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Histamine Contributes to Tissue Remodeling via Periostin Expression

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Histamine is thought to have a critical role in the synthesis of extracellular matrix in skin and may be involved in tissue remodeling of allergic diseases. Recent studies revealed that periostin, a matricellular protein, contributed to tissue remodeling; however, a link between periostin and histamine remains unproven. We investigated whether periostin was involved in histamine-induced collagen production. Cultured dermal fibroblasts derived from wild-type (WT) or periostin knockout ($PN^{-/-}$) mice were stimulated with histamine, and then collagen and periostin production was evaluated. Histamine induced collagen gene expression in WT fibroblasts in the late phase but not in the early phase, whereas no effect on collagen expression was observed in histamine-stimulated $PN^{-/-}$ fibroblasts. In WT fibroblasts, histamine directly induced periostin expression in a dose-dependent manner, and an H1 receptor antagonist blocked both periostin and collagen expression. Histamine activated extracellular signal-regulated kinase 1/2 (ERK1/2) through the H1 receptor. Periostin induction was inhibited by either H1 antagonist or ERK1/2 inhibitor treatment *in vitro* and was attenuated in $H1R^{-/-}$ mice. Elevated expression of periostin was found in lesional skin from atopic dermatitis patients. These results suggest that histamine mediates periostin induction and collagen production through activation of the H1 receptor-mediated ERK1/2 pathway; furthermore, histamine may accelerate the chronicity of atopic dermatitis.

Journal of Investigative Dermatology advance online publication, 3 April 2014; doi:10.1038/jid.2014.120

INTRODUCTION

Tissue remodeling is both a cause and a consequence of allergic inflammation and is considered a target for therapeutic intervention.

Understanding the underlying mechanisms that cause tissue remodeling is progressing gradually, and certain factors that are correlated with allergic inflammation have been found to be involved in tissue remodeling. Among them, histamine has been found to induce *de novo* synthesis of collagen from fibroblasts in both *in vivo* and *in vitro* experiments (Sandberg, 1962, 1964; Cohen *et al.*, 1972; Murota *et al.*, 2008). Furthermore, it is well known that antihistamine drugs used for the treatment of allergic disorders improve hypertrophic scars (Murakami *et al.*, 1998). These results indicate that histamine may be involved in the mechanism of tissue

remodeling in allergic diseases; however, it remains unknown how histamine contributes to tissue remodeling.

Recent studies showed that expression of periostin, a matricellular protein with profibrogenic function, increased in sera and lesional tissue from patients with allergic diseases, such as allergic rhinitis, asthma, and atopic dermatitis (AD), and that this expression was associated with airway or other tissue remodeling (Takayama *et al.*, 2006; Hur *et al.*, 2012; Masuoka *et al.*, 2012). Furthermore, periostin has also emerged as a key regulator in the development of wound healing and scleroderma (Ontsuka *et al.*, 2012; Yang *et al.*, 2012). To the best of our knowledge, the impact of histamine on the expression level of periostin is unknown. Therefore, in this study, we investigated the correlation between histamine and the fibrotic factor periostin in primary cultured dermal fibroblasts.

RESULTS

Collagen production is induced by histamine stimulation

To investigate whether histamine influences collagen synthesis, primary cultured murine dermal fibroblasts were stimulated with histamine at concentrations ranging from 0 to 100 μ M, as described in our previous report (Murota *et al.*, 2008; Murota and Katayama, 2009).

Histamine-induced type I collagen production was observed with concentrations of histamine from 1 to 100 μ M (Figure 1). Compared with nontreated controls, collagen synthesis was significantly increased after histamine treatment

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Abbreviations: AD, atopic dermatitis; CREB, cAMP response element-binding protein; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H1R, histamine receptor 1; WT, wild type

Received 2 October 2013; revised 3 February 2014; accepted 6 February 2014; accepted article preview online 27 February 2014

