RCT and those of well-designed observational studies on the same topics were compared.[6,7] Benson *et al.* reviewed 136 reports about 19 diverse treatments, such as calcium channel-blocker therapy for coronary artery disease, and hormone-replacement therapy for osteoporosis, and showed that well-designed observational studies and RCT overall produce similar results.[6] Concato *et al.* reviewed 99 reports published in five major journals (*Annals of Internal Medicine*, *The British Medical Journal*, *The Journal of the American Medical Association*, *The Lancet*, and *The New England Journal of Medicine*) about five clinical topics and showed that results of RCT are inconsistent in some series. In contrast, results of well-designed observational studies are mostly consistent.[7] In view of the reproducibility of study results, observational studies were superior. How can we account for these results? McKee *et al.* pointed out that RCT have been conducted using very small groups and that subjects excluded from an RCT tend to have a poorer prognosis than that of subjects included in the trial.[8] RCT definitely rank at the top of all types of clinical studies because they are internally valid. However, the results of RCT are relevant to just a definable group of patients in a particular setting. Therefore, results of RCT cannot be easily overgeneralized.

Reasons for Preoperative Risk Assessment in Surgical Studies

What should we do in order to maximize the therapeutic effect of surgery and minimize its invasiveness? Two strategies are: (i) to remove lymph nodes most likely to harbor disease and spare lymph nodes that are unlikely to be affected; and (ii) to allocate only patients with potential benefit from lymphadenectomy to full lymphadenectomy. The first strategy includes sentinel lymph node (SLN) mapping surgery[9–11] and circumflex iliac nodes distal to the external iliac nodes (CINDEIN)-sparing surgery.[12–14] The second strategy needs preoperative risk assessment. However, it has not been clarified which patients have potential benefit from lymphadenectomy. In this session, we focus on the second strategy. GOG #33 showed that there was no case with nodal metastasis in the low-risk group defined as having no myometrial invasion, grade 1 endometrioid histology, and no intraperitoneal disease.[15] Mariani *et al.* confirmed a low-risk group with grade 1 to 2 endometrioid histology, depth of invasion of ≤50%, and tumor size of ≤2 cm.[16] They concluded that lymphadenectomy does not benefit patients in the low-risk group (so-called Mayo criteria). Milam *et al.* also demonstrated that these criteria led to a rate of nodal metastasis of only 0.8% in the low-risk group of the Mayo criteria.[17] However, all of these criteria depend on surgicopathologic findings. There have been only a few studies that aimed to establish preoperative risk assessment for predicting lymph node metastasis in endometrial cancer.[18,19] The results of these studies are shown in Table 1. In 2007, Todo *et al.* proposed a low-risk group with grade 1 to 2 endometrioid histology by endometrial biopsy, volume index of ≤36 by magnetic resonance imaging (MRI), and low cancer antigen (CA)-125 level (70 U/mL for patients aged less than 50 years and 28 U/mL for patients aged 50 years or over) before surgery; only 2.1% of the patients in the group had lymph node metastasis at the assumed prevalence of nodal metastasis of 10%.[18] In 2012, Kang *et al.* confirmed a low-risk group with endometrioid histology by endometrial biopsy, <50% myometrial invasion with no extension beyond the corpus and no enlarged lymph nodes by MRI, and CA-125 level ≤35 U/mL before surgery; only 1.3% of the patients in the group had lymph node metastasis when assuming that the prevalence of lymph node metastasis is 10% in the target patient cohort.[19] As many physicians are not familiar with measuring tumor volume of endometrial cancer, volume index could not be easily used as a factor of preoperative risk assessment. On the other hand, myometrial invasion assessment by MRI has a problematic issue, namely, interobserver inconsistency or variability. MRI-based evaluation of deep myometrial invasion in a multi-institutional cooperative study showed sensitivity of 54% and specificity of 89%, indicating that results of previous single institutional studies might have been biased.[20] There would be some occasions where attending physicians have difficulty in judging myometrial invasion using MRI. Although each set of criteria have their merits and demerits, it is possible to reconcile these criteria. When it is difficult to judge myometrial invasion using MRI, volume index could be used as a substitute index. When planning a prospective clinical trial on the therapeutic significance of lymphadenectomy, an adequate population is needed to assess the full benefit of lymphadenectomy. If a population comprises a large proportion of low-risk patients, the significance of lymphadenectomy would be underestimated because low-risk patients do not benefit from lymphadenectomy.

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Table 1 Results of preoperative risk assessment for excluding lymph node metastasis in endometrial cancer

Author	Todo *et al.*[18]		Kang *et al.*[19]	
Journal	*Gynecol Oncol* (2007)		*J Clin Oncol* (2012)	
Study design	Retrospective cohort study		Retrospective cohort study	
Study aim	Model Derivation	Validation	Model Derivation	Validation
Cases (*n*)	214	211	360	180
Median age (range)	56 (23–80)	57 (24–77)	53 (29–76)	54 (31–82)
FIGO stage (1988)	I: 68% II: 5% III/IV: 27% Unknown: 0%	I: 64% II: 8% III/IV: 28% Unknown: 0%	I: 71% II: 7% III/IV: 20% Unknown: 2%	I: 76% II: 5% III/IV: 19% Unknown: 0%
Histological subtype	Endometrioid: 97% Non-endometrioid: 3%	Endometrioid: 94% Non-endometrioid: 6%	Endometrioid: 94% Non-endometrioid: 6%	Endometrioid: 94% Non-endometrioid: 6%
LNM (rate)	14.5%	17.1%	12.5%	12.8%
PANM (rate)	8.9%	12.3%	NA	NA
Number of lymph nodes harvested (median)	70	77	27	22
Para-aortic node dissection (rate)	99%	100%	61%	51%
Low-risk criteria for LNM	Histologic subtype/grade (endometrial biopsy): endometrioid G1 or G2 Tumor volume (MRI): <36 cm³ CA-125: <70 U/mL (less than 50 years), <28 U/mL (50years or over)		Histologic subtype (endometrial biopsy): Endometrioid Myometrial invasion (MRI): <1/2 Extension beyond uterine corpus (MRI): none Lymph node size (MRI): <1 cm in short axis CA-125: <35 U/mL	
Proportion of patients in the low-risk group	54%	45%	53%	43%
LNM (false negative) rate in the low-risk group	3.6%	3.2%	1.7%	1.4%
Bayesian-adjusted LNM (false negative) rate in the low-risk group†	2.5%	1.9%	1.4%	1.1%

†Adjusted rate at the prevalence of nodal metastasis of 10%. CA-125, cancer antigen 125; FIGO, International Federation of Gynecology and Obstetrics; LNM, lymph node metastasis; MRI, magnetic resonance imaging; NA, not available; PANM, para-aortic node metastasis.

