or 3 hr of additional incubation in cisplatin-free medium (Fig. 4D). According to the above results, the platinum resistance of ANXA4 seemed to be related to the calcium-binding site of the annexin repeat next to the N-terminal domain.

The calcium-binding site of the annexin repeated sequence is required for the resistance to platinum-based drugs *in vivo*

To determine whether the annexin deletion mutants of ANXA4 influenced the sensitivity to cisplatin *in vivo*, we inoculated ICR *nu/nu* mice with NC-14, FL-22, R1-12 or R1(E70A)-95 cells. Mice in each group were randomised into 2 subgroups and received either cisplatin at 3 mg/(kg·d) or PBS *i.p.* once a week for 3 weeks. Cisplatin markedly decreased tumour volume in mice injected with NC-14 and R1(E70A)-95 cells, whereas the treatment effect was relatively smaller in mice injected with FL-22 and R1-12 cells (Fig. 5A). Consistent with the tumour volume, tumour growth was significantly inhibited by cisplatin in mice inoculated with NC-14 (96.5 ± 1.3%) and R1(E70A)-95 cells (87.8 ± 11.4%) compared with those injected with FL-22 (48.5 ± 11.7%) and R1-12 cells (37.7 ± 9.8%; $p < 0.01$; Fig. 5B). These *in vivo* results
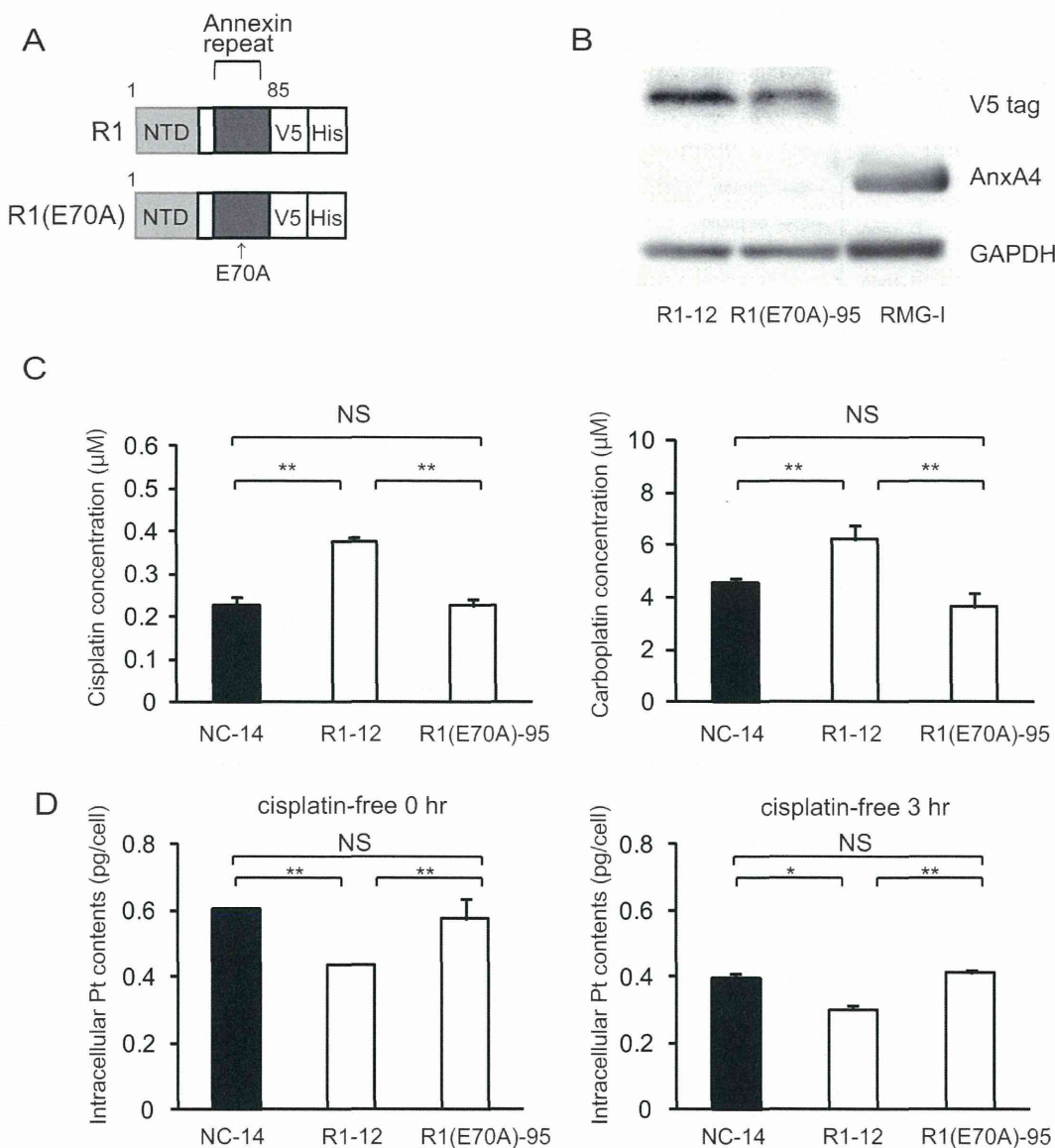

Fig.4: **The calcium-binding site of the annexin repeat is responsible for induction of the platinum resistance.** (A) A structural map of R1 and R1(E70A) variants without a function for the calcium-binding site. (B) Western blotting confirmed the mutant established cell lines. (C) Unlike R1-12, the R1(E70A)-95 cell clone was not resistant to either cisplatin or carboplatin. (D) Intracellular platinum accumulation in R1(E70A) cells was the same as in NC14 cells both with or without additional 3 hr of incubation in cisplatin-free medium. Data are presented as mean ± SD (*$p < 0.05$, **$p < 0.01$).

105

were consistent with those obtained *in vitro*.