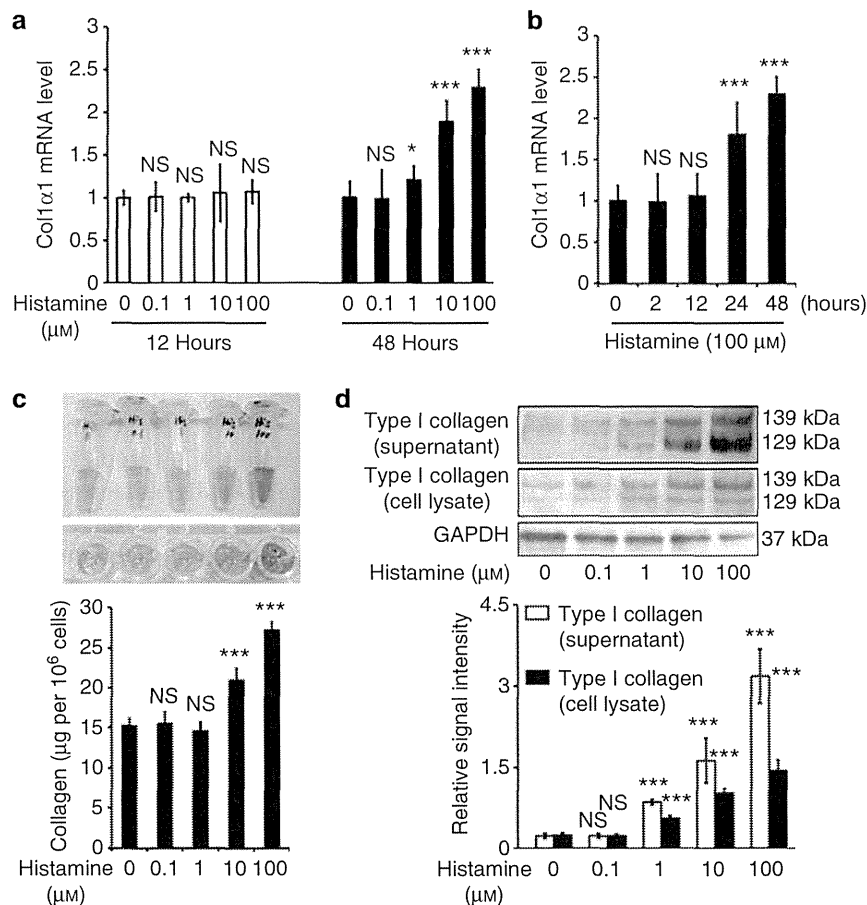


Figure 1. Collagen production is induced by histamine stimulation in cultured wild-type (WT) fibroblasts. (a) Effect of histamine on collagen type-I alpha 1 (Col1α1) mRNA expression was assessed by quantitative real-time reverse transcriptase-PCR (qRT-PCR) at 12 hours (white bar) and 48 hours (black bar) after the addition of histamine at the indicated concentration. (b) Effect of histamine on Col1α1 mRNA expression after the stimulation with histamine (100 μM) for the indicated time periods. (c) Soluble collagen content in the supernatants of WT fibroblasts that had been stimulated with histamine for 48 hours. (d) Representative western blotting and quantitative analyses of signal density on blots from three independent experiments analyzing collagen protein expression in response to 48 hours of histamine stimulation (using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control). Values were derived from three independent experiments using WT fibroblast cultures. Values represent the mean ± SD for the three independent experiments in each condition. **P* < 0.05; ****P* < 0.001; NS, no significance, compared with control (0 μM histamine) by one-way analysis of variance (ANOVA) followed by Dunnett's test.

in a dose-dependent manner (Figure 1a, c and d). No significant increase was observed at 2 and 12 hours, whereas collagen was markedly induced by histamine 24 and 48 hours after addition (Figure 1a and b). In our previous study (Yang *et al.*, 2012), the mRNA expression of type I collagen was found to be significantly increased in cultured mouse dermal fibroblasts after a 2-hour stimulation with recombinant periostin alone. Therefore, these late responses in the present study may be a result of *de novo* synthesis of certain second messengers.

Periostin is upregulated upon histamine stimulation in dermal fibroblasts

Next, to investigate whether histamine affects the expression level of periostin, we stimulated wild-type (WT) primary dermal fibroblasts with histamine at the indicated concentrations (Figure 2). As expected, two hours of incubation with histamine produced a significant dose-dependent increase of

periostin mRNA expression in dermal fibroblasts, as assessed by reverse transcriptase-PCR and quantitative real-time reverse transcriptase-PCR (Figure 2a and b). After 24 hours of incubation with histamine, the periostin protein levels increased in the culture supernatant and cell lysates (Figure 2 c). These results suggest that histamine may directly upregulate the transcription and synthesis of periostin.

Histamine upregulates periostin expression via histamine receptor 1 (H1R)

To identify the histamine receptor subtype responsible for the histamine-induced periostin expression, antagonists for H1R, H2R, and H4R were tested *in vitro*. The effects of histamine on periostin mRNA and protein levels were evaluated at 2 hours or 24 hours, respectively, in dermal fibroblasts after histamine stimulation following preincubation with or without these histamine receptor antagonists (Figure 3a and b). Histamine-induced periostin expression was blocked by H1R antagonist

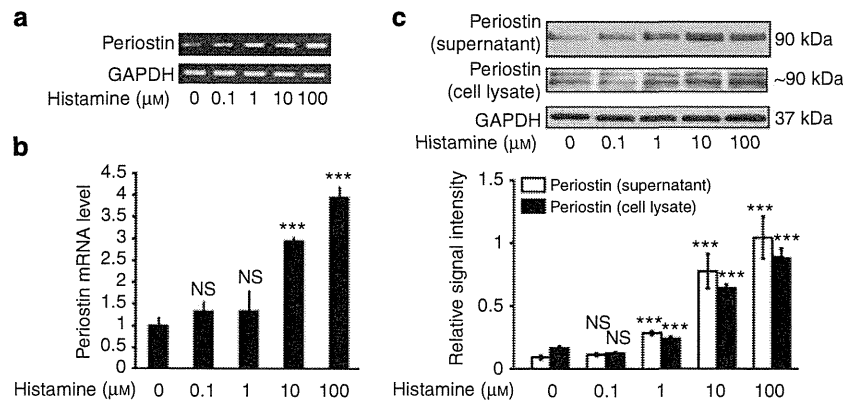


Figure 2. Periostin is upregulated upon histamine stimulation in cultured wild-type (WT) fibroblasts. WT fibroblasts were stimulated with histamine at the indicated concentrations for 2 hours (a, b) or 24 hours (c). Periostin mRNA expression was determined by reverse transcriptase–PCR (RT-PCR) analysis (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* (Derf1) 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.