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Disclosure

The author declares no conflicts of interest.

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Proposal of a concept and design of a randomized phase III trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer

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Abstract

Although prospective studies have failed to show the therapeutic effect of lymphadenectomy in the surgical treatment of endometrial cancer, several retrospective studies including the SEPAL study revealed the survival effect of lymphadenectomy. To prospectively investigate the survival benefit of para-aortic lymphadenectomy shown in the SEPAL study, we are proposing a new concept of a randomized phase III trial. An appropriate study population will be selected according to the preoperative assessments (evaluation of myometrial invasion and cervical invasion with magnetic resonance imaging, extrauterine spread with computed tomography, and histological type and grade by pathological evaluation) to estimate the risk of lymph node metastasis. Patients relevant to potential International Federation of Gynecology and Obstetrics (2008) stage IB, II and III diseases will be eligible, and randomly assigned to two arms: pelvic lymphadenectomy alone (control), or pelvic and para-aortic lymphadenectomy (experimental). After initial surgery, patients with postoperative pathological risk factors for recurrence will receive adjuvant chemotherapy. Because we aim to investigate the therapeutic significance of primary treatments, including surgery and adjuvant chemotherapy, the primary end-point could be recurrence-free survival. One of the most important issues to successfully perform this prospective study is to assure the quality of lymphadenectomy (extent and area), which could be evaluated based on the number of harvested nodes and objective evaluation of dissected area by videos and/or photos.

Key words: endometrial cancer, lymphadenectomy, prospective study, SEPAL study, survival.

Introduction

Despite the diagnostic role of lymphadenectomy in endometrial cancer, there has been controversy and debate about the therapeutic relevance of systematic pelvic and para-aortic lymphadenectomy. The current recommendation of the National Comprehensive Cancer Network (NCCN),[1] and Japan Society of Gynecologic Oncology[2] is to perform systematic lymphadenectomy rather than merely nodal sampling. Furthermore, the International Federation of Gynecology and Obstetrics (FIGO) staging system in endometrial cancer has recently been changed.[3] In the revised FIGO staging system, para-aortic node (PAN) involvement has been separated from the single substage IIIC. Stage IIIC is now categorized as IIIC1 (indicating positive pelvic lymph node [PLN]) and IIIC2 (indicating positive PAN with or without positive PLN) and IIIC2

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shows worse prognosis than IIIC1.[4] However, in clinical practice, it is not clearly defined who should benefit from a systematic para-aortic lymphadenectomy.

Complete systematic pelvic and para-aortic lymphadenectomy has been routinely performed in all operable patients with endometrial cancer in Hokkaido University Hospital, because: (i) nodal status is the most important prognosticator; and (ii) results of lymphadenectomy allow tailoring of postoperative adjuvant treatment.[5,6] Consequently, the retrospective cohort study (SEPAL study) has recently demonstrated that para-aortic lymphadenectomy combined with pelvic node dissection improves survival of endometrial cancer patients with postoperative intermediate risk/high risk for recurrence, but not for patients with low risk for recurrence.[7] Recently, two randomized clinical trials, however, indicated that routine lymphadenectomy provided no survival benefit in endometrial cancer.[8,9] Taken together, we can conclude that no survival benefit of routine lymphadenectomy has been established for patients with postoperative low risk for recurrence. However, survival benefit of lymphadenectomy including para-aortic lymphadenectomy remains controversial for the patients with postoperative intermediate risk/high risk for recurrence, thus prospective study is mandatory to investigate the survival benefit of para-aortic lymphadenectomy shown in the SEPAL study. We are currently proposing a concept and design of a randomized phase III trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer. In this article, we would like to discuss the important issues to definitively prove the potential survival advantage associated with lymphadenectomy in endometrial cancer.

Conclusions from Recent Clinical Studies Investigating Therapeutic Role of Lymphadenectomy

Even after the negative results of two randomized trials from Europe (ASTEC trial and Italian study),[8,9] the latest NCCN guideline[1] still recommends systematic pelvic and para-aortic lymphadenectomy for early stage endometrial cancer. In the discussion, they describe the reasons for not changing their guidelines. It is stated that two randomized clinical trials from Europe have reported that lymph node dissection does not improve outcomes in endometrial cancer patients; however, lymphadenectomy did identify those with nodal disease. To avoid over-interpretation of these results, it is important to address the limitations of these randomized studies, including patient selection, extent of lymph node dissection and standardization of postoperative therapy. Other concerns regarding these trials include the lack of central pathology review, the subspecialty of surgeons and inadequate statistical power. It is also stated that there is a high rate of lymphatic metastasis above the inferior mesenteric artery, suggesting a need for systematic pelvic and para-aortic lymphadenectomy. However, in these two European randomized trials, para-aortic lymphadenectomy was performed at the discretion of the surgeon. Clearly, the standardization of surgical effort to include systematic para-aortic lymphadenectomy may be important to definitively prove the potential survival advantage associated with lymphadenectomy.

The SEPAL study has shown that para-aortic lymphadenectomy significantly improved the survival of the endometrial cancer patients at intermediate risk/high risk for recurrence, but not patients at low risk for recurrence.[7] There are several possible reasons for the positive results of the SEPAL study. First, surgeons were familiar with lymphadenectomy, and the lymph node count is high, because the median number of nodes removed was 34 in the pelvic lymphadenectomy alone group, and 82 nodes in the pelvic and para-aortic lymphadenectomy group. However, the SEPAL study also has some limitations; these include the fact that it was a retrospective cohort study, and adjuvant therapy was not uniformly given. Indeed, in the pelvic lymphadenectomy group, 46% received adjuvant radiotherapy, whereas 98% received adjuvant chemotherapy in the pelvic and para-aortic lymphadenectomy group. Comparison of overall survival (OS) among intermediate risk patients receiving adjuvant chemotherapy, para-aortic lymphadenectomy did not significantly improve the survival.

