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Mini Review Open Access

Circulating MicroRNAs in Sarcoma: Potential Biomarkers for Diagnosis and Targets for Therapy

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Abstract

The importance of microRNAs (miRNAs) in tumor biology has been recognized over the past several years. Recently, evidence of circulating miRNAs in both healthy and unhealthy individuals has been accumulated, and is accelerating their potential to transform clinical diagnostics and therapeutics. Since there is a lack of useful biomarkers for bone and soft tissue sarcomas, the discovery of novel biomarkers that can be used at early disease stages to detect tumors or predict tumor response to chemotherapy or the chance of survival is one of the most important challenges in sarcoma management. Further more highly, sensitive and specific biomarkers might help diagnostic classification, since some cases are unclassifiable using modern diagnostic modalities. In this review, we summarize the emerging evidence of circulating miRNAs in sarcoma and discuss their potential as novel biomarkers and therapeutics.

Sarcoma Needs Novel Biomarkers

Sarcomas are malignant neoplasms originating from transformed cells of mesenchymal origin and are different from carcinomas that are malignant neoplasms originating from epithelial cells. The word "sarcoma" is derived from the Greek word *sarkoma* meaning "fleshy outgrowth," and present as either a bone sarcoma or a soft tissue sarcoma [1]. Malignant primary bone sarcomas constitute 0.2% of all malignancies in adults and approximately 5% of childhood malignancies, for which data were obtained in one large series [2]. Cancer registry data with histological stratification indicate that osteosarcoma is the most common primary malignant bone tumor, accounting for approximately 35% of all cases, followed by chondro sarcoma (25%), Ewing sarcoma (16%), and chordoma (8%) [3]. Soft tissue sarcomas constitute fewer than 1% of all malignancies, 50 per million population [2,4]. According to the results of the Surveillance, Epidemiology, and End Results study (http://seer.cancer.gov/data/), which included 26,758 cases from 1978 to 2001, leiomyosarcoma was the most common sarcoma, accounting for 23.9% of all cases. Other major histological types included malignant fibrous histiocytoma (MFH; 17.1%), liposarcoma (11.5%), dermatofibrosarcoma (10.5%), rhabdomyosarcoma (RMS; 4.6%), and malignant peripheral nerve sheath tumor (MPNST; 4.0%) [5]. Although MFH was the second most common sarcoma in this series, the diagnostic term MFH is now replaced for pleomorphic sarcomas without defined differentiation. Therefore, the incidence rates of MFH will be updated in future studies based on changes in diagnostic criteria that parallel advancements in the understanding of MFH etiology.

According to histological type, treatment options for most patients with sarcoma include surgical resection followed by limb or trunk reconstruction, and pre-operative (neoadjuvant) and/or post-operative (adjuvant) chemotherapy and radiotherapy. Although surgical resection is the mainstay of treatment for musculoskeletal sarcomas, chemotherapy also has a proven role in the primary therapy of certain types of bone sarcomas and a potential role in some patients with soft tissue sarcomas [6]. Despite the development of combined modality treatments, a significant proportion of patients with sarcoma respond poorly to chemotherapy, leading to local relapse or distant metastasis. The main cause of death due to sarcoma is lung metastasis, for which prognosis is extremely poor [7,8]. Therefore, early detection of recurrent or metastatic diseases or early decision-making according to tumor response to chemotherapy could improve patient prognosis. However, there are currently no effective biomarkers in such situations, thus imaging methods, such as X-ray, computed tomography (CT), positron emission tomography-CT, magnetic resonance imaging, and scintigraphy, are mostly used to detect or monitor tumor development. Indeed, only few studies have reported the usefulness of serological markers such as alkaline phosphatase (ALP) [9], lactic dehydrogenase (LDH) [10,11], and CA125 [12] in the patients with osteosarcoma, Ewing sarcoma, and epithelioid sarcoma, respectively. Therefore, the discovery of novel biomarkers to detect tumors or predict drug sensitivity is one of the most important challenges in sarcoma management.

Circulating MicroRNAs (miRNAs) As Potential Biomarkers and Treatment Targets

miRNAs are small non-coding RNA molecules that modulate the expression of their multiple target genes and play important roles in various physiological and pathological processes, such as development, differentiation, cell proliferation, apoptosis, organogenesis, and homeostasis [13-15]. A variety of miRNAs have been investigated in various human cancers over the past several years [16]. Aberrant miRNA expression has been shown to contribute to cancer development through various mechanisms, including deletions, amplifications, and mutations involving miRNA loci, epigenetic silencing, dysregulation of transcription factors that target specific miRNAs, or the inhibition of miRNA processing [17,18]. Growing evidence has revealed that miRNAs are frequently upregulated or downregulated in various

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tumors and indicated that miRNAs act as either an oncogenes or a tumor suppressors [18,19].

