313–21
- Okamoto M, Hoshino T, Kitasato Y *et al.* (2011) Periostin, a matrix protein, is a novel biomarker for idiopathic interstitial pneumonias. *Eur Respir J* 37:1119–27
- Oku E, Kanaji T, Takata Y *et al.* (2008) Periostin and bone marrow fibrosis. *Int J Hematol* 88:57–63
- Ontsuka K, Kotobuki Y, Shiraishi H *et al.* (2012) Periostin, a matricellular protein, accelerates cutaneous wound repair by activating dermal fibroblasts. *Exp Dermatol* 21:331–6
- Russel JD, Russell SB, Trupin KM (1977) The effect of histamine on the growth of cultured fibroblasts isolated from normal and keloid tissue. *J Cell Physiol* 93:389–93
- Sandberg N (1962) Accelerated collagen formation and histamine. *Nature* 194:183
- Sandberg N (1964) Enhanced rate of healing in rats with an increased rate of histamine formation. *Acta Chir Scand* 127:9–21
- Shimazaki M, Kudo A (2008) Impaired capsule formation of tumors in periostin-null mice. *Biochem Biophys Res Commun* 367:736–42
- Shimazaki M, Nakamura K, Kii I *et al.* (2008) Periostin is essential for cardiac healing after acute myocardial infarction. *J Exp Med* 205:295–303
- Siriwardena BS, Kudo Y, Ogawa I *et al.* (2006) Periostin is frequently overexpressed and enhances invasion and angiogenesis in oral cancer. *Br J Cancer* 95:1396–403
- Spector SL, Shikar R, Harding G *et al.* (2007) The effect of fexofenadine hydrochloride on productivity and quality of life in patients with chronic idiopathic urticaria. *Cutis* 79:157–62
- Takayama G, Arima K, Kanaji T *et al.* (2006) Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *J Allergy Clin Immunol* 118:98–104
- Terao M, Murota H, Kitaba S *et al.* (2010) Tumor necrosis factor-alpha processing inhibitor-1 inhibits skin fibrosis in a bleomycin-induced murine model of scleroderma. *Exp Dermatol* 19:38–43
- Thompson AK, Finn AF, Schoenwetter WF (2000) Effect of 60 mg twice-daily fexofenadine HCl on quality of life, work and classroom productivity, and regular activity in patients with chronic idiopathic urticaria. *J Am Acad Dermatol* 43:24–30
- Topol BM, Lewis VL, Benveniste K (1981) The use of antihistamine to retard the growth of fibroblasts derived from human skin, scar, and keloid. *Plast Reconstr Surg* 68:227–32
- Trautmann A, Toksoy A, Engelhardt E *et al.* (2000) Mast cell involvement in normal human skin wound healing: expression of monocyte chemoattractant protein-1 is correlated with recruitment of mast cells which synthesize interleukin-4 *in vivo*. *J Pathol* 190:100–6
- Wahlgen CF (1999) Itch and atopic dermatitis: an overview. *J Dermatol* 26:770–9
- Weller K, Foitzik K, Paus R *et al.* (2006) Mast cells are required for normal healing of skin wounds in mice. *FASEB J* 20:2366–8
- Wood SH, Clements DN, Ollier WE *et al.* (2009a) Gene expression in canine atopic dermatitis and correlation with clinical severity scores. *J Dermatol Sci* 55:27–33
- Wood SH, Ke X, Nuttall T *et al.* (2009b) Genome-wide association analysis of canine atopic dermatitis and identification of disease related SNPs. *Immunogenetics* 61:765–72
- Yang L, Serada S, Fujimoto M *et al.* (2012) Periostin facilitates skin sclerosis via PI3K/Akt dependent mechanism in a mouse model of scleroderma. *PLoS One* 7:e41994

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

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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 Com-

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
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pared 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 Ca²⁺-dependent manner, self-associates on phospholipid

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- κ B 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% CO₂. 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 μ g/ml of Geneticin (Invitrogen). Clones were maintained in 250 μ g/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 IC₅₀ 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 μ M), cisplatin (0–100 μ M) or 5-fluorouracil (5-FU) (0–50 μ M) 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 (IC₅₀ 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 GCUCCAGAGUUUATT-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 μ l 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 mm³. 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 (mm³) = $W \times L \times D$. 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 μ M cisplatin for 4 hr and those pretreated with 50 μ M 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 μ M cisplatin or 50 μ M 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. 1a). 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 OWISE) used as positive controls (Fig. 1b). Significantly higher IC₅₀ values for cisplatin were observed in HEC1-A25 (32.1 μ M, $p < 0.01$), HEC1-A43 (23.8 μ M, $p < 0.01$), HEC1-A63 (34.9 μ M, $p < 0.01$) and HEC1-A77 cells (17.3 μ M, $p < 0.01$) compared with HEC1 (9.8 μ M) and HEC1-CV cells (8.4 μ M) (Fig. 1c). Similarly, IC₅₀ values for carboplatin were significantly increased in HEC1-A25 (194.6 μ M, $p < 0.01$), HEC1-A43 (153.3 μ M, $p < 0.01$), HEC1-A63 (371.5 μ M, $p < 0.01$) and HEC1-A77 cells (158.1 μ M, $p < 0.01$) compared with HEC1 (59.1 μ M) and HEC1-CV cells (60.9 μ M) (Fig. 1c). Thus, Anx A4 overexpression conferred platinum resistance in HEC1 cell lines.