Based on the results obtained from previous clinical studies, we conclude that patients at 'low-risk for lymph node metastasis' should not be included in future prospective trials to investigate the therapeutic role of lymphadenectomy. The survival effect of para-aortic lymphadenectomy should be investigated for the patients at 'risk of lymph node metastasis' by prospective studies based on the positive results obtained from the SEPAL study. Patient selection and quality assurance of lymphadenectomy should be discussed and, finally, when considering the results of phase III trials,[10–12] adjuvant chemotherapy should be uniformly given in future lymphadenectomy trials.

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Study Design and End-points

We are proposing a new trial concept and design to prospectively investigate the survival effect of para-aortic lymphadenectomy in endometrial cancer (Fig. 1). This new concept is a randomized phase III trial and patients will be randomly assigned to undergo pelvic lymphadenectomy alone or pelvic and para-aortic lymphadenectomy. Adjuvant chemotherapy will be given to cases at postoperative risk for recurrence. The trial schema is shown in Figure 2. First of all, we will estimate the risk of lymph node metastasis preoperatively. After getting informed consent from all eligible cases, they will be randomly assigned to two arms. For cases at intermediate risk/high risk for recurrence confirmed by postoperative pathological examination, adjuvant chemotherapy will be given. After completing the initial treatment, we will follow-up participants until recurrence. Because we aim to investigate the therapeutic significance of primary treatments, including surgery and adjuvant chemotherapy, the primary end-point could be recurrence-free survival (RFS). In addition, RFS would be a surrogate for OS in endometrial cancer. Secondary end-points include OS, relationship between number of harvested nodes and recurrence rate, concordance rate of pre- and postoperative assessments (e.g. imaging, pathological diagnosis, grade, histology), predictive value of the combination of preoperative risks for lymph node metastasis, intraoperative tumor size and lymph node metastasis, initial failure site, and perioperative, chemotherapy-related adverse events. At the same time, we can create a scoring system to select patients who are at risk of lymph node metastasis and in whom pelvic and/or para-aortic lymphadenectomy can be safely omitted.[13,14]

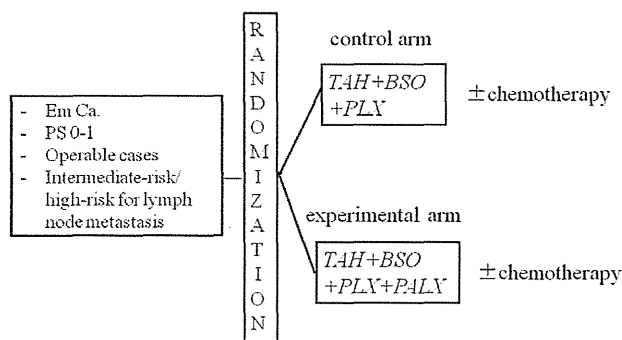

Figure 1 Proposal of a design of a future prospective trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer. BSO, bilateral salpingo-oophorectomy; PALX, para-aortic lymphadenectomy; PLX, pelvic lymphadenectomy; PS, performance status; TAH, total abdominal hysterectomy.

Important Issues Which Need to be Discussed to Finalize the New Concept

First, we have to consider what the control arm for lymphadenectomy should be. Should it be pelvic lymphadenectomy alone or pelvic and para-aortic lymphadenectomy? The second point is how to select the study population. We need to estimate risk for lymph node metastasis preoperatively, and need to exclude inappropriate cases. Third, one of the most important issues is quality assurance of lymphadenectomy and, as such, we need to define the adequate extent and appropriate area of lymphadenectomy. Next we must consider which chemotherapeutic regimen should be given.

What is a control arm for lymphadenectomy in surgical treatment for endometrial cancer?

The latest NCCN guidelines[1] and Japanese guidelines[2] both recommend pelvic and para-aortic lymphadenectomy for staging purposes, but not for therapeutic

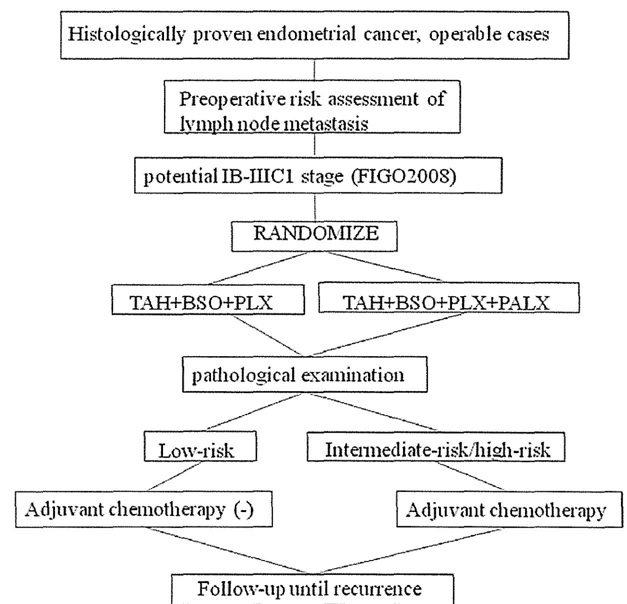

Figure 2 Study schema of a randomized phase III trial investigating the survival effect of para-aortic lymphadenectomy in endometrial cancer. BSO, bilateral salpingo-oophorectomy; PALX, para-aortic lymphadenectomy; PLX, pelvic lymphadenectomy; TAH, total abdominal hysterectomy.

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purposes. According to a previous Japanese survey, most Japanese institutions perform pelvic lymphadenectomy routinely, but perform para-aortic lymphadenectomy selectively depending on the risk for lymph node metastasis.[3] Because 'standard' lymphadenectomy has not been established yet, either arm could be a control arm, but pelvic lymphadenectomy alone seems to be a more plausible control arm.