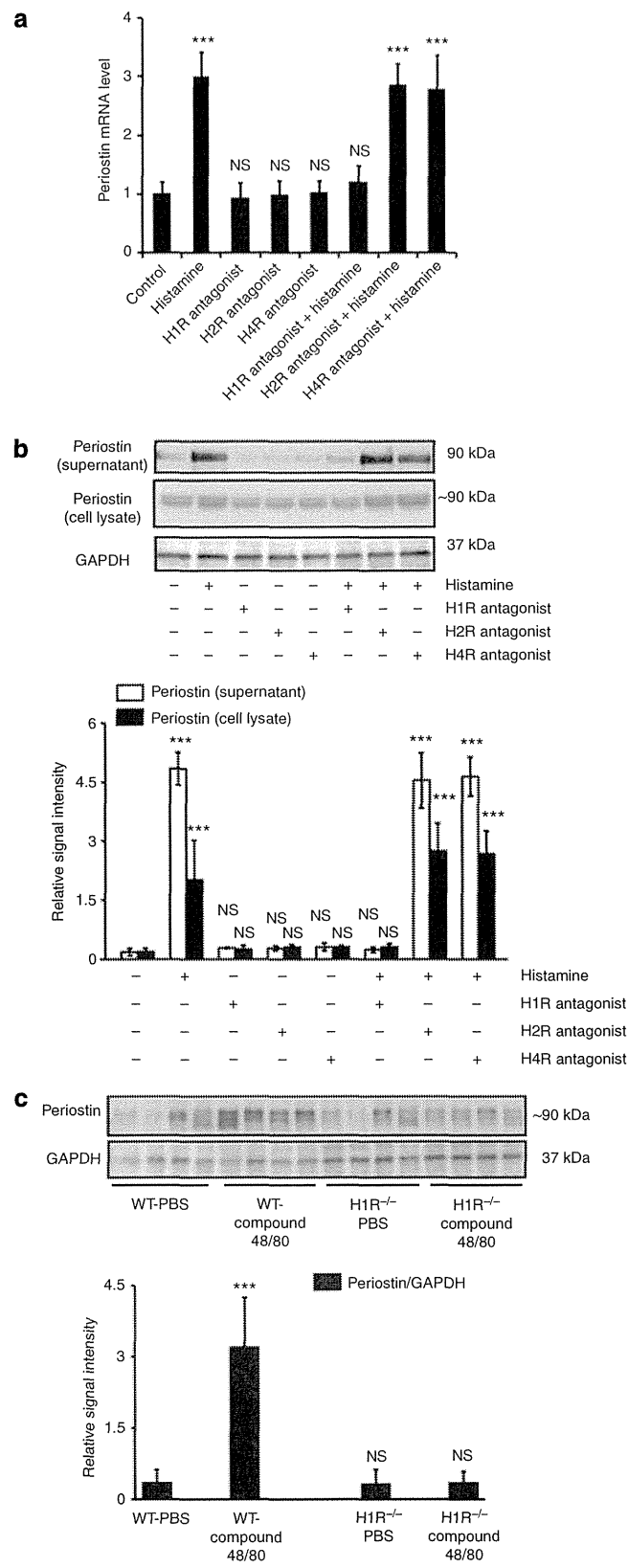


Figure 3. Histamine upregulates periostin expression via histamine receptor 1 (H1R) *in vitro* and *in vivo*. *In vitro*, wild-type (WT) fibroblasts were either treated with histamine antagonists (H1R, H2R, or H4R; 100 μ M) or left untreated for 2 hours, and then cells were stimulated with histamine (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.

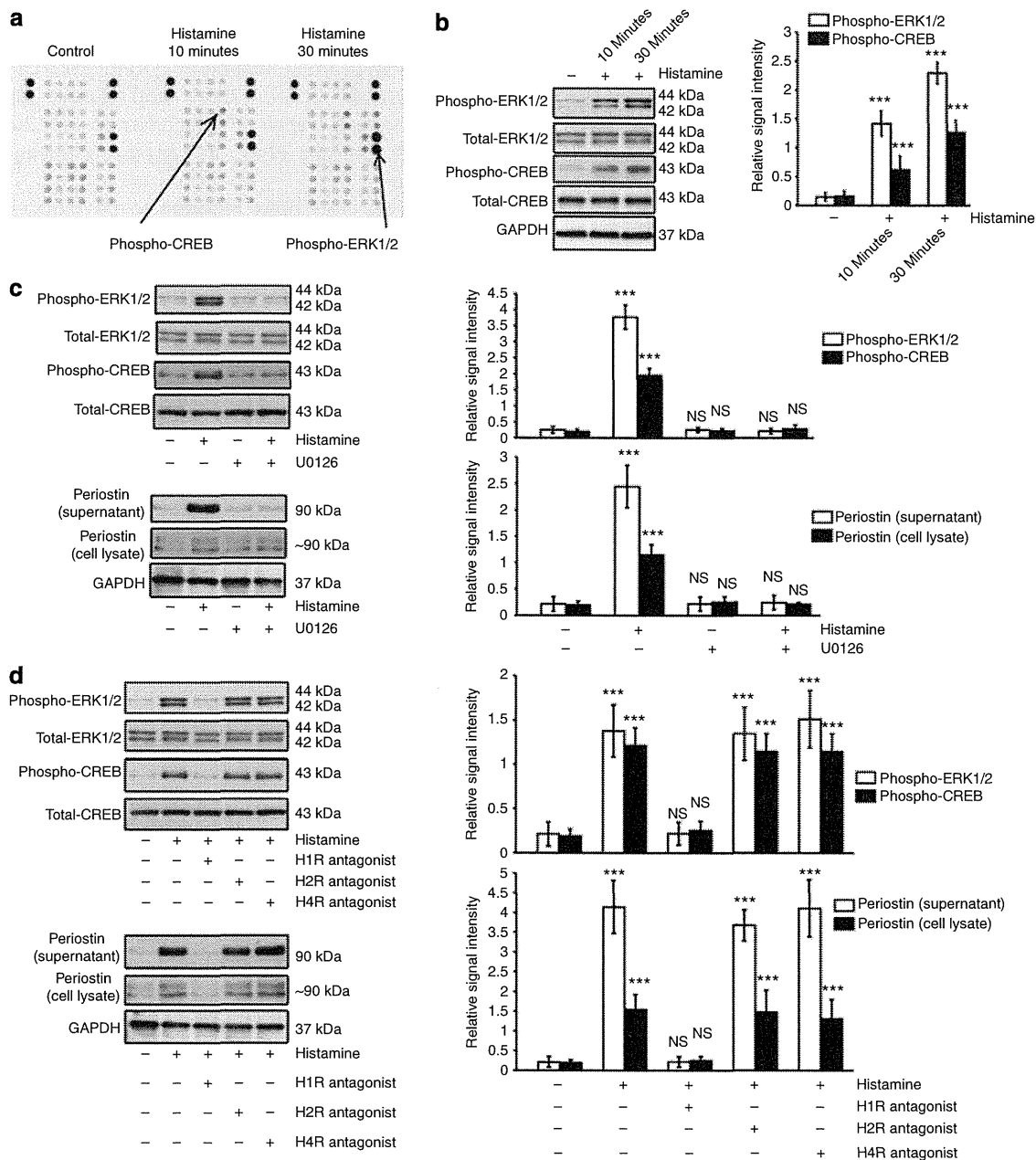
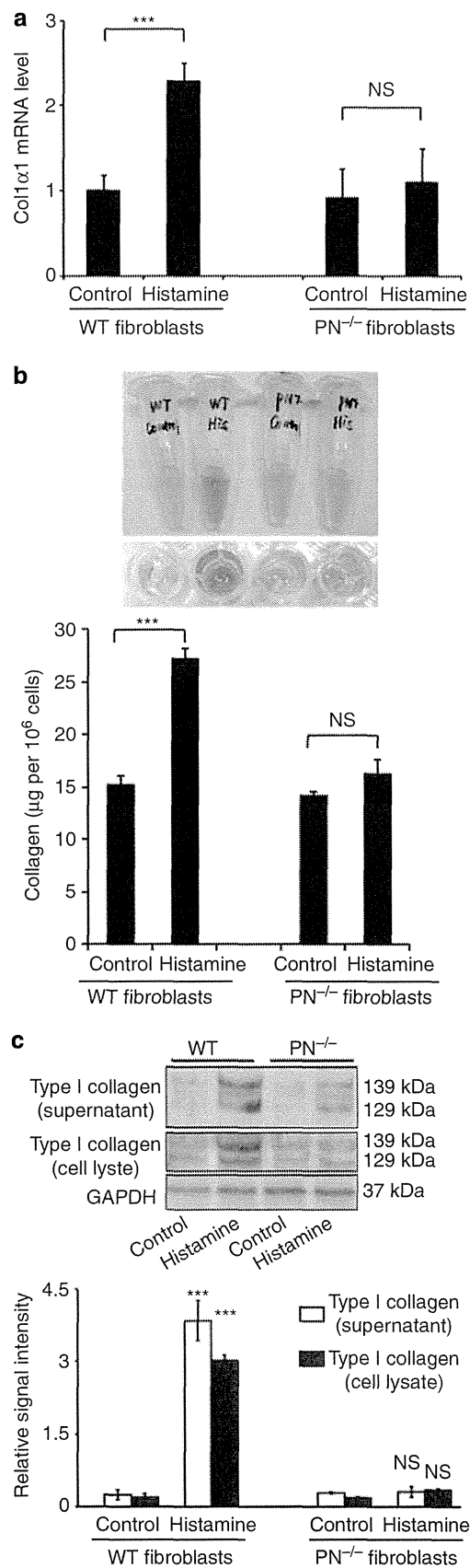


