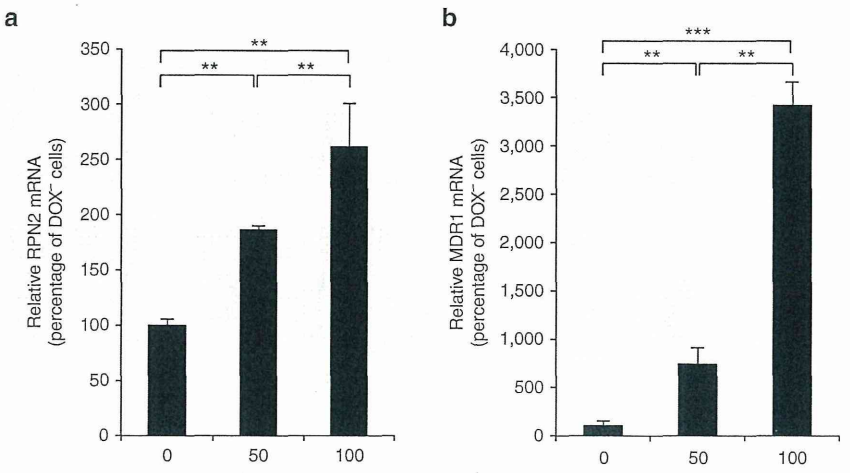

Figure 3 Induction of RPN2 and MDR1 expression by doxorubicin treatment. (**a**) RPN2 expression levels induced by doxorubicin treatment in 143B cells. The data shown are from 48 hours after doxorubicin treatment. Data are presented as mean ± SD ($n = 3$ per group). $^{**}P < 0.01$; Student's *t*-test. (**b**) MDR1 expression levels induced by doxorubicin treatment in 143B cells. The data shown are from 48 hours after doxorubicin treatment. Data are presented as mean ± SD ($n = 3$ per group). $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test.

for survival, and 143B-shRPN2-bearing mice showed longer survival than 143B-shNC-bearing mice (log-rank test, $P = 0.020$) (**Figure 4e**), suggesting that decreased RPN2 expression provided a survival advantage on osteosarcoma-bearing mice.

Discussion

An enormous body of research has been directed to overcome the lethal phenotypes of malignant neoplasms. Recent progress in targeted therapies has opened a new avenue in the treatment of sarcomas. However, little have been proven to be more effective than conventional therapies.[21,22] Therefore, there is an urgent need to develop novel treatments for osteosarcoma. In this context, we have shown that RNA interference for RPN2 suppresses cell proliferation, sphere formation ability, and invasiveness, and increases the sensitivity of osteosarcoma cells to a wide range of chemotherapeutic drugs *in vitro*. Notably, RPN2 silencing inhibited tumor growth as well as lung metastasis formation, leading to a survival advantage of osteosarcoma-bearing mice. Furthermore, we found a close correlation between RPN2 expression and the clinicopathological features such as metastatic status and prognosis.

Using gene expression profiling of breast cancer biopsy samples between responders and nonresponders to docetaxel, Iwao-Koizumi *et al.* devised a diagnostic system that was able to predict the clinical response to docetaxel treatment, and identified molecular targets for therapy.[23] As an extension of their report, we previously performed a study of RNAi-induced gene knockdown in docetaxel-resistant breast cancer cells, and identified the RPN2 gene, which is part of the N-oligosaccharyl transferase complex, as a new target for overcoming the drug resistance of breast cancer. Specifically, silencing of RPN2 reduced the glycosylation of the P-glycoprotein and decreased its membrane localization, thereby sensitizing cancer cells to docetaxel. A recent study by Kurashige *et al.* has shown that RPN2 expression is also able to predict the docetaxel response of esophageal squamous cell carcinoma. Silencing of RPN2 increased the sensitivity of esophageal cancer cells to docetaxel. However, the function and correlation of RPN2 expression with the clinical features of other malignancies, including mesenchymal neoplasms, remains to be elucidated. In this study of osteosarcoma cells, we demonstrated that silencing of RPN2 increased cell sensitivity to doxorubicin, methotrexate, and docetaxel. Doxorubicin and methotrexate are standard drugs for treatment of osteosarcoma, the former being especially effective.[24] Since osteosarcoma patients who show a poor response to these drugs have a poor prognosis,[10,18] silencing of RPN2 in osteosarcoma tissue would improve prognosis by sensitizing the cancer cells to these drugs. Furthermore, studies of second-line chemotherapy for osteosarcoma have made progress in recent years.[25] In phase 2 trials with gemcitabine or docetaxel alone, up to 8% of patients with bone or soft tissue sarcomas showed objective responses.[26,27] When gemcitabine was combined with docetaxel in a series of 10 patients with recurrent or progressive osteosarcoma, three patients showed partial responses and one showed stable disease.[28] Since silencing of RPN2 could sensitize osteosarcoma to docetaxel, this approach might also be effective for patients with recurrent or progressive osteosarcoma. Collectively, the RPN2 gene may represent a novel target for RNAi therapeutics against a wide range of malignant neoplasms.