Selection of study population for lymphadenectomy trial in endometrial cancer

To select an appropriate study population, we need to estimate risk for lymph node metastasis preoperatively. We should assess myometrial invasion and cervical invasion with magnetic resonance imaging (MRI), histological subtype and grade by pathological examination, and extrauterine spread with enhanced computed tomography and/or MRI. Based on these evaluations, we should exclude potential FIGO (2008) stage IA cases (myometrial invasion <1/2) with any grade and subtype, which shows extremely low risk for lymph node metastasis (<3%),[15] cases with carcinosarcoma or sarcoma. Potential FIGO (2008) stage IV disease (peritoneal metastasis, bladder/rectum invasion and distant metastasis) by imagings will be excluded because positive node status does not affect their staging and probably survival. Cases with swelling of para-aortic nodes will not be eligible, because they will have great chance of positive nodes in the para-aortic area. Thus, an appropriate study population includes potential FIGO (2008) stage IB, II and III (IIIA, IIIB and IIIC1) disease.

Quality assurance of lymphadenectomy

To investigate the therapeutic role of lymphadenectomy, the extent and area of lymphadenectomy should be defined, because pelvic lymphadenectomy alone does not have any therapeutic role, but para-aortic lymphadenectomy combined with pelvic lymphadenectomy has survival benefits, and systematic dissection, but not sampling, is appropriate for therapeutic purposes. Indeed, when we compare the area and number of lymph nodes removed among the three recent clinical studies, systematic para-aortic lymphadenectomy was performed in the SEPAL study only, and the lymph node count in the SEPAL study was higher than the other two randomized studies, which is one of the main reasons why the SEPAL study could show a survival effect of lymphadenectomy (Table 1). In addition, other retrospective data has also demonstrated that the extent of lymphadenectomy (over 20 nodes removed) significantly improves disease-specific survival for intermediate risk/high risk patients, but not for low risk patients.[16]

To define the appropriate area for lymphadenectomy, we analyzed the distribution of lymph node metastasis sites in node-positive cases at our institution among cases undergoing systematic pelvic and para-aortic lymphadenectomy. We found that for nodal disease, para-aortic nodes above and below the inferior mesenteric artery, common iliac nodes, internal iliac nodes, external iliac nodes and obturator nodes were prevalent (unpubl. data). The most prevalent sites of nodal disease were the obturator nodes followed by the para-aortic nodes below the inferior mesenteric artery, para-aortic nodes above the inferior mesenteric artery up to the level of the renal vein, the internal iliac nodes, the common iliac nodes and the external iliac nodes (unpubl. data).

To define the extent of the lymphadenectomy, a lower limit of lymph nodes to be removed should be set. In addition, photos and/or videos of the dissected area should be submitted and inspected.

Which chemotherapeutic regimen should be given as an adjuvant therapy?

From the view point of clinical practice, adjuvant chemotherapy is frequently used in Japanese institutions, and the paclitaxel and carboplatin (TC) regimen is

Table 1 Comparison of recent clinical studies investigating the therapeutic role of lymphadenectomy in endometrial cancer

	ASTEC trial	Italian study	SEPAL study
Recurrence risk	Low–high	Intermediate/high	Low–high
Area of LNX	Pelvic	Pelvic (PAN)	Pelvic (PAN)
Lymph nodes count (median)	12	PLX, 26; PLX + PALX, 30.	PLX, 34; PLX, 59 + PALX, 23 = 82
Adjuvant therapy	RT	RT or CT	RT or CT
Therapeutic role of pelvic LNX	(–)	(–)	Not determined
Therapeutic role of para-aortic LNX	Not determined	Not determined	Low risk (–); intermediate/high (+)

CT, chemotherapy; LNX, lymphadenectomy; PALX, para-aortic lymphadenectomy; PAN, para-aortic node; PLX, pelvic lymphadenectomy; RT, radiotherapy.

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widely given.[17] In the GOG209, randomized phase III trial to compare the efficacy of paclitaxel, adriamycin and cisplatin (TAP) and TC for advanced, recurrent disease, it was shown that TC is not inferior to TAP.[12] Therefore, TC is the current standard regimen of GOG for advanced, recurrent endometrial cancer. We are currently thinking that TC should be given to patients at intermediate risk/high risk for recurrence as an adjuvant chemotherapy in the future clinical trial.

Target Accrual

Concerning target accrual, if we assume that 3-year progression-free survival of the control arm would be 70%, the 3-year progression-free survival of the experimental arm should exceed 10% of that of the control arm, and if we have a significance level of 5% and over 90% statistical power, then 604 cases are necessary. If we estimate a 5% dropout rate, then a sample size of 630 cases (315 in each arm) is necessary.

In the year of 2011, 7273 new cases of endometrial cancer were registered in the Japan Society of Obstetrics and Gynecology. Among them, cases that are classified as FIGO (2008) stage IA (old stage IA and IB) are not eligible for the future clinical trial. Cases that are classified as FIGO (2008) stage IV are also not eligible. Therefore, approximately 40% of all endometrial cancer cases fit the inclusion criteria.

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

Key words: Annexin A4, ATP7A, platinum resistance, platinum transporter, copper transporter
Abbreviations: 5-FU: 5-fluorouracil; Anx A4: Annexin A4; CCC: clear cell carcinoma; D-MEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; PBS: phosphate-buffered saline; SAC: serous adenocarcinoma; siRNA: small interfering RNA
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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-κB transcriptional activity, which is significantly upregulated early after etoposide treatment. Anx A4 translocates to the nucleus together with p50 and imparts greater resistance to apoptotic stimulation by etoposide treatment.[18] Anx A4 may also be associated with drug resistance in other types of tumors; enhanced expression of Anx A4 has been reported in colon, renal, lung and pancreatic cancers.[19–23] However, the details of Anx A4-mediated extracellular efflux of platinum drugs remain unclear.