Recently, tumor cells have been shown to secrete miRNAs into the circulation [20]. Therefore, analysis of circulating miRNA levels in serum or plasma presents a novel approach for diagnostic cancer screening. For example, Lawrie et al. [21] were the first to report that tumor-associated miRNA levels in the serum of patients with cancer were higher than those in healthy individuals, indicating that circulating miRNAs can be used as biomarkers to monitor the existence of cancer cells. This group also demonstrated that high miR-21 expression was associated with relapse-free survival in patients with large B-cell lymphomas [21]. Expression of other circulating serum or plasma miRNAs has been widely reported by other investigators. To date, differential expression of circulating miRNA has been reported in cancers of the breast [22], lung [23], stomach [24], liver [25], kidney [26], bladder [27], prostate [28], and ovaries [20], among others. However, it is possible that measuring these miRNAs in the serum or plasma of cancer patients may yield false-positive results because tumor cells may also change the profile of miRNAs of other circulating cells. Validation studies based on more and larger patient sets would be necessary to focus on key miRNAs with high sensitivity and specificity.

The main issues that remain unresolved in measurement of circulating miRNAs include the normalization, amplification, and contamination [29]. There is no consensus on suitable small RNA reference genes for use as internal controls. Current protocols need correction for technical variability using spiked-in synthetic non-human (*Caenorhabditis elegans*) miRNA as a normalizing control [28-30]. Moreover, there is a higher risk of cellular contamination when preparing plasma as the supernatant is pipetted away from the cellular pellet. Profiling of miRNA by qRT-PCR is also dependent on the type of anticoagulant used, where EDTA and citrate are acceptable, but heparin impedes the qRT-PCR reaction [29]. Given these uncertainties surrounding miRNA analysis, further studies to establish consensus protocols could resolve these issues and accelerate this novel method toward clinical application as a novel approach to monitor or detect tumor development.

Circulating miRNAs in Patients with Sarcoma

The first report of circulating miRNAs as potential diagnostic markers was presented in 2010 by Miyachi et al. [31] who analyzed the expression levels of muscle-specific miRNAs in the sera of rhabdomyosarcoma patients and healthy controls [31]. To date, the evidence is restricted to only three types of sarcomas, i.e., osteosarcoma, rhabdomyosarcoma, and malignant peripheral nerve sheath tumor, as summarized in Table 1.

Osteosarcoma

Osteosarcoma is the most common primary malignancy of the bone and accounts for 60% of all childhood bone malignancies [32,33]. The most common primary sites of osteosarcoma are the distal femur, proximal tibia, and proximal humerus, with approximately 50% of cases originating in the vicinity of the knee. The WHO classification recognizes additional histological variants in addition to the conventional osteosarcomas (osteoblastic, chondroblastic, and fibroblastic types); telangiectatic osteosarcoma, small cell osteosarcoma, low-grade central osteosarcoma, secondary osteosarcoma, parosteal osteosarcoma, periosteal osteosarcoma, and high-grade surface osteosarcoma [34]. Standard treatment of patients with conventional osteosarcoma consists of neoadjuvant chemotherapy, surgical resection, and adjuvant chemotherapy [35]. With this combined treatment, the 5-year overall survival for patients with no metastatic disease at diagnosis is 60%–80% [36-41]. However, a significant proportion of patients with osteosarcoma still respond poorly to chemotherapy and have a greater risk of local relapse or distant metastasis even after curative resection of the primary tumor. Indeed, outcomes are far worse for patients who present with metastatic disease, since the 5-year overall survival is less than 30% [42], and has shown little improvement over the past two decades despite multiple clinical trials with increased intensity. Therefore, the discovery of sensitive and specific minimally invasive biomarkers that could detect osteosarcoma at an early stage would be one of the most important challenges. Moreover, it would be helpful if these biomarkers could predict the chance of survival or response to chemotherapy, especially during early treatment stages before surgery.

Four miRNAs (miR-21, miR-34b, miR-143, and miR-199-3p) have been reported as potential osteosarcoma biomarkers. Yuan et al. [43] investigated serum miR-21 expression levels in 65 patients with osteosarcoma and 30 healthy controls by qRT-PCR and found that serum miR-21 expression levels were significantly higher in patients with osteosarcoma than in the controls [43]. Moreover, increased serum miR-21 levels were significantly correlated with Enneking stage and chemotherapeutic resistance. The mean ΔC_T value of miR-21 in the responder group was significantly higher than that in the nonresponder group. Notably, the upregulation of miR-21 was an independent unfavorable prognostic factor for overall survival [43]. Indeed, it has been reported that miR-21 is aberrantly overexpressed in various cancers and is involved in the pathogenesis of cancers [44,45]. The effects of miR-21 on proliferation, migration, invasion, and apoptosis have already been elucidated in cancers of the breast, liver, and colon [46-48]. In osteosarcoma, Ziyan et al. [49] reported that miR-21 was significantly overexpressed in osteosarcoma tissues, and its knockdown decreased cell invasion and migration of osteosarcama MG-63 cell line. *RECK* (reversion-inducing-cysteine-rich protein with kazal motifs), a tumor suppressor gene, was found to be a direct target that was negatively regulated by miR-21 in an osteosarcoma cell line and human osteosarcoma specimens [49].