Figure 4. Histamine receptor 1 (H1R) activation upregulates periostin expression via the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. (a) The phosphorylation state was detected by R&D Systems Proteome Profiler Phospho-Kinase Array in normal human dermal fibroblasts, which were either untreated 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; Ontsuka *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,

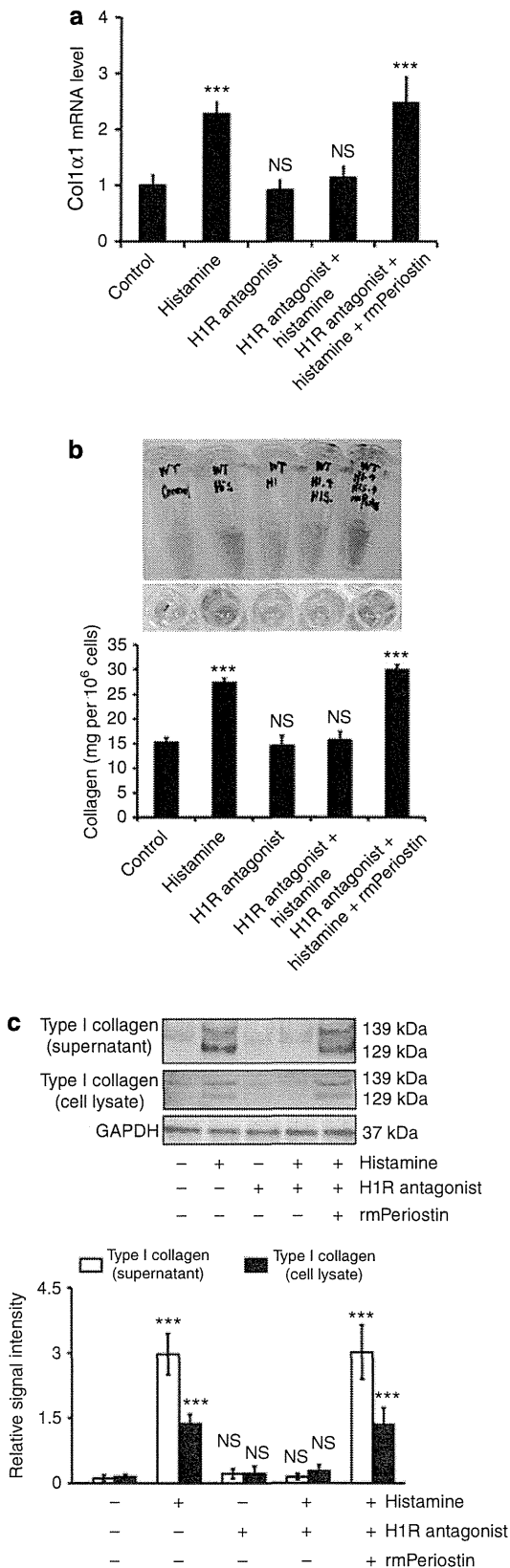


Figure 6. Histamine receptor 1 (H1R)-mediated upregulation of periostin is essential in histamine-induced collagen production. Wild-type (WT) fibroblasts cultured with or without H1R antagonist preincubations were stimulated with histamine ($100 \mu\text{M}$) alone or in the presence of recombinant mouse 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 mM, 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 of 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', anti-sense 5'-GGAGACCTCTTTTGCAAGA-3'; collagen type-I alpha 1 (Col1- α 1), sense 5'-GAGCCCTCGCTTCCGTACTC-3', antisense 5'-TGTTCCCTACTCAGCCGTCTGT-3'; and GAPDH, sense 5'-TGTCATCATACTTGGCAGGTTTCT-3', antisense 5'-CATGGCCTTCCGTGTTCTA-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.

ACKNOWLEDGMENTS

We thank Kenju Nishida for excellent technical support. This study was partly supported by a research grant from the Ministry of Health, Labour and Welfare, Japan.

SUPPLEMENTARY MATERIAL

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

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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-

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

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

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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'-GCGUAGCUCCAGAGGUUUATT-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.