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

Material and Methods

Cell lines

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

Generation of Anx A4 stably transfected cell lines

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

Western blotting

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

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

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

Small interfering RNA transfection

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

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

In vivo model of cisplatin resistance

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

Quantification of intracellular platinum accumulation

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

Preparation of crude membrane fractions

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

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

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

Immunofluorescence for ATP7A and Anx A4

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

Statistical analysis

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

Results

Expression of Anx A4 in endometrial carcinoma cell lines

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

Anx A4 and platinum resistance in HEC1 cell lines

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

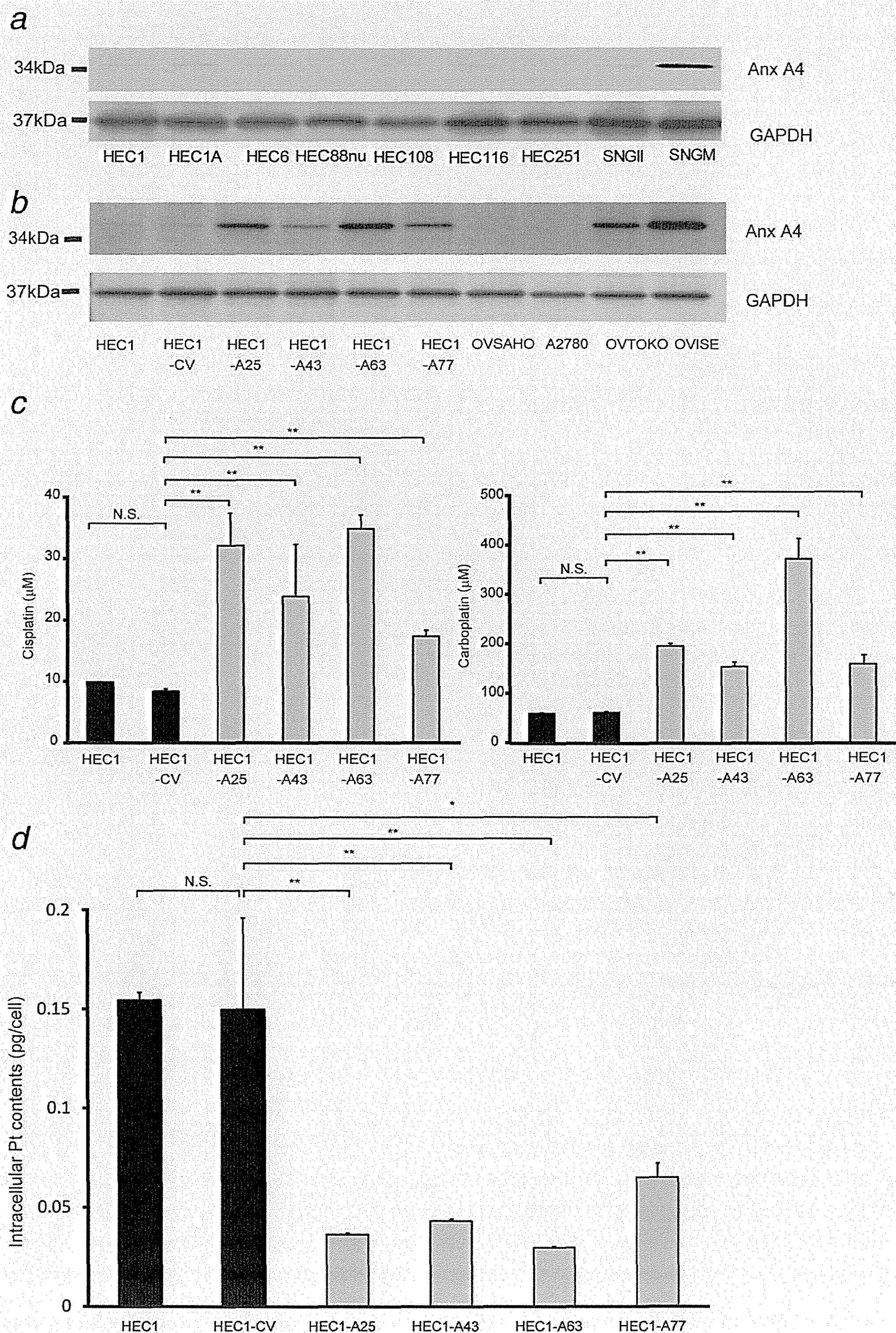

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Figure 1. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vitro*. (*a*) Western blot analysis of nine endometrial carcinoma cell lines. Anx A4 was expressed in one cell line. (*b*) Establishment of an Anx A4-stably-expressing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