Our human study demonstrated that high expression of RPN2 in biopsy samples of osteosarcoma was significantly correlated with patient prognosis. Immunohistochemically, however, all specimens in this sample set were moderately to strongly positive for RPN2 protein. Therefore, we were unable to predict metastatic ability or prognosis on the basis of immunohistochemical staining for RPN2 protein. However, this result indicated that silencing of RPN2 may contribute to sensitization of osteosarcoma cells to chemotherapeutics in all patients. Among 16 high-RPN2 patients who showed a tumor response to neoadjuvant chemotherapy, 13 (81%) were poor responders. These data suggested that patients

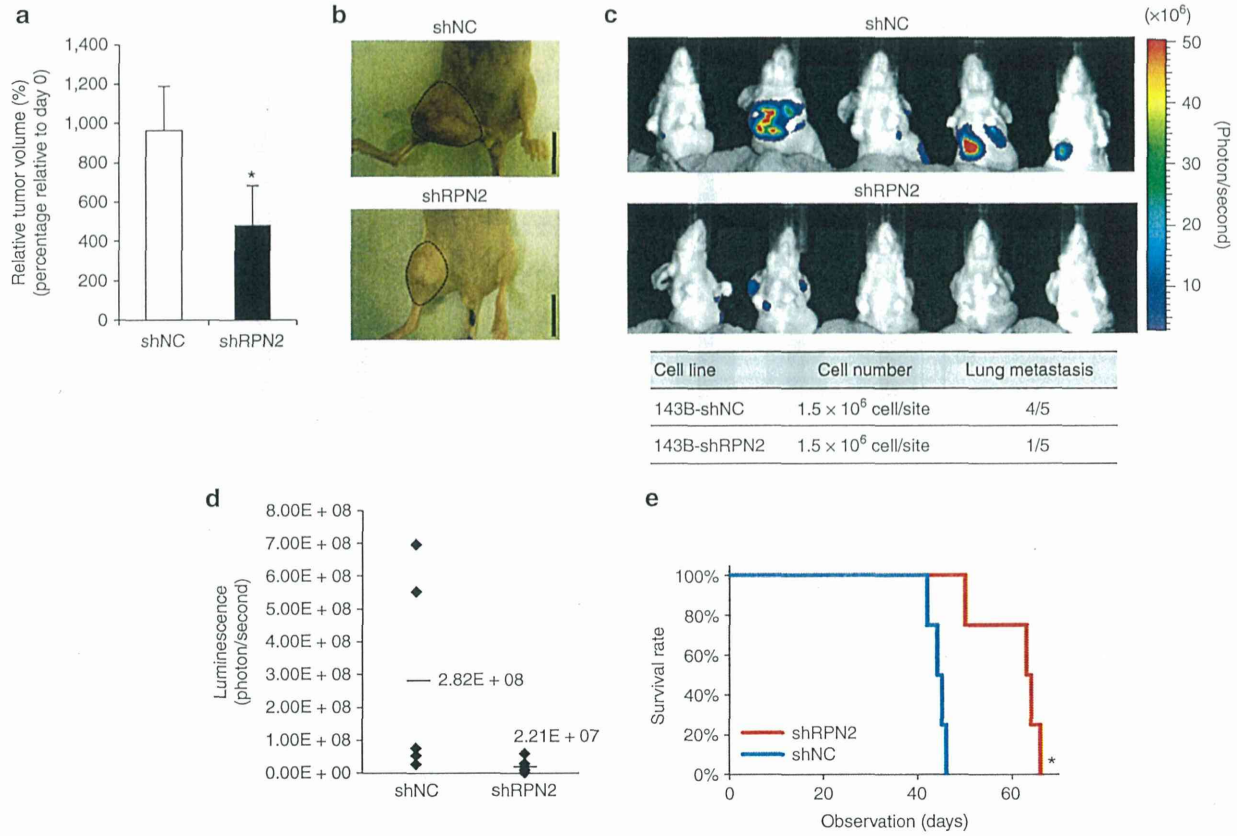

Figure 4 Role of RPN2 in osteosarcoma primary tumor growth and metastasis. (**a**,**b**) Tumors at the primary site of each group measured at 3 weeks after inoculation. The size of each tumor in mice was measured (**a**). Data are presented as mean ± SD ($n = 5$ per group). *$P < 0.05$; Student's *t*-test. The macroscopic appearances of 143B-shRPN2 and 143B-shNC tumors are shown (**b**). The tumor masses are outlined by a dotted line. Scale bars, 10 mm. (**c**,**d**) The lung metastases of each group measured on day 22 using an *in vivo* imaging system (**c**). The luminescence of the chest lesions in each group of mice was determined (**d**). (**e**) Survival curves for each group of mice by Kaplan–Meier analysis. Log-rank test was performed between the two groups (*$P = 0.020$).

showing higher expression of RPN2 in osteosarcoma might tend to be poor responders to neoadjuvant chemotherapy, although the difference between these groups was not statistically significant. We considered that the correlation between high-RPN2 expression in biopsy samples and prognosis might have been due to the metastatic expression of osteosarcoma, since RPN2 expression was significantly correlated with clinical metastasis. Therefore, we analyzed the correlation between RPN2 expression and cell invasion, which is one of the important phenotypes associated with metastasis.