Increase of the intracellular chloride concentration is related to cisplatin resistance

To elucidate the molecular mechanisms of chemoresistance induced by ANXA4, we focused on the chloride channel because one of the functions of ANXA4 is inhibition of calcium-dependent chloride conductance[32]. According to the literature, treatment with cisplatin induces an increase of the intracellular Ca^{2+} concentration [46], which is an important ion for the phospholipid membrane-binding activity of ANXA4. In contrast, cisplatin exposure also induces an elevation of the intracellular chloride concentration: $[Cl^-]_i$ [47]. Elevation of $[Cl^-]_i$ has been shown to prevent the aquation

of 1 or 2 of the 2 chloride coordination sites in cisplatin, and only the aquated forms of cisplatin covalently bind to DNA. Nevertheless, the mechanisms of $[Cl^-]_i$ elevation because of cisplatin treatment have not been fully elucidated. We hypothesised that an increase in intracellular Ca^{2+} concentration after cisplatin exposure would result in translocation of ANXA4 from the cytosol to plasma membrane, which leads to $[Cl^-]_i$ accumulation through inhibition of the chloride channel by the Ca^{2+}-bound ANXA4. To confirm this hypothesis, we quantified $[Cl^-]_i$ after cisplatin treatment using MAQE fluorescence, a fluorescent Cl^- indicator. Relative fluorescence was substituted for $[Cl^-]_i$ as previously reported [48].

We monitored MQAE fluorescence in control cells (NC-14), in cells overexpressing full-length ANXA4 (FL-22) and in 2 deletion mutants (R1-12 and R1[E70A]-95). The relative fluorescence ratio before (F0) and after
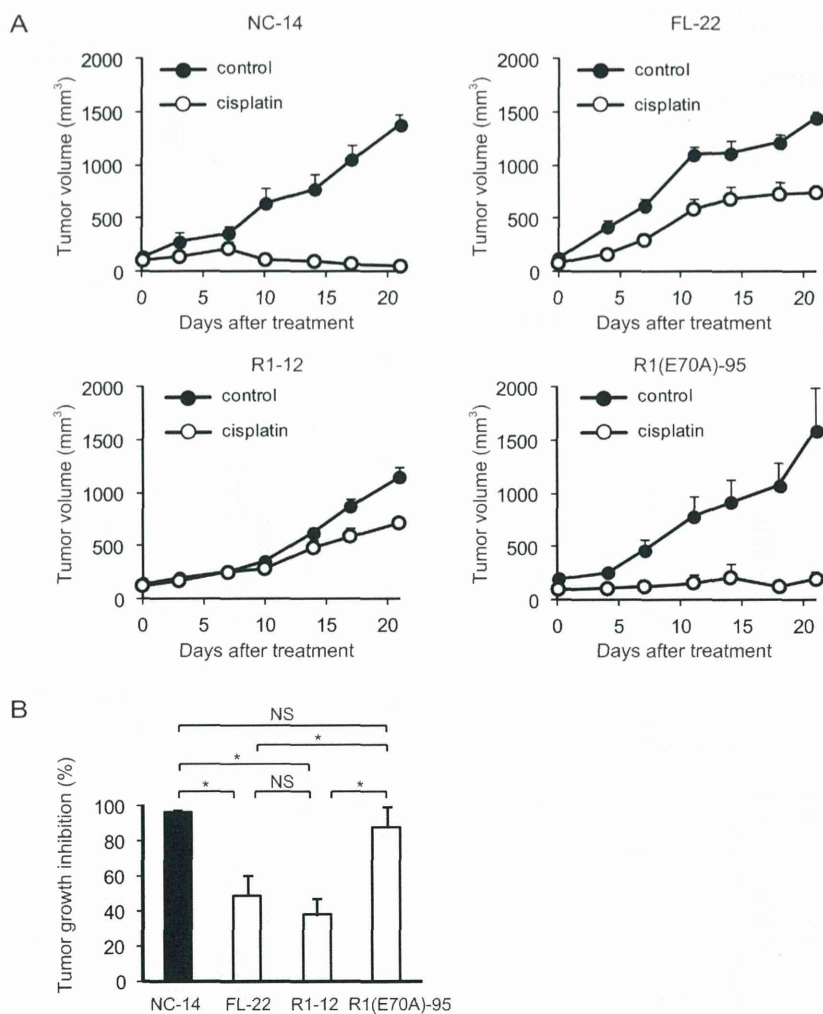

Fig.5: The calcium-binding site of the annexin repeat is required for platinum drug resistance *in vivo.* Female ICR *nu/nu* mice were subcutaneously inoculated with NC14, FL-22, R1-12 or R1(E70A)-95 cells and given PBS (control group: *filled circles*) or cisplatin i.p. (3 mg/kg; treatment group: *open circles*) once a week for 3 weeks (n = 6 per group). (A) Growth curves of tumours of each cell. The mean volume (points) ± SE (bars) is shown. (B) Comparison of the cisplatin-induced growth inhibition of tumours 28 days after treatment. The average (columns) ± SE (bars) are shown; *$p < 0.01$.

106

treatment with cisplatin for 30 min (F30) is shown in Figure 6. The inverse ratio of MQAE fluorescence $1/(F30/F0)$, which is directly proportional to the increase in $[Cl^-]_i$, was significantly elevated in the platinum-resistant cell clones FL-22 (1.12 ± 0.03) and R1-12 (1.12 ± 0.01) compared with sensitive clones, NC-14 (1.06 ± 0.01) and R1(E70A)-95 (1.06 ± 0.02; $p < 0.01$). Thus, the increase in $[Cl^-]_i$ is likely to be involved in cisplatin resistance.