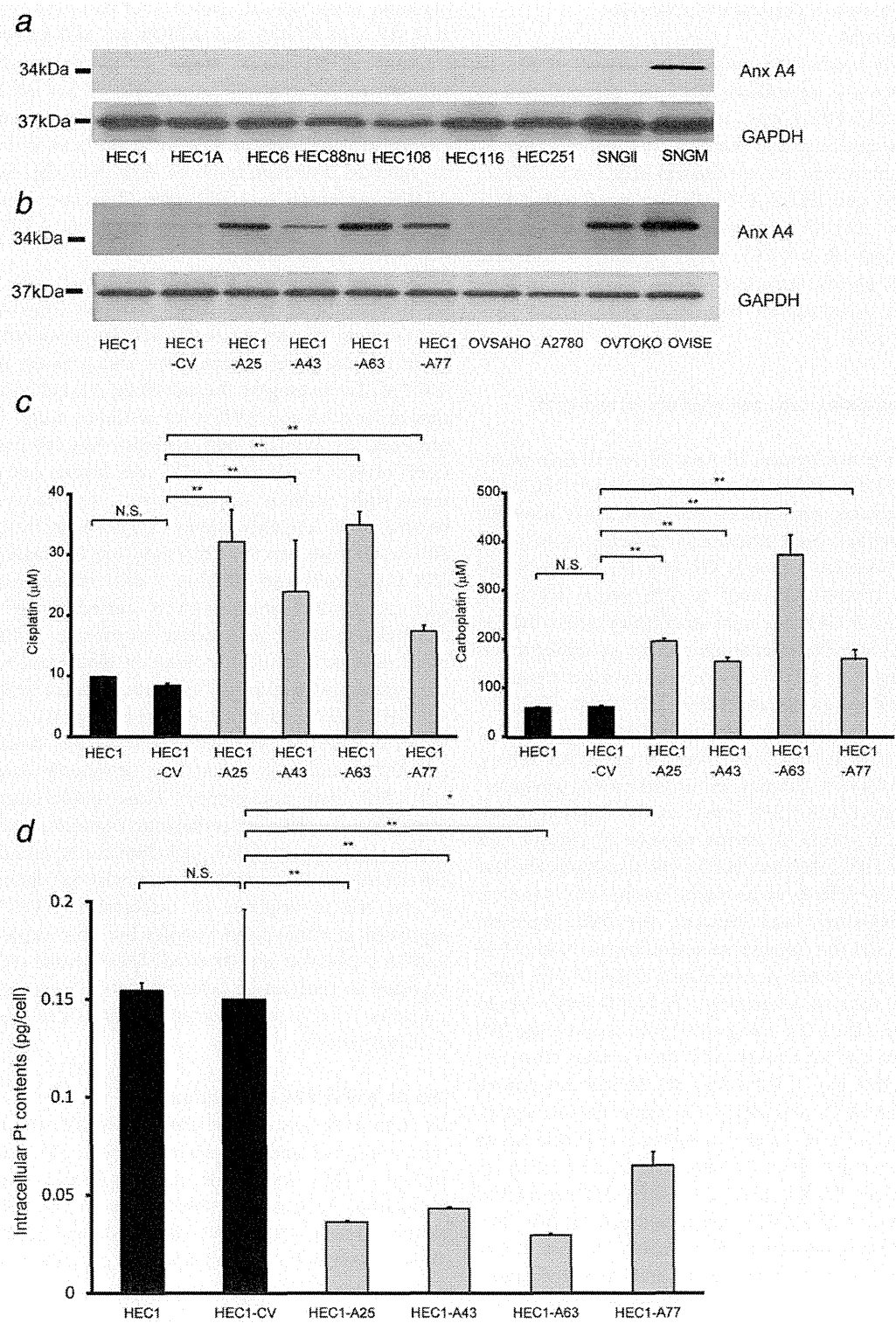


Figure 1. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vitro*. (a) Western blot analysis of nine endometrial carcinoma cell lines. Anx A4 was expressed in one cell line. (b) Establishment of an Anx A4-stably-expressing HEC1 cell line by transfection with the pcDNA3.1-Anx 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