Intracellular platinum accumulation in Anx A4-overexpressing cells

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

Anx A4-overexpressing cells and cisplatin in xenograft models

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

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

Translocation of Anx A4 and ATP7A after platinum exposure

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

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

Anx A4 and ATP7A localization

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

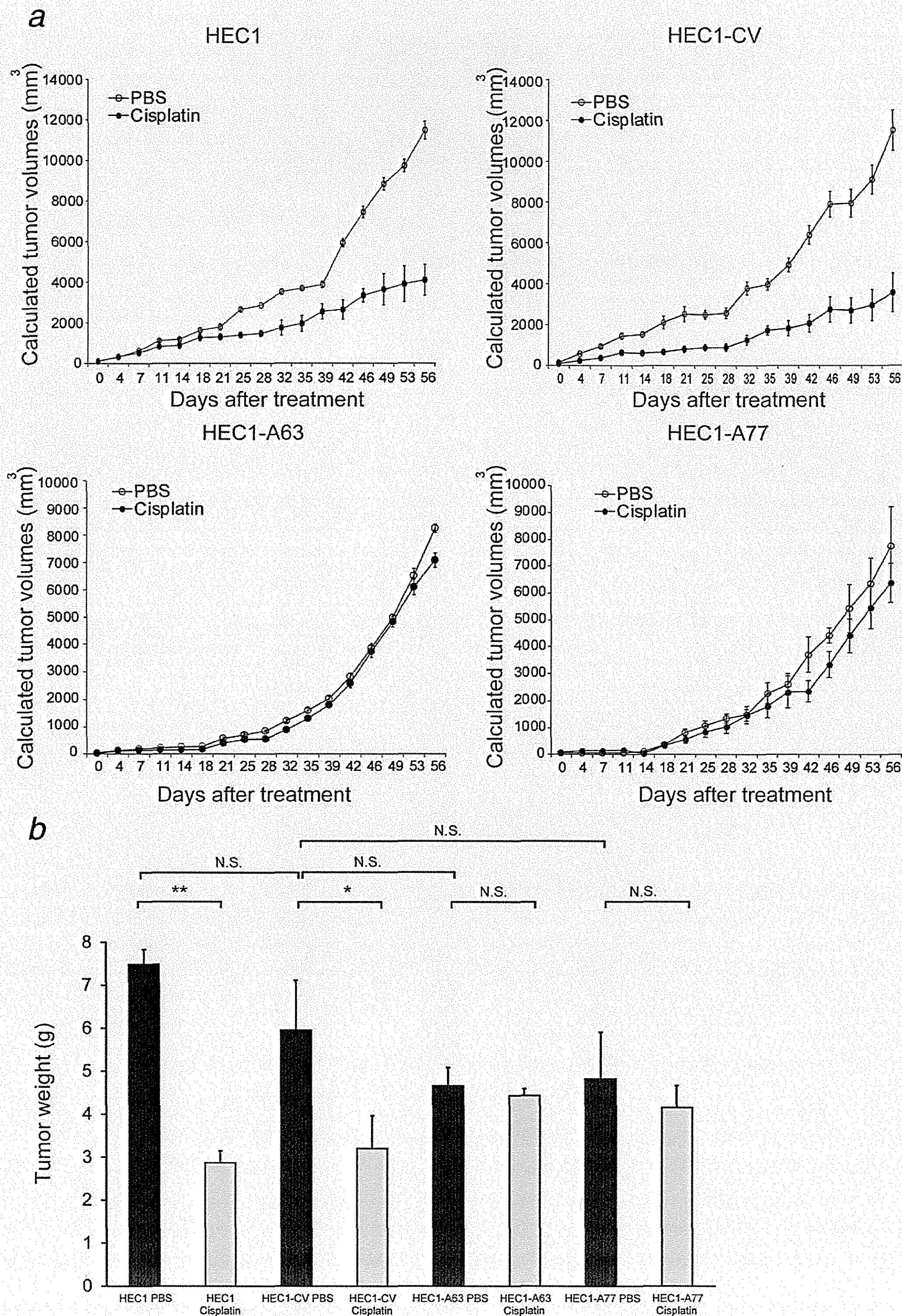

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Figure 2. Enforced expression of Anx A4 in HEC1 cells confers platinum resistance *in vivo*. Analysis of Anx A4 as a platinum-resistant protein *in vivo*. (*a*) To determine the resistance of Anx A4-stably-expressing HEC1 cells to platinum *in vivo*,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

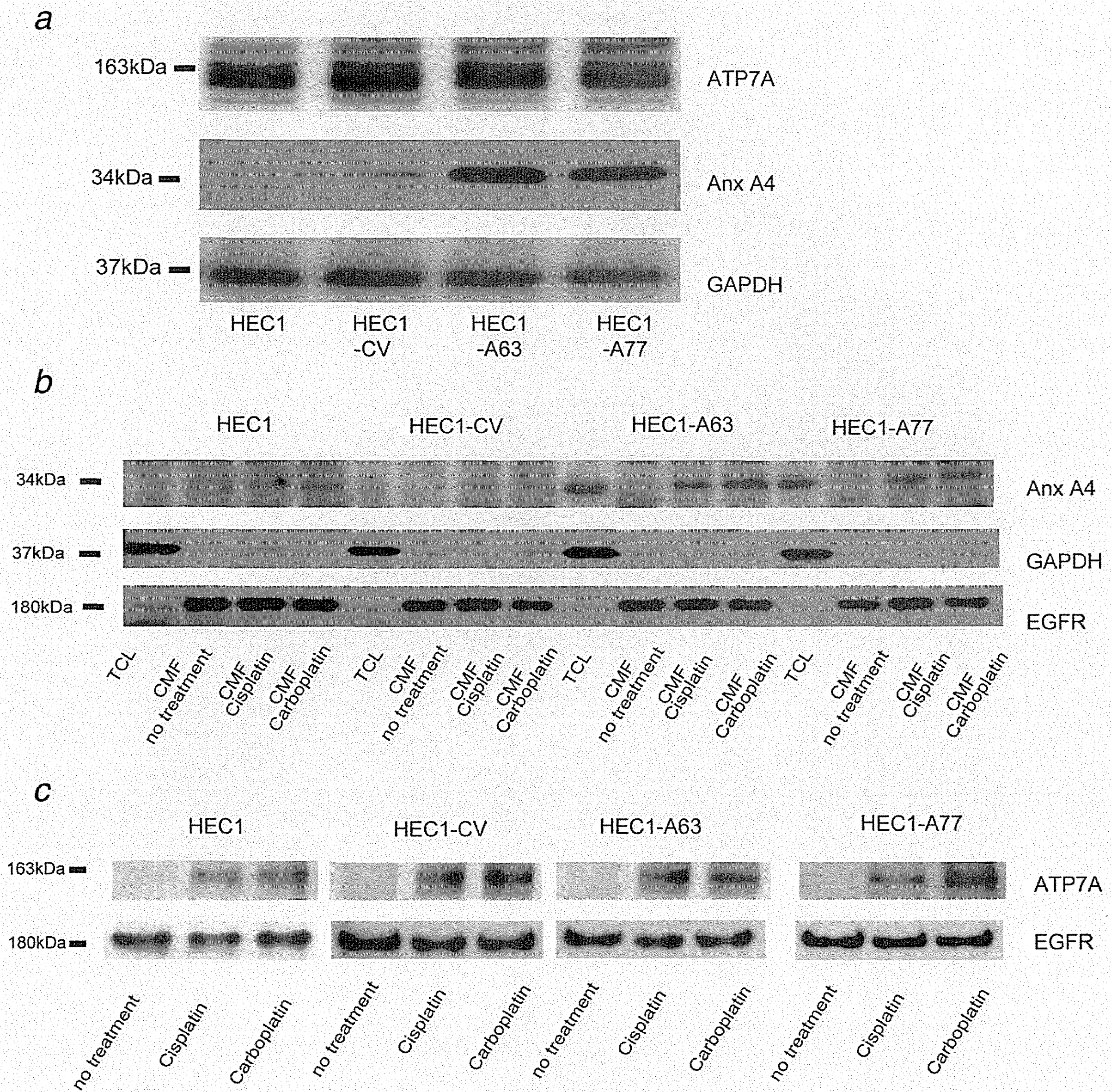

Figure 3. Localization of Anx A4 and ATP7A was investigated using Western blot analysis. The localization of Anx A4 and ATP7A was investigated using two techniques: orthogonal crude membrane fractions and biotinylation of cell surface proteins. (*a*) No significant change in expression levels of ATP7A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