DISCUSSION

ANXA4 has been reported to be strongly expressed and involved in chemoresistance in various cancers. The factors associated with ANXA4-induced chemoresistance have been reported, such as NF-κB [45] and ATP7A [44], but the structure of the protein, i.e. annexin repeats and calcium-binding sites in the annexin repeated sequence, has not been taken into account in relation to the ANXA4-induced chemoresistance. In this study, we showed that ANXA4 knockdown improved sensitivity to platinum drugs, and annexin repeats were involved in chemoresistance.

We first confirmed ANXA4 expression in various ovarian adenocarcinoma cell lines. As previously reported [28, 49], ANXA4 is significantly upregulated in clear cell carcinoma cell lines (OVTOKO, OVISE and RMG-I) compared with other histological types (serous and mucinous adenocarcinoma cell lines: A2780, OVCAR3, OVSAHO and MCAS). It has been previously demonstrated that enhanced ANXA4 expression induces platinum resistance both *in vitro* and *in vivo* [28, 44], but whether ANXA4 knockdown attenuates platinum resistance has been unknown thus far. Mogami et al. recently reported that an ANXA4 knockdown improves sensitivity to carboplatin *in vitro* using 2 cell lines of ovarian clear cell carcinoma, OVTOKO and OVISE. To the best of our knowledge, ours is the first study to show that ANXA4 knockdown markedly improves the sensitivity to platinum-based drugs not only *in vitro* but also *in vivo* (Figs. 1 and 2).

The result that ANXA4 knockdown improves sensitivity to platinum-based drugs suggests that ANXA4 is a good therapeutic target. To identify the functional domain(s) of ANXA4 that could be a promising therapeutic target, we focused on annexin repeats and constructed 4 deletion mutants (R3, R2, R1 and R1[E70A]). Resistance to platinum drugs was enhanced in cells transfected with mutants possessing at least 1 intact annexin repeat. In contrast, the sensitivity to platinum-based drugs improved among the R1(E70A)-transfected clones because in those cells, the calcium-binding site did not function properly (Figs. 3C and 4C). This result implies that the ANXA4-induced chemoresistance to platinum-based agents is calcium dependent. It has been reported that cisplatin induced increase of intracellular calcium concentration in chemosensitive cells, but not in resistant cells [46, 50].

Together with this and the data by Chan et al., elevation of intracellular calcium concentration induced by cisplatin treatment may translocate Ca^{2+} bound form of ANXA4 from cytosol to plasma membrane, which results in platinum-resistance[32]. We are currently investigating on further analysis.

By analysing the intracellular platinum accumulation, we attempted to elucidate the mechanism of the platinum resistance induced by ANXA4 and its deletion mutants. When intracellular platinum contents were quantitated just after exposure to cisplatin or 3 hr incubation with cisplatin-free medium after exposure to cisplatin, significantly less platinum accumulated in cells transfected with the full-length ANXA4 (FL-22) and 3 deletion mutants (R3-6, R2-13 and R1-12), all of which enhanced the resistance to the platinum-based drugs. In contrast, R1(E70A)-transfected cells (R1[E70A]-95), which did not induce chemoresistance, had the same amount of platinum accumulation as the control cells (Figs. 3D and 4D). These results suggest that the resistance to the platinum-based drugs is mediated by the decrease in intracellular platinum accumulation, which is calcium dependent. The annexin repeats, especially their calcium-binding sites, may be involved in inhibition of the influx, promotion of the efflux or both of platinum drugs. Recently, Cu transporters (CTR1 for the uptake and ATP7A and ATP7B for the efflux) have been reported to be involved in resistance to both cisplatin and carboplatin [14, 44, 51]. In addition, ANXA4 likely enhance platinum efflux through the interaction with ATP7A [44]. The possible mechanisms of inhibition of the influx mediated by ANXA4 remains unclear and further analyses are needed.
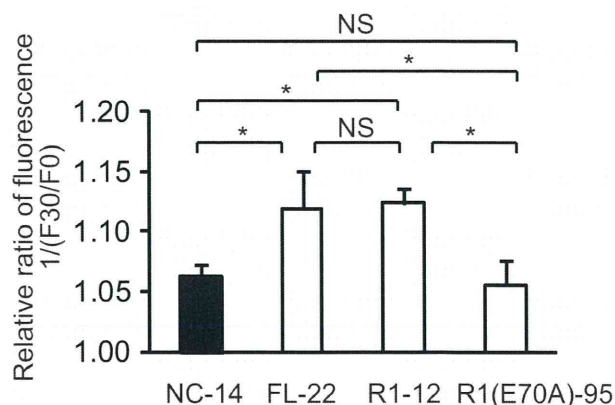

Fig.6: The increase of intracellular chloride concentration is related to cisplatin resistance. ANXA4 deletion mutant series cells (NC-14, FL-22, R1-12 and R1[E70A]-95) loaded with N-ethroxycarbonymethyl-6-methoxyquinolinium bromide (MQAE) were exposed to 100 μM cisplatin. The fluorescence pre-treatment and during treatment (30 min exposure) was compared in each cell clone. Data are presented as mean ± SD (*$p < 0.01$).

Subsequently, the question regarding the involvement of calcium-binding site in the platinum resistance arose. To answer this question, we measured $[Cl^-]_i$ after cisplatin exposure. The significant increase in $[Cl^-]_i$ was observed in the cells with platinum resistance, FL-22 and R1-12, compared with cell clones without platinum resistance, NC-14 and R1(E70A)-95. In a previous study, higher $[Cl^-]_i$ was observed in cisplatin-resistant cells compared with sensitive cells, whereas the intracellular cisplatin accumulation showed the opposite pattern [47]. These results, in addition to the results of decreased platinum accumulation in resistant mutants, indicate that ANXA4 induces platinum resistance through cellular drug efflux partly by elevating the intracellular chloride concentration. We report 'partly' because only cisplatin, not carboplatin, was tested in our $[Cl^-]_i$ assay. Cisplatin becomes intracellularly activated by the aquation of 1 or 2 of the 2 chloride coordination sites, but carboplatin does not contain any chloride coordination sites [1, 52-54]. Thus, this mechanism of resistance through elevation of $[Cl^-]_i$ may be specific to cisplatin and may not be true of carboplatin resistance. In this study, 3 cell clones overexpressing a deletion mutant (R3-6, R2-13, and R1-12) show stronger tolerance to cisplatin than to carboplatin in terms of their IC_{50}; a 1.7- to 2.2-fold increase for cisplatin and only a 1.4- to 1.7-fold increase for carboplatin (Fig. 3C). It is assumed that the increase in $[Cl^-]_i$ is one of the factors inducing cisplatin resistance.