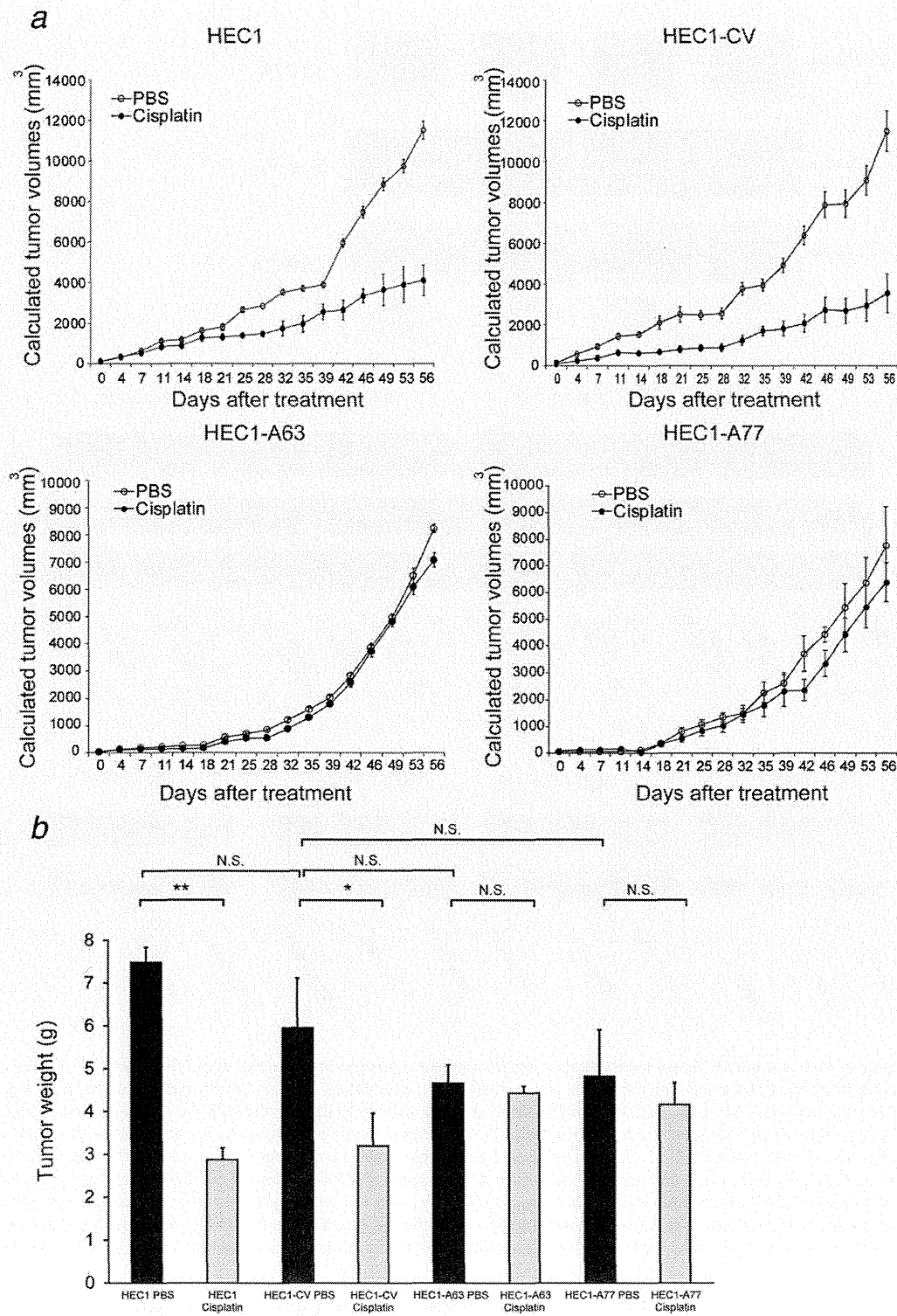


Figure 2. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vivo*. Analysis of Anx A4 as a platinum-resistant protein *in vivo*. (a) To determine the resistance of Anx A4-stably-expressing HEC1 cells to platinum *in vivo*, parent HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were 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).

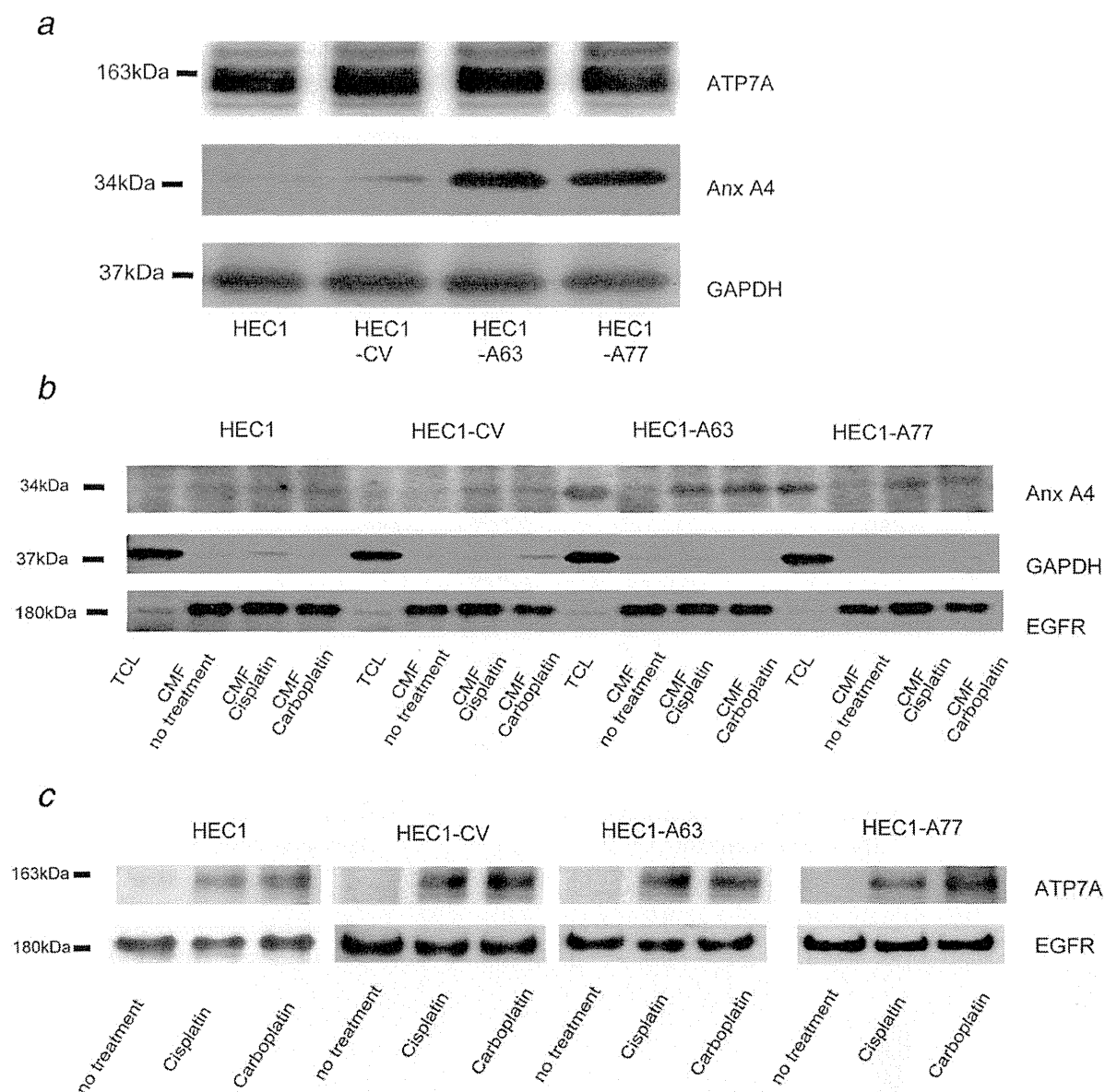


Figure 3. Localization of Anx A4 and ATP7A was investigated using Western blot analysis. The localization of Anx A4 and ATP7A was investigated using two techniques: orthogonal crude membrane fractions and biotinylation of cell surface proteins. (a) No significant change in expression levels of ATP7A 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