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

Effect of ATP7A expression on resistance to platinum drugs

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

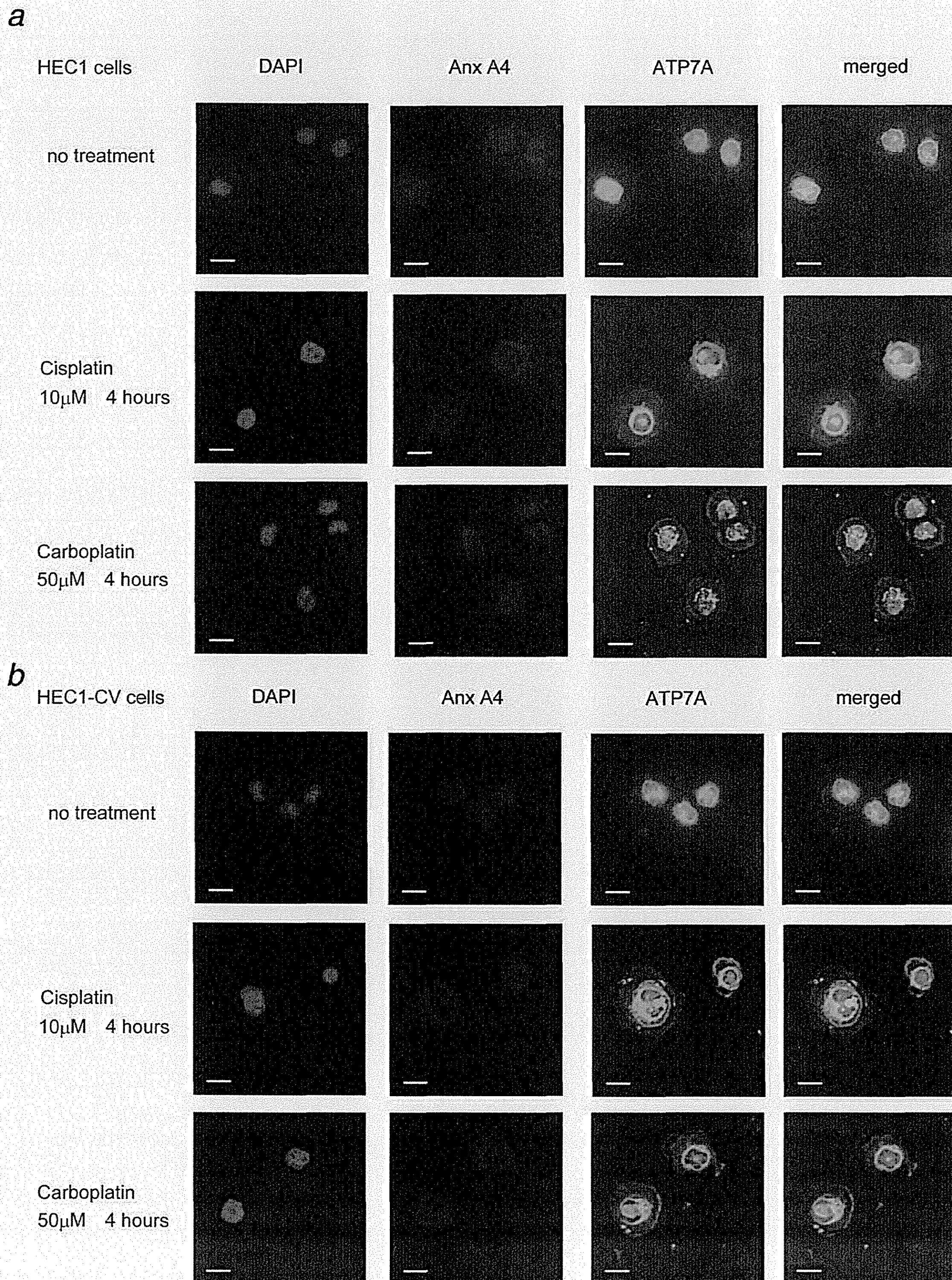

Figure 4. Immunofluorescence staining for ATP7A and Anx A4. HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells were divided into three groups: the no treatment, cisplatin exposure and carboplatin exposure groups. (*a*) HEC1 cells, (*b*) HEC1-CV cells, (*c*) HEC1-A63 cells and (*d*) HEC1-A77 cells. Cells were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