We found that RPN2 expression also regulates the invasiveness of osteosarcoma cells, representing a novel function of RPN2. Although the molecular mechanisms responsible for regulation of invasiveness via RPN2 protein are unclear, previous reports have demonstrated that N-linked glycosylation correlates with tumor cell invasion or metastatic phenotypes.[29–35] N-glycosylation of integrins plays an important role in their biological functions.[31–33] Integrins, cell surface transmembrane glycoproteins that function as adhesion receptors between cell and extracellular matrix and link matrix proteins to the cytoskeleton, play an important role in cytoskeletal organization and in the transduction of intracellular signals, regulating various processes such as proliferation, differentiation, apoptosis, and cell migration.[32] Reportedly, in comparison with the non-metastatic WM35 melanoma cell line, α1 and β3 subunits, expressed by the metastatic A375 melanoma cell line, carry β1,6 GlcNAc branched structures, suggesting that these cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand-binding properties of α1β3 integrin.[35] Another report has shown that N-glycosylation is essential for the function of integrin α5β1, and that any alteration in the expression of N-glycans in α5β1 integrin would contribute to the adhesive and metastatic properties of tumors. When NIH3T3 cells were transformed with the oncogenic *Ras* gene, cell spreading on fibronectin was greatly enhanced due to an increase in β1,6 GlcNAc branched tri- and tetra-antennary oligosaccharides in α5β1 integrin.[29] Indeed, α5β1 integrin is related to tumor cell invasion and metastatic potential in osteosarcoma cells.[36] Therefore, inhibition of osteosarcoma cell invasion was caused by RPN2 silencing via alteration of N-glycosylation status of this molecule. Furthermore, a novel function of RPN2-mediated tumor cell malignancy was recently reported. RPN2 silencing resulted in reduced CD63 glycosylation and deregulated localization in tumor cells, which regulates drug resistance and tumor cell invasion. Collectively, the glycosylation status

of several molecules associated with tumor cell invasion may be regulated by RPN2 expression.[37] Moreover, considering the regulation of sphere formation ability of osteosarcoma cells, RPN2 might be correlated with cancer stem cell properties of osteosarcoma, which was also indicated in breast cancer cells.[37] Since the direct interaction of these phenotypes with RPN2 in osteosarcoma has not been elucidated, further study is needed to clarify the molecular mechanisms underlying the tumor-suppressive function by RPN2 silencing.

In this study, we found that RPN2 silencing contributed to inhibition of tumor growth and lung metastasis formation *in vivo*. Previously, we showed that atelocollagen-mediated RPN2 small interfering RNA (siRNA) delivery markedly reduced tumor growth in murine breast cancer models.[20] In recent years, RNA interference (RNAi) therapeutics, most notably with lipid nanoparticle-based delivery systems, have advanced to the human clinical trial stage.[38–41] One of the most advanced trials included a study from the United States in 2010 demonstrating that systemic administration of siRNA via targeted nanoparticles reduced the levels of both specific mRNA (the M2 subunit of ribonucleotide reductase; RRM2) and protein (RRM2) in melanoma biopsy specimens, representing the first human phase 1 clinical trial for patients with solid cancers.[38] Our preclinical trial of RPN2 silencing suggests that it would be worth evaluating the efficacy of siRNA administration, which is our next goal. In fact, a clinical phase 1 study of siRNA targeting RPN2 is now in the preliminary stage at our institution, and it is anticipated that this will yield novel information on treatments for solid cancers.

Our study had several limitations that warrant consideration. First, the number of patients in our clinical cohort was relatively small. For this reason, we were unable to draw any clear conclusions about the correlation between RPN2 expression in biopsy samples and tumor response to neoadjuvant chemotherapy, while most patients in the high-RPN2 group were poor responders. A larger series with more patients will therefore be needed to validate these results, and for this purpose we are continuing to collect biopsy specimens from osteosarcoma patients. Second, as RPN2 regulates the membrane localization of P-glycoprotein via N-linked glycosylation, the glycosylation status of the invasion-related molecules might also be affected, which we plan to elucidate. Third, the molecular mechanisms underlying RPN2 upregulation in highly malignant cells or during a drug response have not been elucidated. Therefore, we plan to further investigate the molecular mechanisms underlying RPN2 upregulation and the interactions of N-linked glycosylation with invasion-related molecules with RPN2 in osteosarcoma cells.

In summary, we have shown that the RPN2 gene is moderately to strongly expressed in all osteosarcomas, and that higher RPN2 expression is significantly correlated with clinical metastasis and poor patient survival. Furthermore, silencing of RPN2 contributes to reduction of cell proliferation, sphere formation, and invasiveness, and sensitizes osteosarcoma cells to standard chemotherapeutic regimens, thus providing a survival advantage on osteosarcoma-bearing mice. These data indicate that the RPN2 gene may represent a novel target for RNAi therapeutics against osteosarcoma.

MATERIALS AND METHODS

Cells and cell culture. Three human osteosarcoma cell lines (SaOS2, HOS, and 143B) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA). All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), penicillin (100 U/ml), and streptomycin (100 µg/ml), and the cells were maintained under 5% CO_2 in a humidified incubator at 37 °C.

Lentiviral shRNA transduction. Cell lines stably expressing RPN2 shRNA or control non-target shRNA were established using a vector-based shRNA technique (**Supplementary Figure S1**). Human RPN2 shRNA targets 5′-GGAGGAGA TTGAGGACCTTGT-3′ (shRPN2-site1), 5′-GCCACTTTGAA GAACCCAATC-3′ (shRPN2-site2), 5′-TCCAGATTGTAGTT ATACTTC-3′ (shRPN2-UTR), and control shRNA targets 5′-GAAATGTACTGCGCGTGGAGAC-3′. Briefly, each fragment was subcloned into pGreenPuro (System Biosciences, Tokyo, Japan). Recombinant lentiviruses were produced in accordance with the manufacturer's instructions. In knockdown experiments, 143B cells were infected with recombinant lentiviruses expressing control shRNA (shNC) or shRNA against RPN2 (shRPN2).[37]

RNA isolation and qRT-PCR. We purified total RNA from tumor cells and tissues using the RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN, Tokyo, Japan). For qPCR of mRNAs, cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). For each qPCR, equal amounts of cDNA were mixed with Platinum SYBR Green qPCR SuperMix (Life Technologies) and the specific primers (**Supplementary Table S1**). We normalized gene expression levels to β-actin or *GAPDH*.