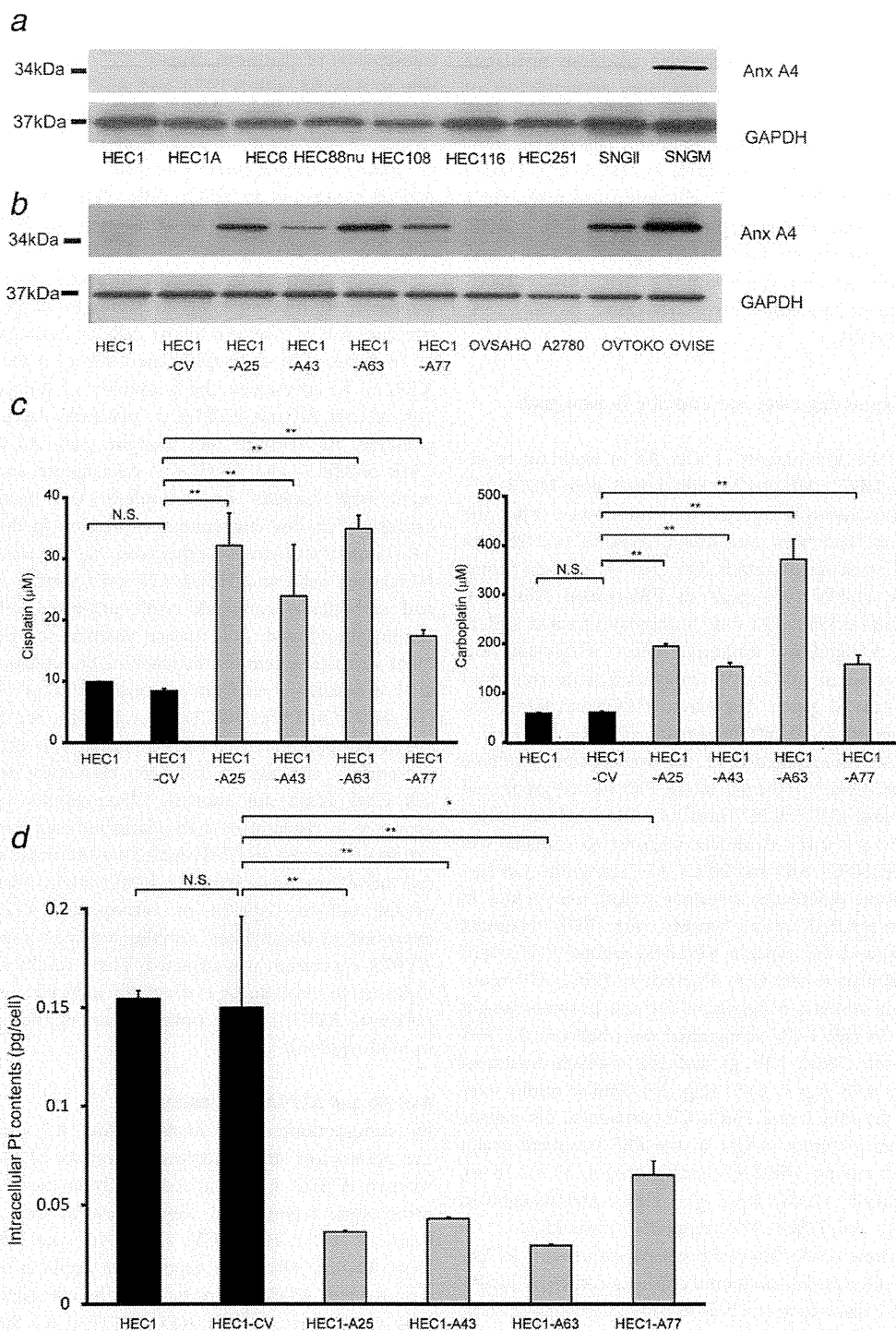


Figure 1. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vitro*. (a) Western blot analysis of nine endometrial carcinoma cell lines. Anx A4 was expressed in one cell line. (b) Establishment of an Anx A4-stably-expressing HEC1 cell line by transfection with the pcDNA3.1-Anx 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 IC₅₀ 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. 1d). 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 $11,496 \pm 950 \text{ mm}^3$ in PBS-treated HEC1-CV control mice and $3,554 \pm 872 \text{ mm}^3$ 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. 2a; $p < 0.01$).