In this study, the calcium-binding site in the annexin repeat next to the N terminus was observed to be responsible for the resistance to the platinum drugs. Nevertheless, the role of the other 3 calcium-binding sites has not yet been investigated. The roles of individual calcium-binding sites were demonstrated using site-directed mutagenesis by Nelson and Creutz regarding calcium-dependent membrane binding and aggregation [30]. The mutations in each domain had different effects on the binding or aggregating activities, i.e. a mutation in the first or fourth domain had a greater effect on membrane binding. A mutation in the second domain had a stronger effect on membrane aggregation, whereas the mutation of the third domain was almost silent. Although the mechanisms involved in membrane binding/aggregation and the mechanisms of chemoresistance are likely different, our data could provide some clues to understanding the function of each annexin repeat and each calcium-binding site in chemoresistance.

ANXA4 has been shown to induce resistance to paclitaxel and platinum-based drugs [55]. The effect of ANXA4 knockdown on paclitaxel sensitivity was assessed in a previous study. The effect of sensitivity to paclitaxel varied among different cell clones: ANXA4 knockdown in the OVTOKO cell line with acidic isoelectric point (IEPs) did not improve the sensitivity to paclitaxel, whereas OVISE cell lines with basic IEPs showed improved sensitivity to paclitaxel [43]. In our own preliminary data,

significant chemosensitisation to paclitaxel and etoposide was confirmed in RMG-I Y4 and R5 (data not shown). Further studies are required to identify the detailed mechanism.

In summary, in this study, we observed the annexin repeat, especially its calcium binding site, was associated with platinum-resistance induced by ANXA4, and it happened in calcium-dependent manner. Our findings may help to understand the mechanisms of platinum resistance induced by other annexin family proteins, which possesses the same annexin repeat structure, and offer new strategies for the treatment of chemoresistant cancers.

METHODS

Cell lines and culture

The human ovarian serous adenocarcinoma cell line (OVSAHO), human ovarian mucinous adenocarcinoma cell line (MCAS), human ovarian clear cell carcinoma cell lines (OVTOKO, OVISE and RMG-I) and human gastric cancer cell line (NUGC3) were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). A2780 cells from the human ovarian serous adenocarcinoma were obtained from the European Collection of Animal Cell Culture (Salisbury, Scotland) and OVCAR-3 cells from another human ovarian serous adenocarcinoma were from American Type Culture Collection (Manassas, VA). MCAS cells were maintained in the DMEM medium and the others in the RPMI medium, all supplemented with 10% foetal bovine serum (FBS; Serum Source International, NC, USA) and 1% penicillin–streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C in a humidified atmosphere with 5% CO_2. All the cell lines were tested and authenticated.

Generation of ANXA4 knockdown cell lines

To generate stable ANXA4 knockdown cell lines, RMG-I cells were transfected with a commercial plasmid vector expressing short heparin RNA (shRNA) that targeted ANXA4 mRNA or a negative control nonspecific shRNA (SuperArray Bioscience Corp., KH06928N; Frederick, MD, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. After selection using a culture medium containing geneticin (600 µg/mL; Invitrogen), stable clones were maintained in 250 µg/mL geneticin. Two stable RMG-I-ANXA4 shRNA cell clones were established, designated Y4 and R5 cells. In addition, we transfected the empty vector into the RMG-I cell line using the same procedure to generate control cells, designated NC7.

108

Construction of ANXA4 deletion mutants and gene transfection

Using pcDNA3.1-ANXA4 as a template, full-length cDNA of ANXA4 was amplified using KOD-plus (Toyobo Co. Led., Osaka, Japan) with the primers forward 5′-TTGACCTAGAGTCATGGCCA-3′, reverse 5′-ATCATCTCCTCCACAGAGAA-3′ and subsequently ligated into the pcDNA3.1/V5-His-TOPO vector in-frame with the C-terminal V5 and 6× His tag. To generate ANXA4 deletion mutants, annexin repeat domains were deleted one by one from the C-terminal site. Three deletion mutants named R3, R2 and R1 were generated and similarly amplified (an Arabic number shows the number of annexin repeat domains). The nucleotide sequences of the forward primers for PCR were the same as those described above for all the deletion mutants, and the reverse primers were as follows: R3 5′-TATAGCCAGCAGAGCATCTT-3′, R2 5-CAGAGACACCAGCACTCGCT-3′ and R1 5′-CATCCCCACAATCACCTGCT-3′. We subsequently set out to generate an R1 mutant (the E70A mutation), whose calcium-binding site does not work because of the change of a negatively charged carboxyl group to a neutral side chain, as previously described [30]. The site-directed mutagenesis was performed using the KOD-Plus-Mutagenesis kit (Toyobo), according to the manufacturer's protocol. These cDNA fragments, including full-length gene, were subsequently inserted between the *Bgl* II and *EcoR* I sites of the pIRES2AcGFP vector (Clontech, Palo Alto, CA). The sequences of all the mutants were confirmed using the ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, USA).

Full-length ANXA4, each ANXA4 deletion mutant construct and the empty vector were transfected into NUGC3 cells using Lipofectamine 2000 (Invitrogen). Stable transfectants designated FL-12, R3-6, R2-13, R1-12, R1(R70A)-95 and NC-14 were obtained by selection in a medium containing geneticin and were maintained in the same manner described above.