Western blotting. Western blotting was performed as described previously.[20] The membranes were blotted with a rabbit polyclonal antibody against human RPN2 antigen (1:100 dilution, H-300, Santa Cruz Biotechnology, Santa Cruz, CA), or with a monoclonal antibody against β-actin (1:2,000, AC-15, Sigma, St Louis, MO). Signals were visualized with an enhanced chemiluminescence system (ECL Detection System; Amersham Pharmacia Biotech Piscataway, NJ).

Cell proliferation and cytotoxicity assays. The cell proliferation rates and cell viability were determined using the TetraColor ONE Cell Proliferation Assay (Seikagaku, Tokyo, Japan) or Cell proliferation kit 8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Cells growing in the logarithmic phase were seeded in 96-well plates (5×10^3/well), allowed to attach overnight, and then treated with varying doses of doxorubicin (Sigma-Aldrich, St. Louis, MO), cisplatin (Enzo Life Sciences, Farmingdale, NY), methotrexate (Sigma-Aldrich), and docetaxel (Sigma-Aldrich) for 72 hours. Triplicate wells were used for each treatment group. The absorbance was measured at 450 nm with a reference wavelength at 650 nm using EnVision (Perkin-Elmer, Waltham, MA).

The relative number of viable cells was expressed as a percentage of the total number.

Sphere formation. Osteosarcoma cells were plated at 100 cells/well in 300 µl of serum-free DMEM/F12 medium (Life Technologies) supplemented with 20 ng/ml human recombinant EGF (Sigma-Aldrich), 10 ng/ml human recombinant bFGF (Life Technologies), 4 µg/ml insulin (Sigma-Aldrich), B27 (1:50; Invitrogen), 500 units/ml penicillin (Life Technologies), and 500 µg/ml streptomycin (Life Technologies). The cells were cultured in suspension in 24-well ultra-low attachment plates (Corning, Corning, NY), and replenished with 30 µl of new medium every second day. The spheres were counted on day 5 in triplicate wells. Cell culture was maintained at 37 °C in a 5% CO_2 humidified incubator.

Invasion assays. Invasion assays were performed using 24-well BD BioCoat Invasion Chambers with Matrigel (Becton-Dickinson, Tokyo, Japan). A total of 1×10^5 cells were suspended in 500 µl DMEM medium without fetal bovine serum and added to the upper chamber. DMEM medium with 10% fetal bovine serum was added to the lower chamber. After incubation for 24 hours, the cells on the upper surface of the filter were completely removed by wiping with cotton swabs. The filters were fixed in methanol and stained with 1% toluidine blue in 1% sodium tetraborate (Sysmex, Kobe, Japan). The filters were then mounted onto slides, and the cells on the lower surfaces were counted.

Animal experiments. Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Four- to six-week-old female Balb/c athymic nude mice (CLEA Japan, Tokyo, Japan) were anesthetized by exposure to 3% isoflurane for injections and *in vivo* imaging. On day 0, the mice were anesthetized with 3% isoflurane, and the right leg was disinfected with 70% ethanol. The cells were aspirated into a 1-ml tuberculin syringe fitted with a 27-G needle. The needle was inserted through the cortex of the anterior tuberosity of the tibia with a rotating movement to avoid cortical fracture. Once the bone was traversed, the needle was inserted further to fracture the posterior cortex of the tibia. A 100 µl volume of solution containing 1.5×10^6 cells of 143B-shRPN2 ($n = 5$) or 143B-shNC ($n = 5$) cells was injected while slowly removing the needle. Tumor size was monitored by measuring tumor length and width using calipers. The volumes of 143B-shRPN2 or 143B-shNC tumors were calculated using the formula: $(L + W) \times L \times W \times 0.2618$, where L is the length and W is the width of each tumor as reported previously.[42] To evaluate lung metastases, mice were injected with D-luciferin (150 mg/kg, Promega, Tokyo, Japan) by intraperitoneal injection. After 10 minutes, the photons from the firefly luciferase were counted using the *in vivo* imaging system (Xenogen, Alameda, CA) according to the manufacturer's instructions. Data were analyzed using LIVINGIMAGE 4.3.1 software (Xenogen).

Human samples. The osteosarcoma tissue samples were obtained from diagnostic incisional biopsies of primary osteosarcoma sites before the start of neoadjuvant chemotherapy at the National Cancer Center Hospital, Tokyo, between 1997 and 2010. Patients older than 40 years of age or patients who had primary tumors located outside the extremities were excluded. Each fresh tumor sample was cut into two pieces; one piece was immediately cryopreserved in liquid nitrogen, and the other piece was fixed in formalin. The diagnosis of osteosarcoma and the histological subtypes were determined by certified pathologists. The response to chemotherapy was classified as good if the tumor necrosis was 90% or greater. All patients provided written informed consent authorizing the collection and use of their samples for research purposes. The study protocol for obtaining clinical information and collecting samples was approved by the Institutional Review Board of the National Cancer Center of Japan.