In HEC1-A63-xenografted mice, the average tumor volume on Day 56 was $8,245 \pm 160 \text{ mm}^3$ in the PBS-treated group and only slightly less ($7,078 \pm 257 \text{ mm}^3$) in the cisplatin-treated group (Fig. 2a; $p = 0.42$). 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 ($4.66 \pm 0.42 \text{ g}$) and the cisplatin treatment groups ($4.43 \pm 0.16 \text{ g}$) (Fig. 2b). 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 ($5.95 \pm 1.16 \text{ g}$) and the cisplatin treatment groups ($3.20 \pm 0.76 \text{ g}$; $p < 0.05$) (Fig. 2b). 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 ($5.95 \pm 1.16 \text{ g}$), HEC1-xenografted ($7.48 \pm 0.34 \text{ g}$), HEC1-A63-xenografted ($4.66 \pm 0.42 \text{ g}$) and HEC1-A77-xenografted mice ($4.82 \pm 1.08 \text{ g}$) (Fig. 2b). 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. 3a) 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. 3b). 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. 3b). 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. 3c). 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. 4a–4d). 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. 4c). Similar findings were observed in HEC1-A77 cells (Fig. 4d). 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. 4a and 4b). Thus, the results of the immunofluorescence analysis were in accordance with those of both Western blot analysis of CMF preparations and biotinylation