Western blotting

Cells were lysed in RIPA buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1× phosphatase inhibitor cocktail (Nacalai Tesque) and 1× protease inhibitor cocktail (Nacalai Tesque)), followed by centrifugation (13,200 rpm, 4°C, 15 min). Soluble proteins in the supernatant were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis, as described previously [28]. Additional information can be found in the Supporting Information on Material and Methods section.

Measurement of IC_{50} values after treatment with a platinum-based drug

Cells were suspended in the RPMI medium supplemented with 10% FBS, seeded in 96-well plates (1,500/well for the RMG-I series and 2,500 cells/well for ANXA4 deletion mutant series), cultured for 24 h and exposed to various concentrations of cisplatin (0–25 µM; Sigma–Aldrich, St Louis, MO) or carboplatin (0–1000 µM; Sigma–Aldrich) for 72 h. Cellular proliferation was subsequently evaluated using the WST-8 assay, i.e. 2-(2-methyosy-4-nitro-phenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophynel)-2H-tetrazolium monosodium salt assay (Cell Counting Kit-SF; Nacalai Tesque) after treatment. 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 the treated samples were expressed as percentages relative to results for untreated controls, and IC_{50} values were calculated.

Measurement of intracellular platinum accumulation

Full-length ANXA4-transfected cells (FL-22), each ANXA4 deletion mutant-transfected cell clone (R3-6, R2-13, R1-12 and R1[E70A]-95), and control cells (NC-14) were cultured up to 80% confluence in 150-mm tissue culture dishes. The cells were then exposed to 100 µM cisplatin for 60 min at 37°C and washed twice with PBS either immediately or after 3 hr of incubation in cisplatin-free RPMI 1640 medium supplemented with 10% FBS. Whole-cell 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, USA).

In vivo model of cisplatin resistance

All animal experiments were conducted in accordance with the Institutional Ethical Guidelines for Animal Experimentation of the National Institute of Biomedical Innovation (Osaka, Japan). Female Institute of Cancer Research (ICR) *nu/nu* mice were obtained from Charles River Japan (Yokohama, Japan). Injection of the ANXA4 knockdown cells was performed as follows: ICR *nu/nu* mice at 4 weeks of age were subcutaneously inoculated (into the flank of the mice; n = 6 per group) with 2.5×10^6 cells of RMG-I NC7 cells or RMG-I-Y4 cells in the total volume of 100 µL of 1/1 (v/v) PBS/Matrigel (Becton Dickinson, Bedford, MA). Injection of ANXA4 mutant-transfected cells, i.e. mice at 5 weeks of age were inoculated with 10^6 cells of NC-14, FL-22, R1-12 or R1(E70A) in the same manner

109

as with the ANXA4 knockdown cells. Treatment with cisplatin (3 mg/kg) or PBS *i.p.* was initiated 1 week after inoculation and administered twice weekly for 4 weeks (ANXA4 knockdown cells) and once a week for 3 weeks (ANXA4 mutant-transfected cells). Tumour volumes were determined twice weekly by measuring length (L), width (W) and depth (D) and using the following formula: tumour volume (mm^3) = W × L × D.

[Cl$^-$]$_i$ measurements

[Cl$^-$]$_i$ was measured using the fluorescent Cl$^-$ indicator N-ethroxycarbonymethyl-6-methoxyquinolinium bromide (MQAE; Dojindo, Kumamoto, Japan). [Cl$^-$]$_i$ is detected by the mechanism of diffusion-limited collisional quenching of MQAE fluorescence. MQAE fluorescence intensity inversely correlates with [Cl$^-$]$_i$. The cells of the ANXA4 deletion mutant series (NC-14, FL-22, R1-12 and R1[E70A]-95) were cultured in 35-mm tissue culture dishes up to 20% confluence and incubated with a medium containing 10 mM MQAE for 4 h at 37°C. After loading, the cells were washed 5 times with Cl$^-$-free buffer and electrically stimulated under a microscope at 37°C in a humidified atmosphere with 5% CO_2. Fluorescence measurements were initiated immediately at the indicated periods using Biozero BZ-9000 (Keyence, Tokyo, Japan) at 510/40 nm excitation and 380/50 nm emission. The fluorescence was quantitated by means of a standardised procedure using a BZ-II Analyser (Keyence), and the data were presented as the reciprocal of the ratio of fluorescence data (F0/F30) to identify possible correlations with the increase in [Cl$^-$]$_i$.

Statistical analysis

All calculations involved one-way analysis of variance (ANOVA) followed by Dunnett's analysis to evaluate the significance of differences. In all experiments, p value of <0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

REFERENCES

1. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer. 2007; 7(8):573-584.

2. Loriot Y, Mordant P, Deutsch E, Olaussen KA and Soria JC. Are RAS mutations predictive markers of resistance to standard chemotherapy? Nat Rev Clin Oncol. 2009; 6(9):528-534.

3. Alva AS, Matin SF, Lerner SP and Siefker-Radtke AO. Perioperative chemotherapy for upper tract urothelial cancer. Nat Rev Urol. 2012; 9(5):266-273.

4. Bookman MA. First-line chemotherapy in epithelial ovarian cancer. Clin Obstet Gynecol. 2012; 55(1):96-113.

5. Matos CS, de Carvalho AL, Lopes RP and Marques MP. New strategies against prostate cancer--Pt(II)-based chemotherapy. Curr Med Chem. 2012; 19(27):4678-4687.

6. Rossi A, Di Maio M, Chiodini P, Rudd RM, Okamoto H, Skarlos DV, Fruh M, Qian W, Tamura T, Samantas E, Shibata T, Perrone F, Gallo C, Gridelli C, Martelli O and Lee SM. Carboplatin- or cisplatin-based chemotherapy in first-line treatment of small-cell lung cancer: the COCIS meta-analysis of individual patient data. J Clin Oncol. 2012; 30(14):1692-1698.