Immunohistochemistry. To stain RPN2, we prepared slides from clinical samples of osteosarcoma. Endogenous peroxidase was quenched with 1% H_2O_2 (30 minutes). The slides were heated for antigen retrieval in 10 mmol/l sodium citrate (pH 6.0). Subsequently, we incubated the slides with RPN2-specific antibody (1:100 dilution, H-300, Santa Cruz Biotechnology) and isotype-matched control antibodies overnight at 4 °C. Immunodetection was performed using ImmPRESS peroxidase polymer detection reagents (Vector Laboratories, Burlingame, CA) and the Metal-Enhanced DAB Substrate Kit (Thermo Fisher Scientific, Yokohama, Japan) in accordance with the manufacturer's directions. Sections were counterstained with hematoxylin for contrast.

Statistical analysis. All statistical analyses were performed using SPSS Statistics Version 21 software (IBM SPSS, Tokyo, Japan). Student's *t*-test or Welch's *t*-test, was used to determine the significance of any differences between experimental groups. Differences in RPN2 expression among different clinicopathological data were analyzed using the chi-squared (χ^2) test. We performed ROC curve analysis using the SPSS software, and the optimal cutoff points for the expression levels of RPN2 were determined by the Youden index, *i.e.*, J = max (sensitivity + specificity − 1).[43] The Kaplan–Meier method and the log-rank test were used to compare the survival of patients. We defined the survival period as the time from diagnosis until death, whereas living patients were censored at the time of their last follow-up. For all the analyses, we considered a P value of 0.05 or less to be significant.

SUPPLEMENTARY MATERIAL

Figure S1. Diagram of the lentiviral vector utilized in the present study.
Figure S2. Phase-contrast micrograph of 143B-shRPN2 and 143B-shNC cells in the presence of 50 nmol/l methotrexate (**a**), 10 µmol/l cisplatin (**b**), and 10 nmol/l docetaxel (**c**).
Figure S3. Metastasis index (= intensity of lung metastasis luminescence/primary tumor size) in 143B-shRPN2-bearing mice or 143B-shNC-bearing mice at 3 weeks after orthotopic implantation.
Table S1. The sequences of primers used for real-time RT-PCR analysis.

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The authors declare that no conflicts of interest exist with regard to this study.

T.F. initiated the project, performed the experimental work, and wrote the draft of the manuscript. R.T. helped with the experimental work and data analysis. N.K. provided helpful discussion. A.K. and T.O. initiated the project and provided clinical samples. The manuscript was finalized by T.O. with the assistance of all authors.

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Editorial

RPN2: A Promising Therapeutic Target for Breast Cancer?

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Editorial

Ribopholin II (RPN2) is a type I integral membrane protein localized to the rough endoplasmic reticulum [1]. RPN2 is partly involved in the N-lined glycosylation reaction that conjugates high mannose oligosaccharides to the asparagine residues found in the N-X-S/T consensus sequence of nascent polypeptide chains [2]. Changes in glycosylation patterns are common in both normal and abnormal biological processes, including cancer in which aberrant glycosylation is associated with malignant transformation and promotes the acquisition of invasiveness and metastatic ability [3–5].

N-linked glycosylation is modulated by an oligosaccharyl transferase (OST) complex comprising seven subunits: ribophorin I (RPN1), DAD1, N33/IAP, OST4, STT3A/STT3B, Ost48, and RPN2 [2]. RPN1 regulates the glycosylation of several glycoproteins by selectively interacting with, and delivering them to, the catalytic core of the OST complex [6,7]; however, the role of RPN2 is not well characterized. Honma et al. were the first to discover that RPN2 plays an important role in the acquisition of drug resistance by breast cancer cells [8]. They examined the responses of breast cancer patients to docetaxel and found that non-responders showed higher expression of RPN2 than responders [8,9]. In drug-resistant breast cancer, RPN2 modulates the N-linked glycosylation of p-glycoprotein (a major cause of docetaxel resistance), thereby regulating its efflux activity [8]. Since drug resistance is a major focus of current cancer research, targeting p-glycoprotein with small molecules, including natural products and peptides, is a promising approach to overcoming cancer recurrence and a subsequent poor prognosis. Therefore, silencing RPN2 with siRNAs might improve the outcome for drug-resistant breast cancer patients.

Our own group reported that RPN2 is highly expressed in a population of breast cancer stem cells (CSC) expressing the CD44high/CD24low antigen phenotype [10]. In addition, Mani et al. demonstrated that non-CSC acquires the CD44high/CD24low antigen phenotype after epithelial to mesenchymal transition (EMT), resulting in drug resistance and high tumorigenicity [11]; therefore, EMT regulators such as Snail, Slug, and Twist are important factors for CSC generation. RPN2 induces EMT and metastatic activity in highly metastatic breast cancer cells by stabilizing Snail [12]. It does this by inhibiting GSK3β, a serine/threonine protein kinase that normally suppresses Snail expression and destabilizes the Snail protein [12]. Interestingly, Zhu et al. found that RPN2 is also highly expressed in pancreatic CSCs [13].

A recent study shows that RPN2 is associated with the N-linked glycosylation of CD63 [14]. CD63 is a member of the tetraspanin super family [15] and a component of exosomes (small extracellular vesicles containing proteins and small RNAs) [16]. A number of studies report that, in addition to extracellular communication, exosomes play important roles in cancer development and metastasis [17,18]. As RPN2 is highly expressed in breast CSCs and regulates the localization of CD63 at the cell surface, it might be involved in the development of a pre-metastatic niche. Taken together, the results of the tudies discussed herein suggest that identifying the role of RPN2 in cancer biology may shed light on the mechanisms underlying CSC generation and lead to promising approaches to treating intractable cancers.