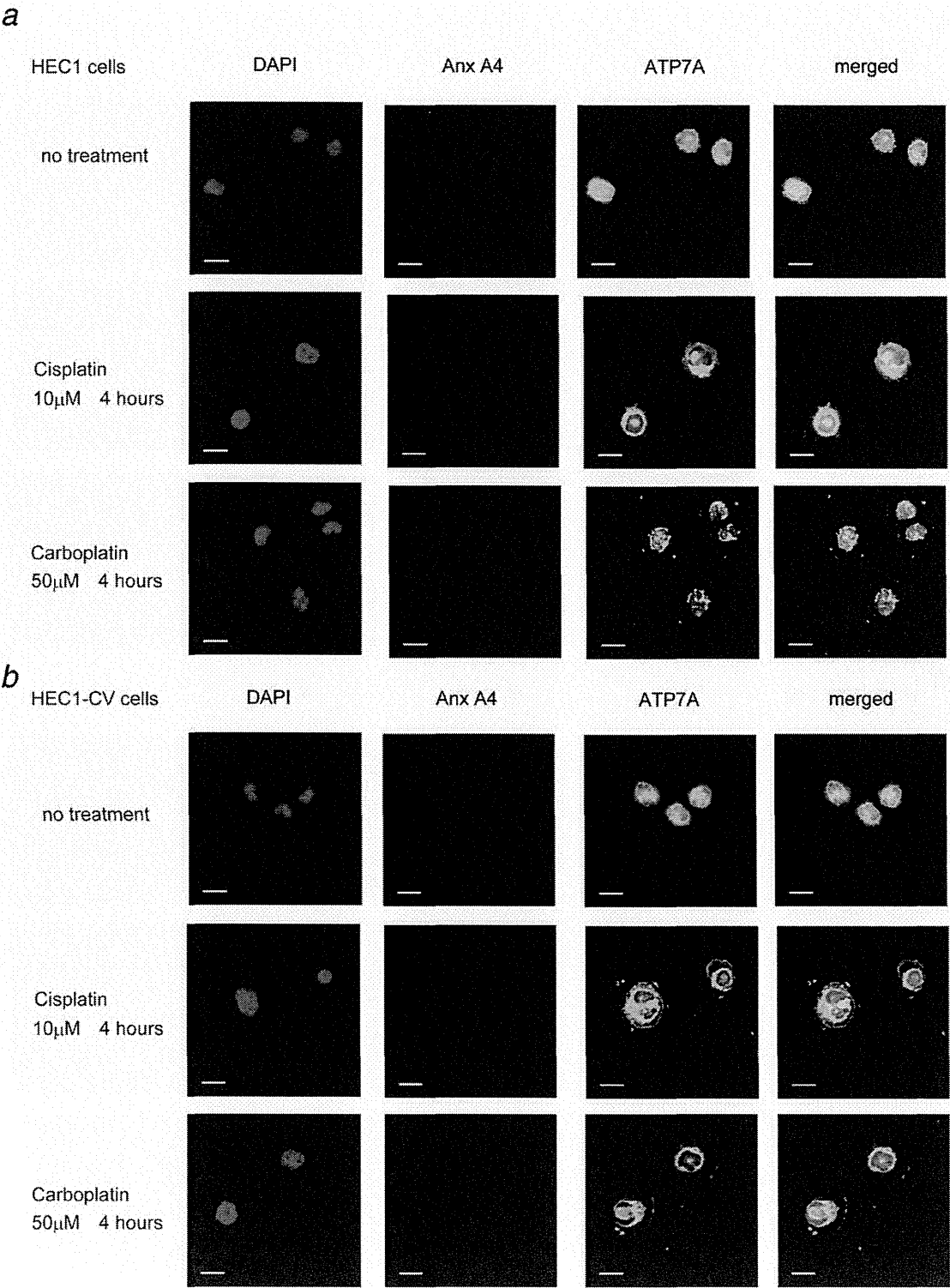
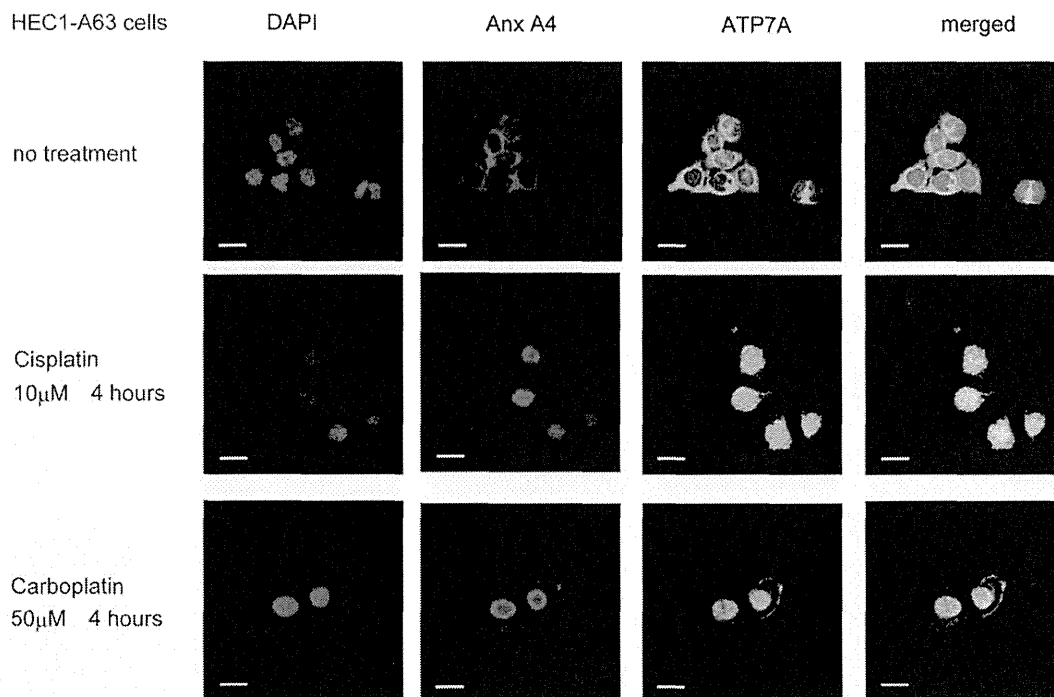


Figure 4. Immunofluorescence staining for ATP7A and Anx A4. HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were divided into three groups: the no treatment, cisplatin exposure and carboplatin exposure groups. (a) HEC1 cells, (b) HEC1-CV cells, (c) HEC1-A63 cells and (d) HEC1-A77 cells. Cells were incubated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