7. Muggia FM and Los G. Platinum resistance: laboratory findings and clinical implications. Stem Cells. 1993; 11(3):182-193.

8. Jassem J. Chemotherapy of advanced non-small cell lung cancer. Ann Oncol. 1999; 10 Suppl 6:77-82.

9. Perez RP. Cellular and molecular determinants of cisplatin resistance. Eur J Cancer. 1998; 34(10):1535-1542.

10. Fuertes MA, Castilla J, Alonso C and Perez JM. Novel concepts in the development of platinum antitumor drugs. Curr Med Chem Anticancer Agents. 2002; 2(4):539-551.

11. Gonzalez VM, Fuertes MA, Alonso C and Perez JM. Is cisplatin-induced cell death always produced by apoptosis? Mol Pharmacol. 2001; 59(4):657-663.

12. Niedner H, Christen R, Lin X, Kondo A and Howell SB. Identification of genes that mediate sensitivity to cisplatin. Mol Pharmacol. 2001; 60(6):1153-1160.

13. Ishida S, Lee J, Thiele DJ and Herskowitz I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci U S A. 2002; 99(22):14298-14302.

14. Safaei R, Holzer AK, Katano K, Samimi G and Howell SB. The role of copper transporters in the development of resistance to Pt drugs. J Inorg Biochem. 2004; 98(10):1607-1613.

15. Katano K, Kondo A, Safaei R, Holzer A, Samimi G, Mishima M, Kuo YM, Rochdi M and Howell SB. Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. Cancer Res. 2002; 62(22):6559-6565.

16. Samimi G, Varki NM, Wilczynski S, Safaei R, Alberts DS and Howell SB. Increase in expression of the copper

transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients. Clin Cancer Res. 2003; 9(16 Pt 1):5853-5859.

17. Rabik CA, Maryon EB, Kasza K, Shafer JT, Bartnik CM and Dolan ME. Role of copper transporters in resistance to platinating agents. Cancer Chemother Pharmacol. 2009; 64(1):133-142.

18. Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M and Kuwano M. A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res. 1997; 57(24):5475-5479.

19. Cui Y, Konig J, Buchholz JK, Spring H, Leier I and Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol. 1999; 55(5):929-937.

20. Liedert B, Materna V, Schadendorf D, Thomale J and Lage H. Overexpression of cMOAT (MRP2/ABCC2) is associated with decreased formation of platinum-DNA adducts and decreased G2-arrest in melanoma cells resistant to cisplatin. J Invest Dermatol. 2003; 121(1):172-176.

21. Ban N, Takahashi Y, Takayama T, Kura T, Katahira T, Sakamaki S and Niitsu Y. Transfection of glutathione S-transferase (GST)-pi antisense complementary DNA increases the sensitivity of a colon cancer cell line to adriamycin, cisplatin, melphalan, and etoposide. Cancer Res. 1996; 56(15):3577-3582.

22. Olaussen KA, Dunant A, Fouret P, Brambilla E, Andre F, Haddad V, Taranchon E, Filipits M, Pirker R, Popper HH, Stahel R, Sabatier L, Pignon JP, Tursz T, Le Chevalier T and Soria JC. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med. 2006; 355(10):983-991.

23. Wang Q, Shi S, He W, Padilla MT, Zhang L, Wang X, Zhang B and Lin Y. Retaining MKP1 expression and attenuating JNK-mediated apoptosis by RIP1 for cisplatin resistance through miR-940 inhibition. Oncotarget. 2014; 5(5):1304-1314.

24. Li H and Yang BB. MicroRNA in drug resistance. Oncoscience. 2014; 1(1):2.

25. Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J, Wenham RM, Coppola D, Kruk PA, Nicosia SV and Cheng JQ. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer Res. 2008; 68(2):425-433.

26. Sorrentino A, Liu CG, Addario A, Peschle C, Scambia G and Ferlini C. Role of microRNAs in drug-resistant ovarian cancer cells. Gynecol Oncol. 2008; 111(3):478-486.

27. Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, Pierotti MA, Miyashita T, Reed JC and Zunino F. Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma

cell systems. Cancer Res. 1996; 56(3):556-562.

28. Kim A, Enomoto T, Serada S, Ueda Y, Takahashi T, Ripley B, Miyatake T, Fujita M, Lee CM, Morimoto K, Fujimoto M, Kimura T and Naka T. Enhanced expression of Annexin A4 in clear cell carcinoma of the ovary and its association with chemoresistance to carboplatin. Int J Cancer. 2009; 125(10):2316-2322.

29. Gerke V and Moss SE. Annexins: from structure to function. Physiol Rev. 2002; 82(2):331-371.

30. Nelson MR and Creutz CE. Combinatorial mutagenesis of the four domains of annexin IV: effects on chromaffin granule binding and aggregating activities. Biochemistry. 1995; 34(9):3121-3132.

31. Kaetzel MA, Hazarika P and Dedman JR. Differential tissue expression of three 35-kDa annexin calcium-dependent phospholipid-binding proteins. J Biol Chem. 1989; 264(24):14463-14470.

32. Chan HC, Kaetzel MA, Gotter AL, Dedman JR and Nelson DJ. Annexin IV inhibits calmodulin-dependent protein kinase II-activated chloride conductance. A novel mechanism for ion channel regulation. J Biol Chem. 1994; 269(51):32464-32468.

33. Kaetzel MA, Mo YD, Mealy TR, Campos B, Bergsma-Schutter W, Brisson A, Dedman JR and Seaton BA. Phosphorylation mutants elucidate the mechanism of annexin IV-mediated membrane aggregation. Biochemistry. 2001; 40(13):4192-4199.