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MOLECULAR CANCER

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RPN2-mediated glycosylation of tetraspanin CD63 regulates breast cancer cell malignancy

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Abstract

Background: The tetraspanin CD63 is a highly N-glycosylated protein that is known to regulate cancer malignancy. However, the contribution of glycosylation of CD63 to cancer malignancy remains unclear. Previously, we reported that ribophorin II (RPN2), which is part of an N-oligosaccharyle transferase complex, is responsible for drug resistance in breast cancer cells. In this study, we demonstrate that cancer malignancy associated with the glycosylation of CD63 is regulated by RPN2.

Results: Inhibition of RPN2 expression led to a reduction in CD63 glycosylation. In addition, the localization of CD63 was deregulated by knockdown of RPN2. Interestingly, multidrug resistance protein 1 (MDR1) localization was displaced from the cell surface in CD63-silenced cells. CD63 silencing reduced the chemoresistance and invasion ability of malignant breast cancer cells. Furthermore, the enrichment of CD63/MDR1-double positive cells was associated with lymph node metastasis. Taken together, these results indicated that high glycosylation of CD63 by RPN2 is implicated in clinical outcomes in breast cancer patients.

Conclusions: These findings describe a novel and important function of RPN2-mediated CD63 glycosylation, which regulates MDR1 localization and cancer malignancy, including drug resistance and invasion.

Background

The tetraspanin family is a group of cell surface proteins that are characterized by four transmembrane domains [1]. It is well known that tetraspanin proteins regulate several types of physiological properties, including cell morphology, motility, invasion, fusion and signaling of tumors, among others [2]. The CD63 gene, which is located on human chromosome 12q13, was the first tetraspanin to be characterized [3]. Recent studies have demonstrated that CD63 interacts with many different proteins, either directly or indirectly, and regulates intracellular transport and localization [4,5]. In addition, an increasing number of studies have indicated that the cell surface expression of CD63 is tightly regulated by glycosylation [6]. In fact, the molecular weight of CD63 has been observed to be 32, 35, or 50 kDa with N-linked glycosylation in western blotting experiments, although the predicted molecular weight of CD63 is 25 kDa [7]. Furthermore, it has been reported that CD63 is associated with the biological behavior of solid tumors, especially those with metastatic potential [8]. However, the contribution of glycosylation of CD63 to cancer malignancy is poorly understood.

Previously, we established that glycosylation in multidrug resistance protein 1 (MDR1, also known as ABCB1) is regulated by ribophorin II (RPN2), which is part of an N-oligosaccharyl transferase complex [9]. RPN2 silencing induced docetaxel-dependent apoptosis and cell growth inhibition of human breast cancer cells through the reduction of P-glycoprotein glycosylation. In addition, *in vivo* delivery of RPN2 siRNA inhibited tumor growth in mice given docetaxel. These observations indicated that RPN2 is a key regulator of N-glycosylation in drug-resistant cancer cells. However, little is currently known regarding the association between RPN2 and specific glycosylated proteins that are related to cancer malignancy. In this study, we demonstrate that RPN2 promotes cancer cell malignancy in breast cancer cells through the regulation of CD63 glycosylation.

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Results

Inhibition of RPN2 expression led to the deregulation of CD63 glycosylation

To investigate whether CD63 was glycosylated by RPN2, MCF7-ADR and MDA-MB-231-luc-D3H2LN (MM231-LN) cells were transiently transfected with siRNA against RPN2, and the glycosylation state of CD63 was examined using western blotting. The reduction in RPN2 expression after transduction with the RPN2 siRNA was confirmed using western blotting (Figure 1A). The RPN2 siRNA had no effect on total CD63 expression in either breast cancer cell line (Figure 1B). However, as shown in Figure 1C, the molecular weight of CD63 decreased in RPN2 siRNA-treated cells compared to control siRNA-treated cells (N.C.) in the MM231-LN (upper panel) and MCF7-ADR (lower panel) cell lines. In addition, to confirm whether the molecular weight of CD63 actually decreased after deglycosylation, N-glycanase was added to cell lysates of MCF7-ADR and MM231-LN cells transfected with control or RPN2 siRNAs. As shown in Figure 1D, the molecular weight of glycosylated CD63 decreased after treatment with N-glycanase in both breast cancer cell lines, suggesting that the smeared band represents the glycosylated form of CD63. Furthermore, a non-glycosylated form of CD63 (25 kDa) and a less glycosylated form of CD63 (35 kDa) emerged from the 50 kDa glycosylated form of CD63 (Figure 1D) [7]. The N-glycanase experiment demonstrated the differences in the molecular weight of various forms of the CD63 protein. These results indicated that RPN2 contributes to the N-glycosylation of CD63 in human breast cancer cells.