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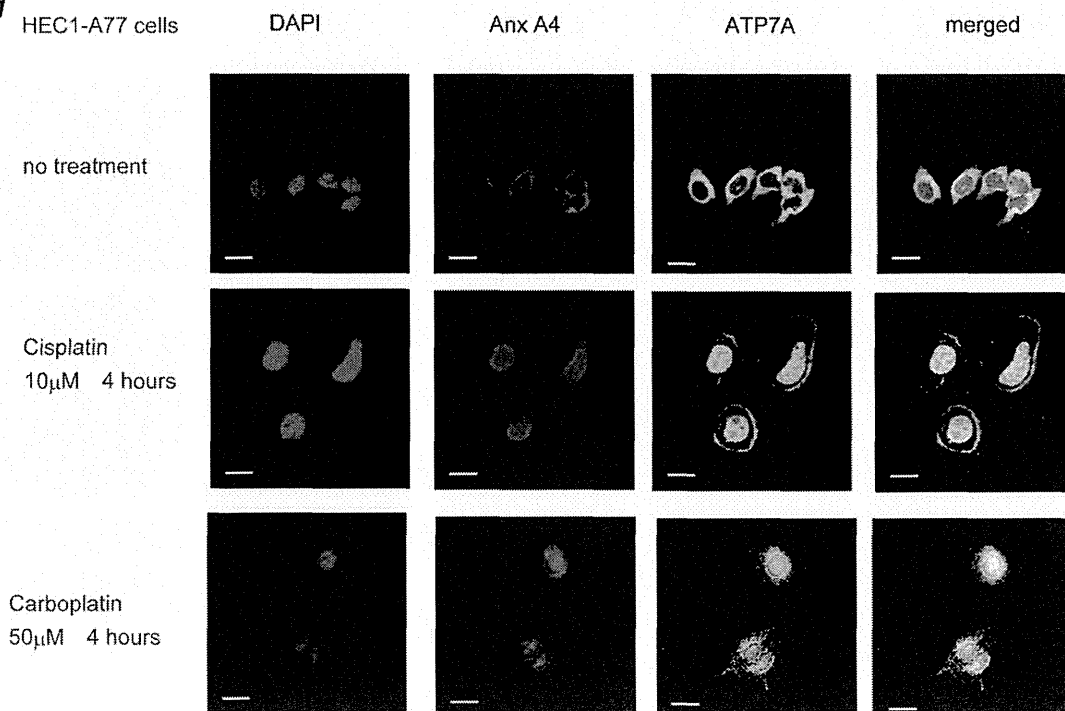


Figure 4. (Continued)

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

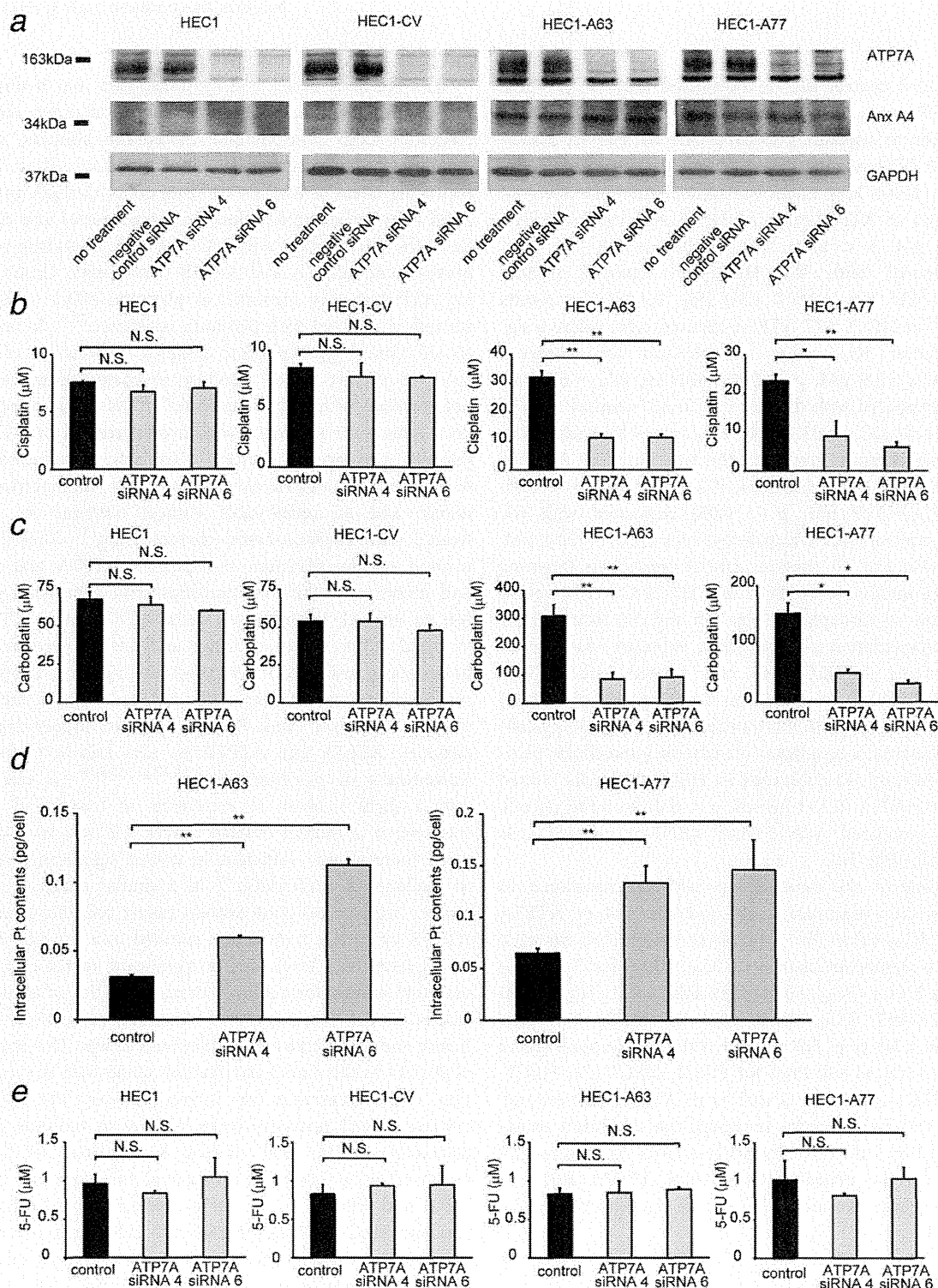


Figure 5. Knockdown of ATP7A expression improves platinum chemosensitivity in Anx A4-overexpressing cells. (a) Knockdown expression of ATP7A by siRNA in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells by Western blot analysis. (b) IC₅₀ values are shown for cisplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells transfected with negative control siRNA and two types of siRNA targeting ATP7A. A significant decrease in IC₅₀ 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) IC₅₀ 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 IC₅₀ 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 IC₅₀ 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).