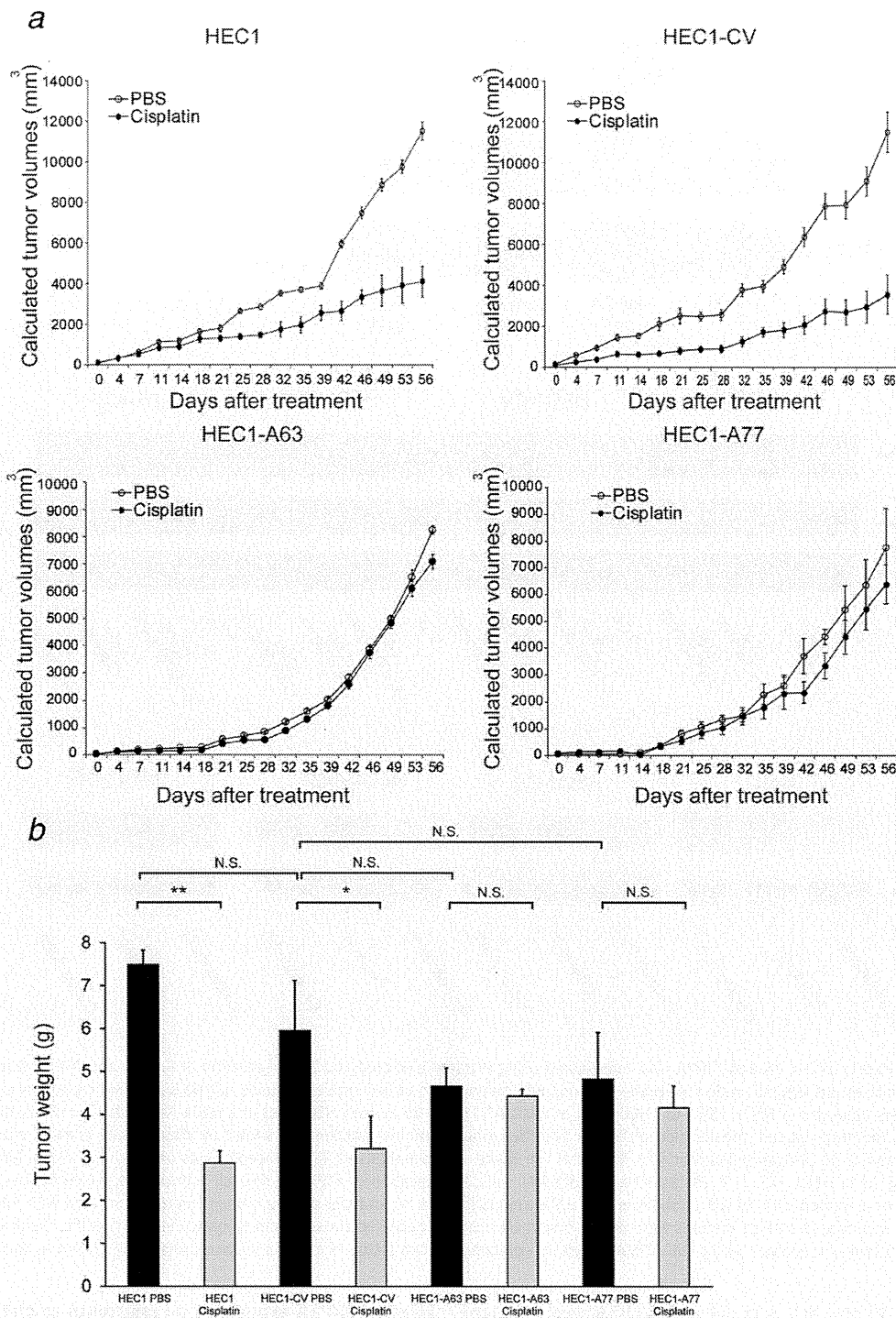


Figure 2. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vivo*. Analysis of Anx A4 as a platinum-resistant protein *in vivo*. (a) To determine the resistance of Anx A4-stably-expressing HEC1 cells to platinum *in vivo*, parent HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were 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 \pm SD (bars). (b) Fifty-six days after implantation, tumors were removed and weighed. Values shown are the means (\pm SD) of five mice. NS: not significant ($*p < 0.05$; $**p < 0.01$; one-way ANOVA, followed by Dunnett's analysis).