34. Dreier R, Schmid KW, Gerke V and Riehemann K. Differential expression of annexins I, II and IV in human tissues: an immunohistochemical study. Histochem Cell Biol. 1998; 110(2):137-148.

35. Wei R, Zhang Y, Shen L, Jiang W, Li C, Zhong M, Xie Y, Yang D, He L and Zhou Q. Comparative proteomic and radiobiological analyses in human lung adenocarcinoma cells. Mol Cell Biochem. 2012; 359(1-2):151-159.

36. Alfonso P, Canamero M, Fernandez-Carbonie F, Nunez A and Casal JI. Proteome analysis of membrane fractions in colorectal carcinomas by using 2D-DIGE saturation labeling. J Proteome Res. 2008; 7(10):4247-4255.

37. Zimmermann U, Balabanov S, Giebel J, Teller S, Junker H, Schmoll D, Protzel C, Scharf C, Kleist B and Walther R. Increased expression and altered location of annexin IV in renal clear cell carcinoma: a possible role in tumour dissemination. Cancer Lett. 2004; 209(1):111-118.

38. Sitek B, Luttges J, Marcus K, Kloppel G, Schmiegel W, Meyer HE, Hahn SA and Stuhler K. Application of fluorescence difference gel electrophoresis saturation labelling for the analysis of microdissected precursor lesions of pancreatic ductal adenocarcinoma. Proteomics. 2005; 5(10):2665-2679.

39. Xin W, Rhodes DR, Ingold C, Chinnaiyan AM and Rubin MA. Dysregulation of the annexin family protein family is associated with prostate cancer progression. Am J Pathol. 2003; 162(1):255-261.

111

40. Duncan R, Carpenter B, Main LC, Telfer C and Murray GI. Characterisation and protein expression profiling of annexins in colorectal cancer. Br J Cancer. 2008; 98(2):426-433.

41. Yamashita T, Nagano K, Kanasaki S, Maeda Y, Furuya T, Inoue M, Nabeshi H, Yoshikawa T, Yoshioka Y, Itoh N, Abe Y, Kamada H, Tsutsumi Y and Tsunoda S. Annexin A4 is a possible biomarker for cisplatin susceptibility of malignant mesothelioma cells. Biochem Biophys Res Commun. 2012; 421(1):140-144.

42. Choi CH, Sung CO, Kim HJ, Lee YY, Song SY, Song T, Kim J, Kim TJ, Lee JW, Bae DS and Kim BG. Overexpression of annexin A4 is associated with chemoresistance in papillary serous adenocarcinoma of the ovary. Hum Pathol. 2013; 44(6):1017-1023.

43. Mogami T, Yokota N, Asai-Sato M, Yamada R, Koizume S, Sakuma Y, Yoshihara M, Nakamura Y, Takano Y, Hirahara F, Miyagi Y and Miyagi E. Annexin a4 is involved in proliferation, chemo-resistance and migration and invasion in ovarian clear cell adenocarcinoma cells. PLoS One. 2013; 8(11):e80359.

44. Matsuzaki S, Enomoto T, Serada S, Yoshino K, Nagamori S, Morimoto A, Yokoyama T, Kim A, Kimura T, Ueda Y, Fujita M, Fujimoto M, Kanai Y and Naka T. Annexin A4-conferred platinum resistance is mediated by the copper transporter ATP7A. Int J Cancer. 2013.

45. Jeon YJ, Kim DH, Jung H, Chung SJ, Chi SW, Cho S, Lee SC, Park BC, Park SG and Bae KH. Annexin A4 interacts with the NF-kappaB p50 subunit and modulates NF-kappaB transcriptional activity in a Ca2+-dependent manner. Cell Mol Life Sci. 2010; 67(13):2271-2281.

46. Al-Bahlani S, Fraser M, Wong AY, Sayan BS, Bergeron R, Melino G and Tsang BK. P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. Oncogene. 2011; 30(41):4219-4230.

47. Salerno M, Yahia D, Dzamitika S, de Vries E, Pereira-Maia E and Garnier-Suillerot A. Impact of intracellular chloride concentration on cisplatin accumulation in sensitive and resistant GLC4 cells. J Biol Inorg Chem. 2009; 14(1):123-132.

48. Chao AC, Dix JA, Sellers MC and Verkman AS. Fluorescence measurement of chloride transport in monolayer cultured cells. Mechanisms of chloride transport in fibroblasts. Biophys J. 1989; 56(6):1071-1081.

49. Masuishi Y, Arakawa N, Kawasaki H, Miyagi E, Hirahara F and Hirano H. Wild-type p53 enhances annexin IV gene expression in ovarian clear cell adenocarcinoma. FEBS J. 2011; 278(9):1470-1483.

50. Splettstoesser F, Florea AM and Busselberg D. IP(3) receptor antagonist, 2-APB, attenuates cisplatin induced Ca2+-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis. Br J Pharmacol. 2007; 151(8):1176-1186.

51. Safaei R. Role of copper transporters in the uptake and efflux of platinum containing drugs. Cancer Lett. 2006; 234(1):34-39.

52. El-Khateeb M, Appleton TG, Charles BG and Gahan LR. Development of HPLC conditions for valid determination of hydrolysis products of cisplatin. J Pharm Sci. 1999; 88(3):319-326.

53. Kelland LR. Preclinical perspectives on platinum resistance. Drugs. 2000; 59 Suppl 4:1-8; discussion 37-38.

54. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene. 2003; 22(47):7265-7279.