CD63 localization was regulated by RPN2

It is well known that glycosylation affects the localization of proteins within the cytoplasm, membranes and pericellular matrix [10]. CD63 is a ubiquitously expressed protein that is localized within the endosomal system [5]. We performed an apoptosis assay using Hoechst staining and a

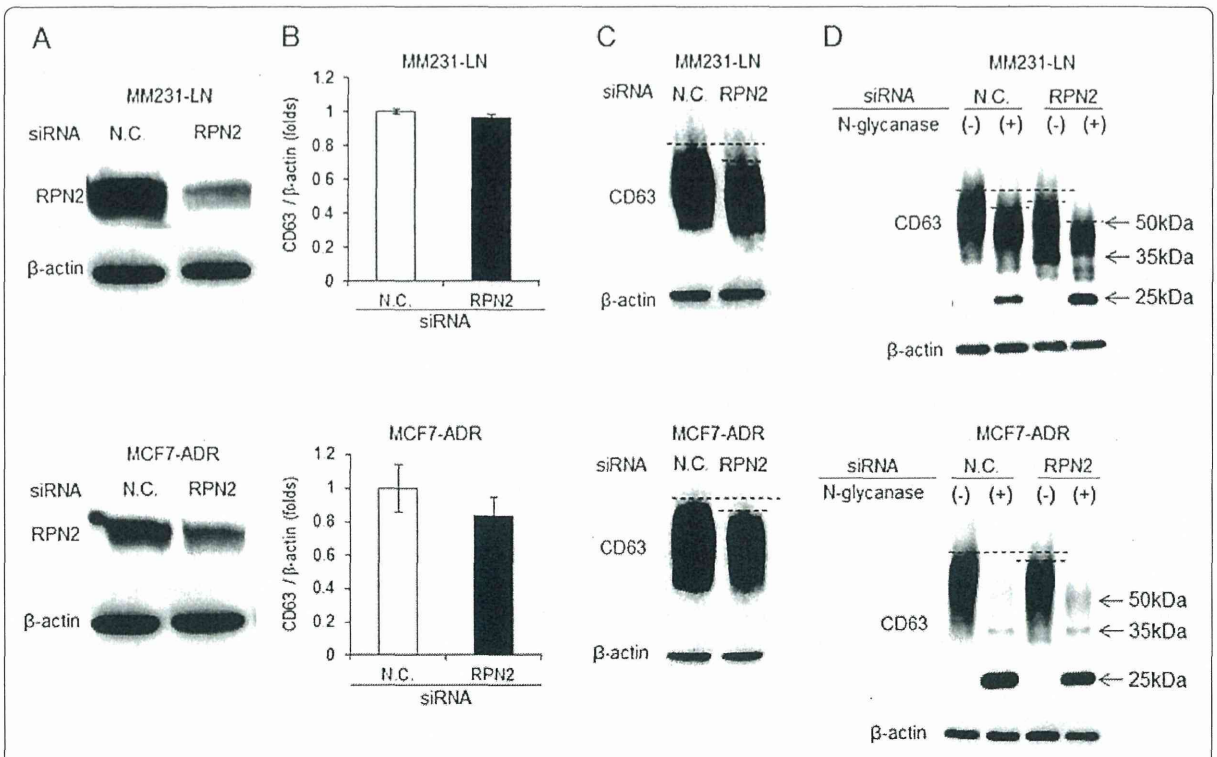

Figure 1 CD63 glycosylation in breast cancer cells. A) MDA-MB-231-luc-D3H2LN (MM231-LN) (upper panel) and MCF7-ADR cells (lower panel) were transiently transfected with control (N.C.) or RPN2 siRNAs. After 2 days in culture, RPN2 expression was detected using immunoblotting. β-actin was used as a loading control. **B)** MM231-LN (upper panel) and MCF7-ADR cells (lower panel) were transiently transfected with the N.C. or RPN2 siRNAs. After 2 days in culture, the cell extracts were subjected to qRT-PCR. The values on the y-axis are plotted relative to the expression level of N.C., which is defined as 1. **C)** MM231-LN (upper panel) and MCF7-ADR cells (lower panel) were transiently transfected with N.C. or RPN2 siRNAs. After 2 days in culture, CD63 expression was detected using immunoblotting. β-actin was used as a loading control. Dashed lines show the difference between glycosylated CD63 in the presence or absence of RPN2 siRNA treatment. **D)** Whole cell lysates were collected from MM231-LN (upper panel) and MCF7-ADR cells (lower panel) that were transiently transfected with N.C. or RPN2 siRNA and treated with PBS for 6 hours at 37°C. N.C. and RPN2 siRNA-transfected cells were treated with N-glycosidase for 6 hours at 37°C. CD63 glycosylation was detected by immunoblotting. β-actin was used as a loading control. The CD63 molecular weights of 25 (non-glycosylated), 35 (lower-glycosylated) and 50 kDa (higher-glycosylated) are indicated with arrows to the right.

caspase-3/7 assay in the MCF7-ADR and MM231-LN cells after RPN2 or CD63 siRNA transfection. As shown in Figure 2A and B, we found that neither RPN2 nor CD63 silencing induced apoptosis. In addition, knockdown of RPN2 or CD63 slightly inhibited cell proliferation in MCF7-ADR and MM231-LN cells (Figure 2C). To determine the localization of CD63, we performed immunofluorescence staining of CD63 in MCF7-ADR and MM231-LN cells after a control or RPN2 siRNA transfection. In MM231-LN and MCF7-ADR cells transfected with control siRNAs, CD63 was localized in the cell membrane, as indicated by PKH26 staining (Figure 2D: MM231-LN and 2E: MCF7-ADR; N.C.). Notably, CD63 aggregated at the nuclear periphery in RPN2-silenced cells more than in control siRNA treated cells (Figure 2D: MM231-LN and 2E: MCF7-ADR; RPN2 siRNA). These results indicate that the localization of CD63 was regulated by RPN2-mediated N-glycosylation.