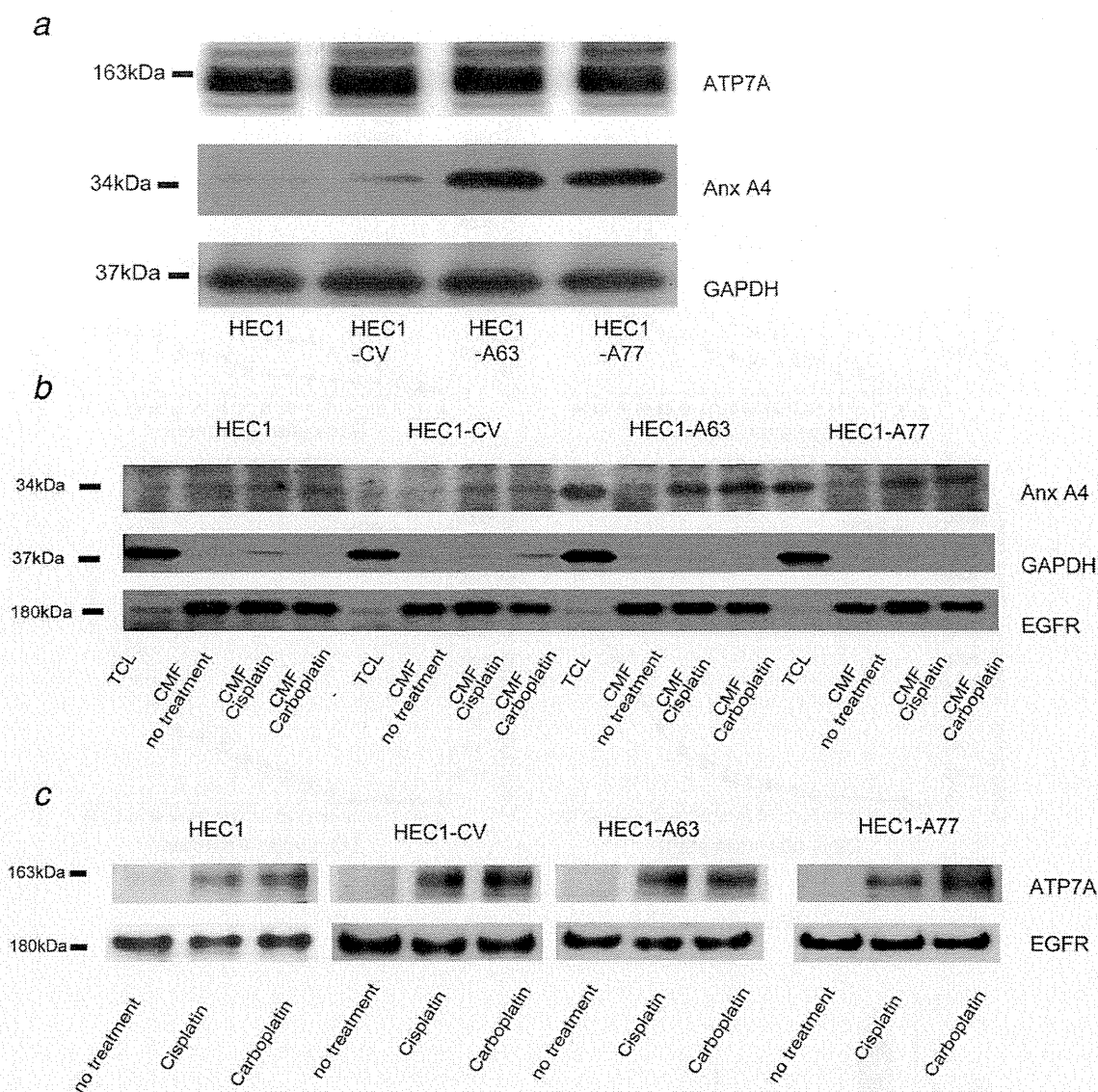


Figure 3. Localization of Anx A4 and ATP7A was investigated using Western blot analysis. The localization of Anx A4 and ATP7A was investigated using two techniques: orthogonal crude membrane fractions and biotinylation of cell surface proteins. (a) No significant change in expression levels of ATP7A 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 μ M cisplatin or 50 μ M 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 μ M cisplatin or 150 μ M carboplatin for 4 hr, and cell surface proteins were biotinylated with 500 μ M sulfo-NHS-SS-biotin. Biotinylated surface proteins were enriched with UltraLink Immobilized Neutravidin (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. 3b and 3c). 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. 5a). Anx A4 expression was unchanged by silencing ATP7A (Fig. 5a). The