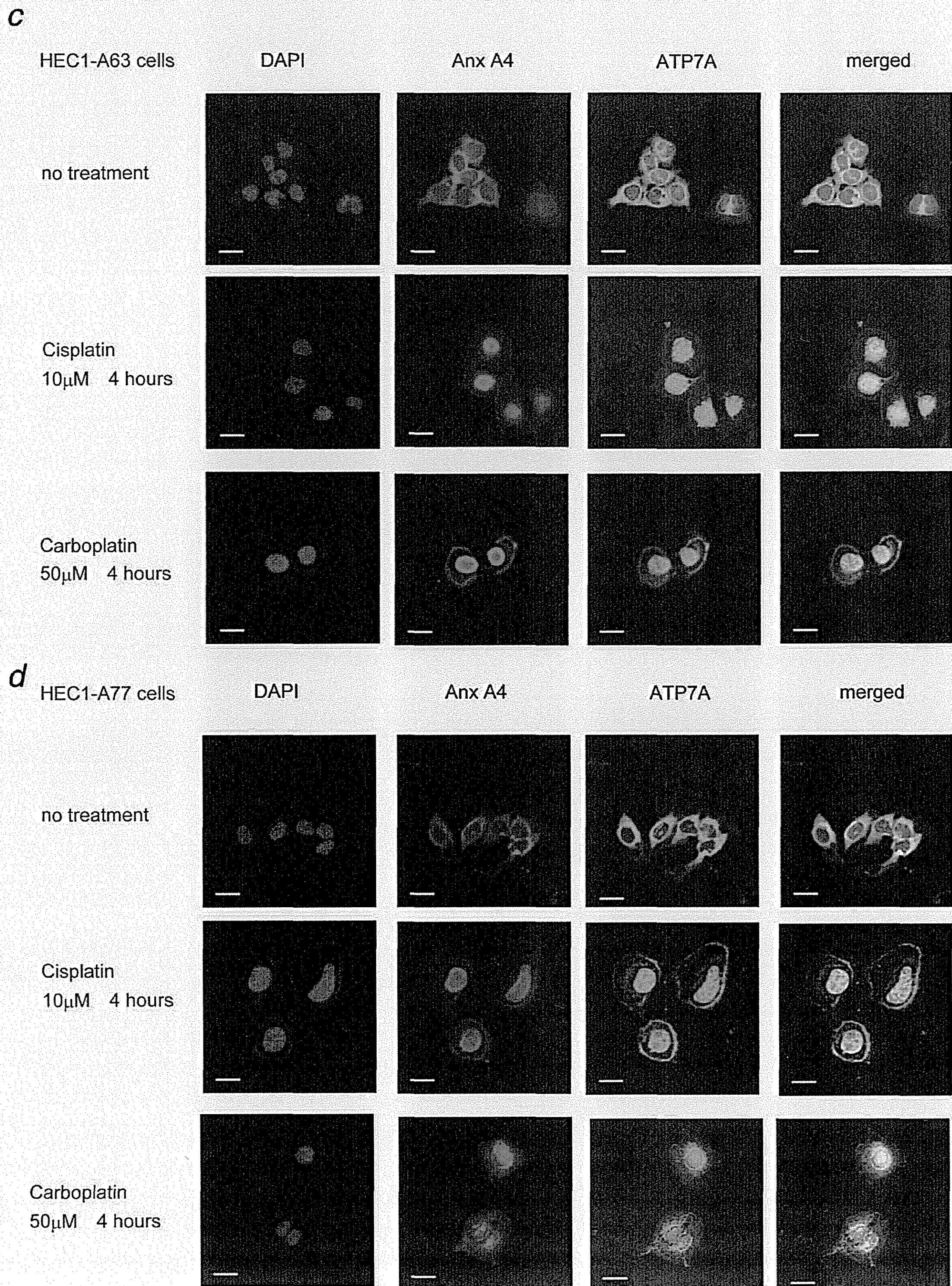

Figure 4. (Continued)

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

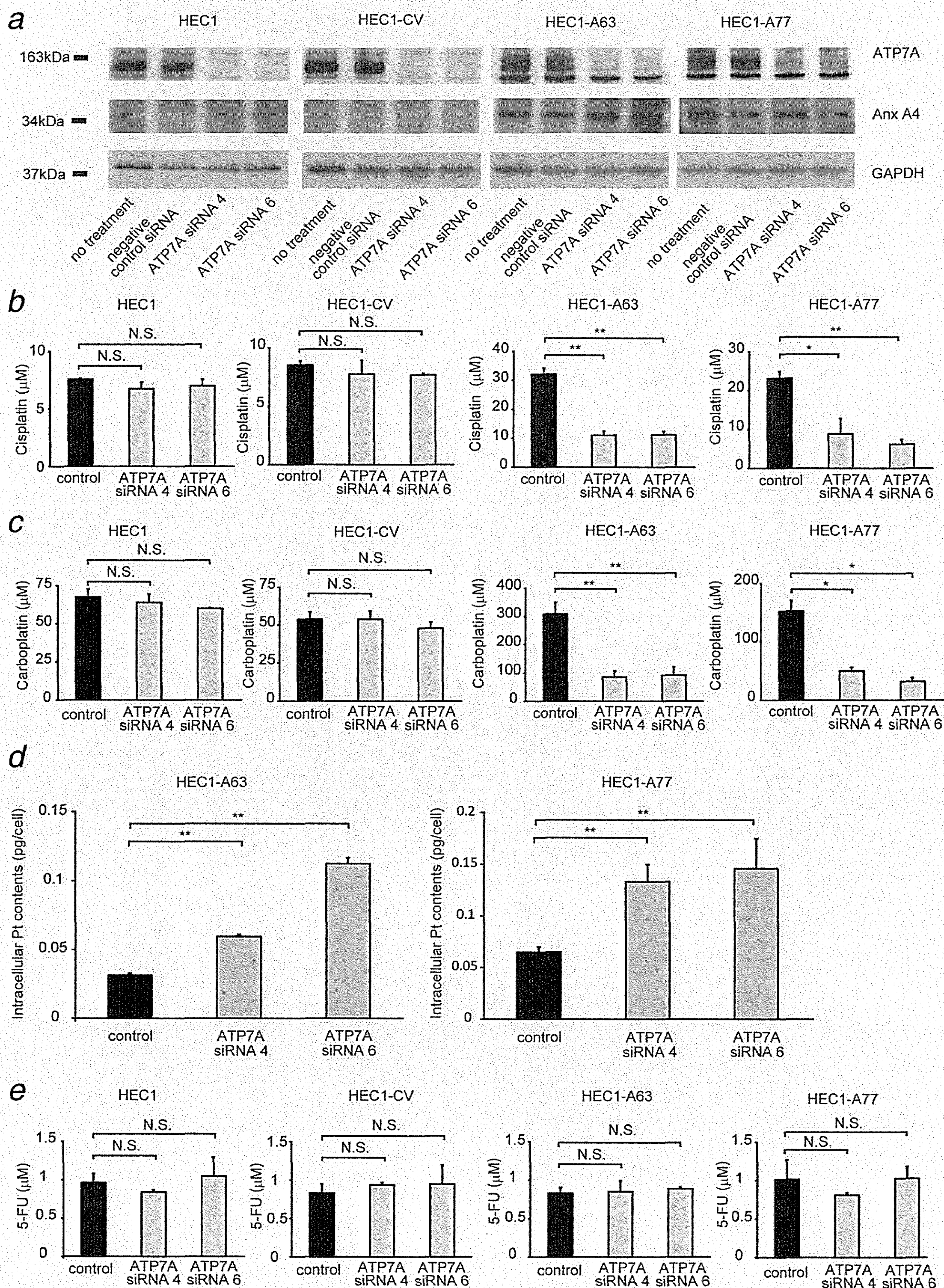

Figure 5. Knockdown of ATP7A expression improves platinum chemosensitivity in Anx A4-overexpressing cells. (*a*) Knockdown expression of ATP7A by siRNA in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77 cells by Western blot analysis. (*b*) IC_{50} values are shown for cisplatin in HEC1, HEC1-CV, HEC1-A63 and HEC1-A77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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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