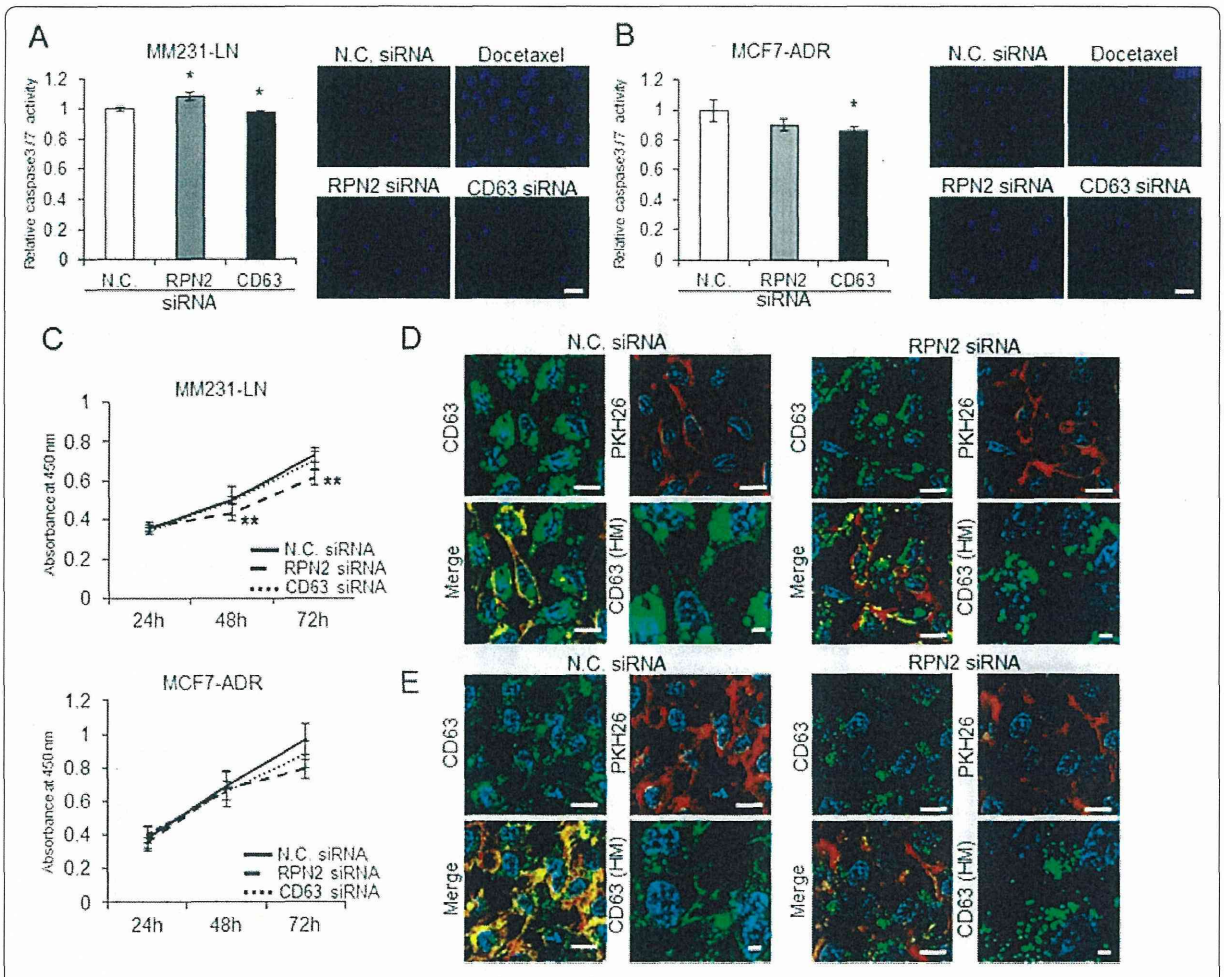

Figure 2 CD63 localization in breast cancer cells. A) MDA-MB-231-luc-D3H2LN (MM231-LN) cells were transiently transfected with control (N.C.), RPN2 or CD63 siRNAs. After 2 days in culture, caspase-3/7 activity was assessed using an Apo-ONE Homogeneous Caspase-3/7 Assay system (left panel). The right panels show a representative image of Hoechst 33258-stained sections after N.C., RPN2 or CD63 siRNA transfection. The size bar indicates 50 μm. **B)** MCF7-ADR cells were transiently transfected with control (N.C.), RPN2 or CD63 siRNAs. After 2 days in culture, caspase-3/7 activity was assessed using an Apo-ONE Homogeneous Caspase-3/7 Assay system (left panel). The right panels show a representative image of Hoechst 33258-stained sections after N.C., RPN2 or CD63 siRNA transfection. The size bar indicates 50 μm. **C)** MM231-LN (upper panel) and MCF7-ADR cells (lower panel) were transiently transfected with N.C., RPN2 or CD63 siRNAs. After 1–3 days in culture, cell growth was assessed by an MTS assay. **D)** MM231-LN cells were grown and transiently transfected with N.C. (left panels) or RPN2 siRNA (right panels). After 2 days in culture, immunofluorescence of CD63 (Green) was detected. The blue color indicates nuclei. Cell membranes were stained PKH26 (Red). A higher magnification view is shown (CD63 HM). The size bar indicates 20 μm. **E)** MCF7-ADR cells were grown and transiently transfected with N.C. (left panels) or RPN2 siRNA (right panels). After 2 days in culture, immunofluorescence of CD63 (green) was detected. The blue color indicates nuclei. Cell membranes were stained PKH26 (red). A higher magnification view is shown (CD63 HM). The size bar indicates 20 μm. All data are shown as the mean ±S.D., *P<0.05, **P<0.01.