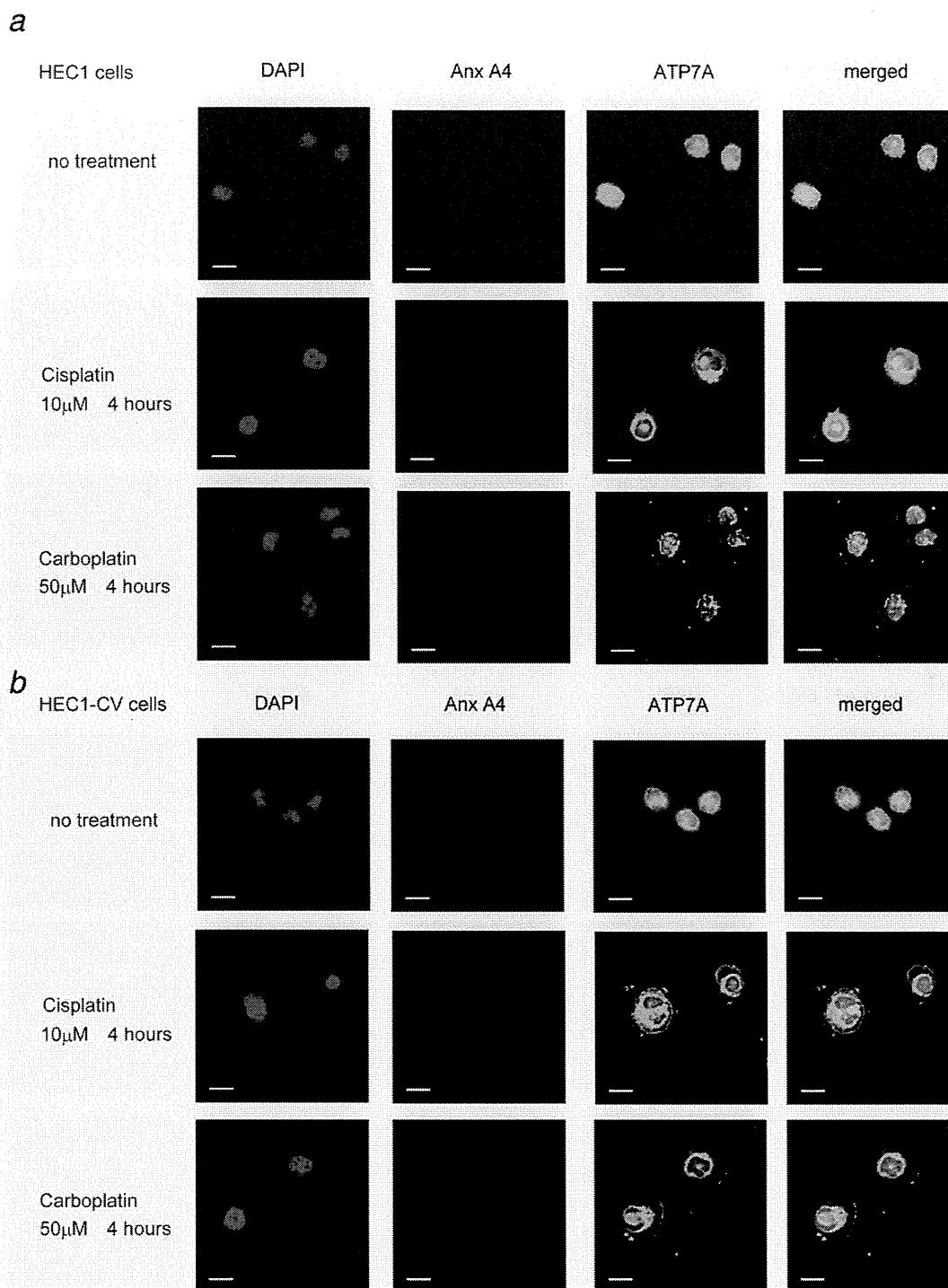


Figure 4. Immunofluorescence staining for ATP7A and Anx A4. HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were divided into three groups: the no treatment, cisplatin exposure and carboplatin exposure groups. (a) HEC1 cells, (b) HEC1-CV cells, (c) HEC1-A63 cells and (d) HEC1-A77 cells. Cells were incubated 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 relocated 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 μ m.

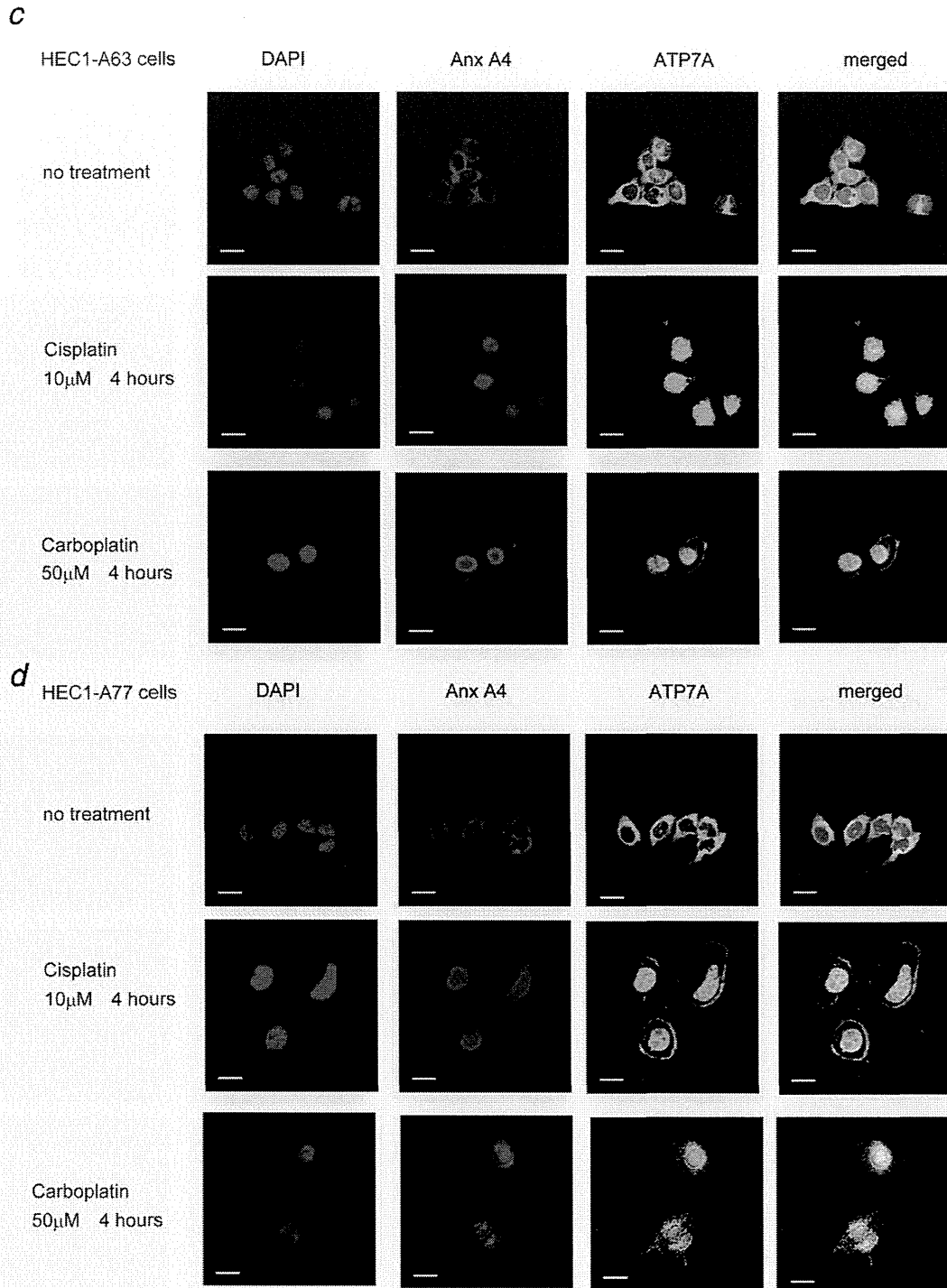


Figure 4. (Continued)

control and commercial siRNAs against ATP7A were transfected and the IC₅₀ values of cisplatin and carboplatin were determined for each cell line. The IC₅₀ value for cisplatin was

significantly lower for the two kinds of ATP7A-silenced HEC1-A63 cells (ATP7A siRNA4, IC₅₀ = 11.0 μ M, $p < 0.01$; ATP7A siRNA6, IC₅₀ = 11.2 μ M, $p < 0.01$) compared with