55. Gaudio E, Paduano F, Spizzo R, Ngankeu A, Zanesi N, Gaspari M, Ortuso F, Lovat F, Rock J, Hill GA, Kaou M, Cuda G, Aqeilan RI, Alcaro S, Croce CM and Trapasso F. Fhit delocalizes annexin a4 from plasma membrane to cytosol and sensitizes lung cancer cells to Paclitaxel. PLoS One. 2013; 8(11):e78610
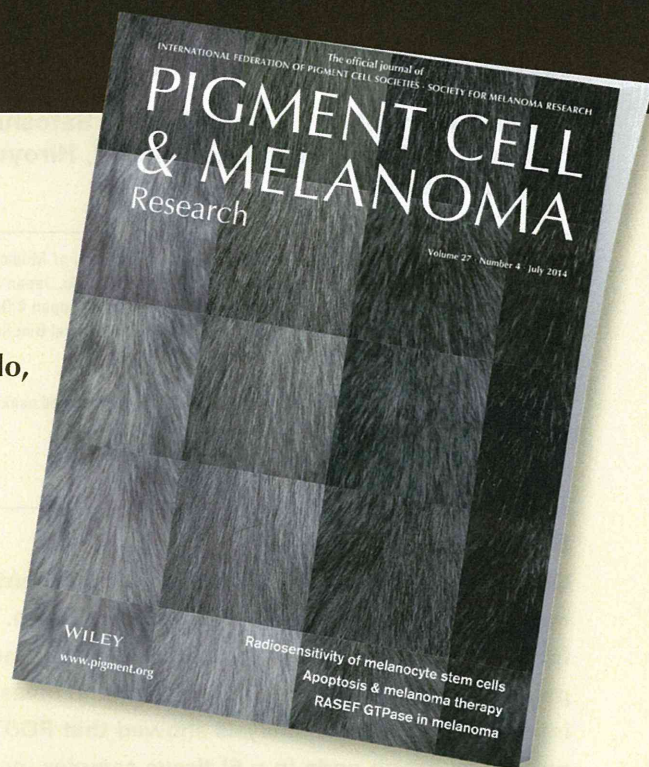
Periostin accelerates human malignant melanoma progression by modifying the melanoma microenvironment

Yorihisa Kotobuki, Lingli Yang, Satoshi Serada,
Atsushi Tanemura, Fei Yang, Shintaro Nomura, Akira Kudo,
Kenji Izuhara, Hiroyuki Murota, Minoru Fujimoto,
Ichiro Katayama and Tetsuji Naka

If you wish to order reprints of this article,
please see the guidelines **here**

Supporting Information for this article is freely available **here**

EMAIL ALERTS

Receive free email alerts and stay up-to-date on what is published
in Pigment Cell & Melanoma Research – **click here**

Submit your next paper to PCMR online at http://mc.manuscriptcentral.com/pcmr

Subscribe to PCMR and stay up-to-date with the only journal committed to publishing
basic research in melanoma and pigment cell biology

As a member of the IFPCS or the SMR you automatically get online access to PCMR. Sign up as
a member today at **www.ifpcs.org** or at **www.societymelanomaresarch.org**

To take out a personal subscription, please click here
More information about Pigment Cell & Melanoma Research at **www.pigment.org**

ORIGINAL ARTICLE

Periostin accelerates human malignant melanoma progression by modifying the melanoma microenvironment

Yorihisa Kotobuki[1,2,a], Lingli Yang[1,a], Satoshi Serada[2], Atsushi Tanemura[1], Fei Yang[1], Shintaro Nomura[3], Akira Kudo[4], Kenji Izuhara[5], Hiroyuki Murota[1], Minoru Fujimoto[2], Ichiro Katayama[1] and Tetsuji Naka[2]

1 Department of Dermatology, Osaka University Graduate School of Medicine, Suita, Japan 2 Laboratory for Immune Signal, National Institute of Biomedical Innovation, Ibaraki, Japan 3 Department of Animal Bioscience, Nagahama Institute of Bio-Science and Technology, Nagahama, Japan 4 Department of Biological Information, Tokyo Institute of Technology, Yokohama, Japan 5 Division of Medical Biochemistry, Department of Biomolecular Sciences, Saga Medical School, Saga, Japan

CORRESPONDENCE Atsushi Tanemura, e-mail: tanemura@derma.med.osaka-u.ac.jp

[a]These two authors contributed equally to this work.

Summary

Given no reliable therapy for advanced malignant melanoma, it is important to elucidate the molecular mechanisms underlying the disease progression. Using a quantitative proteomics approach, the 'isobaric tags for relative and absolute quantitation (iTRAQ)' method, we identified that the extracellular matrix protein, periostin (POSTN), was highly expressed in invasive melanoma compared with normal skin. An immunohistochemical analysis showed that POSTN was expressed in all invasive melanoma (n = 20) and metastatic lymph node (n = 5) tissue samples, notably restricted in their stroma. In terms of the intercellular regulation of POSTN, we found that there was upregulation of POSTN when melanoma cells and normal human dermal fibroblasts (NHDFs) were cocultured, with restricted expression of TGF-β1 and TGF-β3. In a functional analyses, recombinant and NHDF-derived POSTN significantly accelerated melanoma cell proliferation via the integrin/mitogen-activated protein kinase (MAPK) signaling pathway in vitro. The size of implanted melanoma tumors was significantly suppressed in POSTN/Rag2 double knockout mice compared with Rag2 knock-out mice. These results indicate that NHDF-derived POSTN accelerates melanoma progression and might be a promising therapeutic target for malignant melanoma.

Significance

In this study, we found an extracellular matrix protein, periostin (POSTN), increased in invasive melanoma compared with radial growth melanoma a quantitative proteomics approach, the 'isobaric tags for relative and absolute quantitation (iTRAQ)' method. POSTN was exclusively expressed in the tumor-associated stromal tissue not in the tumor cells, suggesting the paracrine effect of POSTN to melanoma cells aggressiveness. As expected, secreted POSTN could augment cell proliferation in melanoma cell lines in vitro. Moreover, we generated of postn and rag2 double knockout mice and showed significant inhibition of human melanoma growth in those KO mice in vivo. This study could give us the cue of therapeutic effect on melanoma growth by an agent controlling tumor microenvironment induced by POSTN.