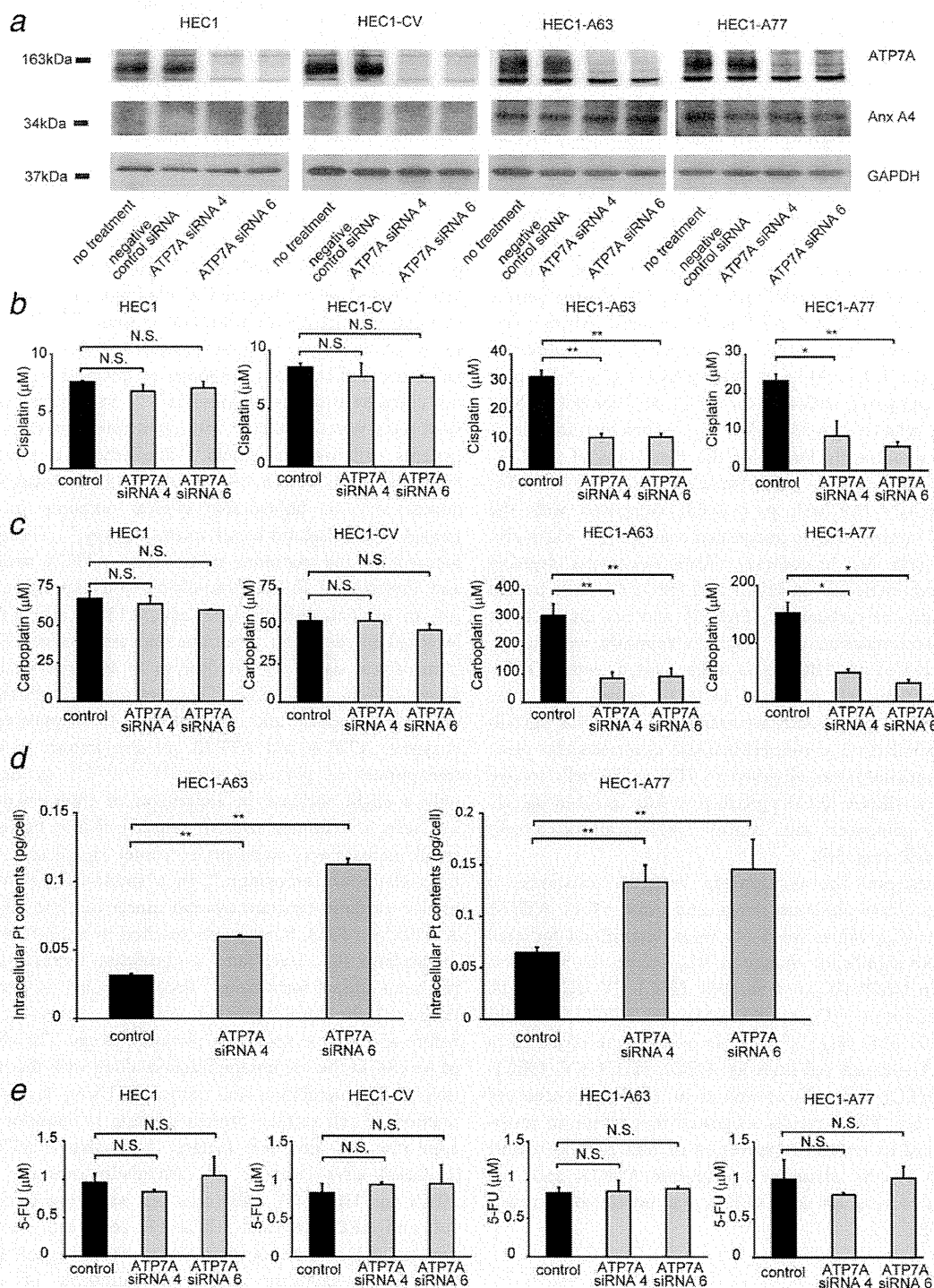


Figure 5. Knockdown of ATP7A expression improves platinum chemosensitivity in Anx A4-overexpressing cells. (a) Knockdown expression of ATP7A by siRNA in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells by Western blot analysis. (b) IC_{50} values are shown for cisplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 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 ($IC_{50} = 32.2 \mu\text{M}$) (Fig. 5b).

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, $IC_{50} = 85.9 \mu\text{M}$, $p < 0.01$; siRNA6, $IC_{50} = 92.8 \mu\text{M}$, $p < 0.01$) compared with the HEC1-A63 control siRNA-transfected cells ($IC_{50} = 300.7 \mu\text{M}$) (Fig. 5c). Similar results were found for HEC1-A77 ATP7A-silenced cells, where a significantly lower IC_{50} value for cisplatin was observed (siRNA4, $IC_{50} = 8.9 \mu\text{M}$, $p < 0.05$; siRNA6, $IC_{50} = 6.2 \mu\text{M}$, $p < 0.01$) compared with that for HEC1-A77 control siRNA-transfected cells ($IC_{50} = 23.3 \mu\text{M}$). IC_{50} values for carboplatin were also significantly lower for the two kinds of ATP7A-silenced HEC1-A77 cells (siRNA4, $IC_{50} = 49.8 \mu\text{M}$, $p < 0.05$; siRNA6, $IC_{50} = 31.9 \mu\text{M}$, $p < 0.05$) compared with the HEC1-A77 control siRNA-transfected cells ($IC_{50} = 152.1 \mu\text{M}$, $p < 0.01$) (Fig. 5c). In contrast, siRNA treatments targeting ATP7A were ineffective in HEC1 and HEC1-CV cells treated with cisplatin or carboplatin (Figs. 5b and 5c). 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. 5d). 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. 5d).

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 ($IC_{50} = 0.96 \mu\text{M}$), HEC1-CV ($IC_{50} = 1.00 \mu\text{M}$), HEC1-A63 ($IC_{50} = 0.83 \mu\text{M}$) or HEC1-A77 cells ($IC_{50} = 1.01 \mu\text{M}$) (Fig. 5e). 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. 5e). 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 Anx A4 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- κ B 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- κ B 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 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} target-

ing Anx A4 may lead to the development of an effective therapy for overcoming chemoresistance in more types of cancer.

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References

- 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.
- Thigpen T, Vance R, Punecky L, et al. Chemotherapy in advanced ovarian carcinoma: current standards of care based on randomized trials. *Gynecol Oncol* 1994;55:S97–S107.
- Vaughan S, Coward JI, Bast RC, Jr, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer* 2011;11:719–25.
- 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.
- 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.
- Obel JC, Friberg G, Fleming GF. Chemotherapy in endometrial cancer. *Clin Adv Hematol Oncol* 2006;4:459–68.
- 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).
- 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.
- Pectasides D, Fountzilias G, Aravantinos G, et al. Advanced stage clear-cell epithelial ovarian cancer: the Hellenic Cooperative Oncology Group experience. *Gynecol Oncol* 2006;102:285–91.
- 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.
- 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.
- 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.
- 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.
- Gerke V, Moss SE. Annexins: from structure to function. *Physiol Rev* 2002;82:331–71.
- 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.
- Kaetzel MA, Mo YD, Mealy TR, et al. Phosphorylation mutants elucidate the mechanism of annexin IV-mediated membrane aggregation. *Biochemistry* 2001;40:A192–9.
- 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.
- 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.
- Alfonso P, Canamero M, Fernandez-Carbonie F, et al. Proteomic analysis of membrane fractions in colorectal carcinomas by using 2D-DIGE saturation labeling. *J Proteome Res* 2008;7:4247–55.
- 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.
- Sitek B, Luttes 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.
- 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.
- 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.
- 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.
- Gourdon P, Liu XY, Skjorringe T, et al. Crystal structure of a copper-transporting PIB-type ATPase. *Nature* 2011;475:59–64.
- 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.
- 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.
- 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.
- 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.
- 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.
- Rabik CA, Maryon EB, Kasza K, et al. Role of copper transporters in resistance to platinating agents. *Cancer Chemother Pharmacol* 2009;64:133–42.
- Galluzzi L, Senovilla L, Vitale I, et al. Molecular mechanisms of cisplatin resistance. *Oncogene* 2012;31:1869–83.
- Kelland L. The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* 2007;7:573–84.
- 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.
- 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.
- Materna V, Plegler 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.
- 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.
- 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.
- Kuo MT, Chen HH, Song IS, et al. The roles of copper transporters in cisplatin resistance. *Cancer Metastasis Rev* 2007;26:71–83.
- 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.
- Mangala LS, Zuzel V, Schmandt R, et al. Therapeutic targeting of ATP7B in ovarian carcinoma. *Clin Cancer Res* 2009;15:3770–80.
- Kalayda GV, Wagner CH, Buss J, et al. Altered localisation of the copper efflux transporters ATP7A and ATP7B associated with cisplatin