Ouyang et al. [50] evaluated the expression levels of six miRNAs (miR-34, miR-21, miR-199-3p, miR-143, miR-140, and miR-132) that had been reported as aberrantly expressed in osteosarcoma using plasma from 40 patients with osteosarcoma and 40 matched healthy controls by qRT-PCR [50]. They found that plasma miR-21 levels were significantly higher in patients with osteosarcoma than in controls, whereas miR-199a-3p and miR-143 were decreased. Furthermore, plasma miR-21 and miR-143 levels were correlated with metastasis and histological subtype, whereas plasma miR-199a-3p correlated with histological subtype. Interestingly, the area under the curve (AUC) value of the combined signature of three miRNAs (miR-21, miR199-3p, and miR-143) was higher than that of bone-specific alkaline phosphatase (0.953 and 0.922, respectively), and the sensitivities and specificities of the combined miRNAs were 90.5 and 93.8%, respectively. The aberrant expression of miR-199-3p in osteosarcoma was first reported by Duan et al. [51] who found that miR-199a-3p, miR-127-3p, and miR-376 were significantly downregulated in osteosarcoma cell lines compared to osteoblasts [51]. Overexpression of miR-199a-3p in osteosarcoma cell lines significantly decreased cell growth and migration. In addition, they identified that miR-199a-3p suppressed the expression of the oncogenic and antiapoptotic proteins mTOR and STAT3. Osaki et al. [52] were the first to demonstrate that the expression of miR-143 was decreased in metastatic osteosarcoma cells. They profiled the miRNA expression in a parental HOS cell line and its

Sarcoma entity	Promising circulating miRNAs		Study design	Sample type	Sample size	Technology	Circulating miRNAs examined	Normalization	Other related clinical factors	Reference
	Upregurated	Downregulated								
Osteosarcoma	miR-21		OS vs normal	Serum	65 patients vs 30 healthy controls	qRT-PCR	1	snRNA U6	①Enneking stage ②Drug resistance ③Prognosis	Yuan et al. [43]
Osteosarcoma		miR-34b	OS vs normal, tissue and plasma	Plasma	133 patients vs 133 healthy controls	qRT-PCR	2	cel-miR-39	Metastasis	Tian et al. [53]
Osteosarcoma	miR-21	miR-143, miR-199a-3p	OS vs normal	Plasma	40 patients vs 40 healthy controls	qRT-PCR	6	cel-miR-39	①Metastasis (miR-21, 143) ②Histological subtype	Ouyang et al. [50]
Rhabdomyosarcoma	miR-206		RMS vs non-RMS vs normal, tissue and plasma	Serum	8 RMS patients vs 23 non-RMS patients vs 17 healthy controls	qRT-PCR	4	miR-16	N.A.	Miyachi et al. [31]
Malignant peripheral nerve sheath tumor	miR-24, miR-801, miR-214		Sporadic MPNST vs NF1 MPNST vs NF1	Serum	Screening: 10 sporadic MPNST vs 10 NF1 MPNST vs 10 NF1 Validation: 83 sporadic MPNST vs 61 NF1 MPNST vs 90 NF1	Solexa sequencing, qRT-PCR	Genome-wide profiling by Solexa sequencing	cel-miR-39	N.A.	Weng et al. [61]

Abbreviations: OS: Osteosarcoma; RMS: Rhabdomyosarcoma; MPNST: Malignant peripheral nerve sheath tumor; NF1: Neurofibromatosis type 1; N.A: not available

Table 1: Differential expression of circulating miRNAs in patients with bone and soft tissue sarcoma.

sub-clone 143B metastatic osteosarcoma cell line, and found that miR-143 was the most downregulated miRNA in 143B cells [52]. Significant inhibition of cell invasion was observed in miR-143-transfected 143B cells. Several genes were identified as probable candidates of miR-143 targets by a comprehensive collection system to detect miRNA-target mRNA. Among them, matrix metalloprotease-13 was one of the most probable targets of miR-143, which was positive in clinical specimens of metastatic cases by immunohistochemistry, but negative in those of at least three cases showing higher miR-143 expression levels in the nonmetastatic group [52].

Tian et al. [53] investigated the associations between plasma miR-34b/c expression levels in osteosarcoma, and found that plasma miR-34b level was significantly lower in osteosarcoma patients than in controls and related with its expression in osteosarcoma tissues [53]. Furthermore, plasma miR-34b expression levels were significantly decreased in patients with metastatic disease compared to patients with nonmetastatic disease, while no significant difference in miR-34b levels was observed between patients with osteoblastic and nonosteoblastic diseases [53]. Indeed, the miR-34 family, which is a direct target of the p53 tumor suppressor gene, are composed of three homologous miRNAs (miR-34a, miR-34b, and miR-34c), and are associated with the tumor growth and metastasis of various human cancers. Previous reports from He et al. [54] and Yan et al. [55] have demonstrated the association of miR-34a with osteosarcoma. They identified decreased miR-34a expression levels in tumor samples and found that miR-34a overexpression could inhibit the tumor growth and metastasis by downregulation of the proto-oncogene *c-Met* [54,55].

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in childhood, representing 5%–8% of all pediatric malignancies [56]. Histopathologically, RMS is classified into embryonal (eRMS), alveolar (aRMS), and pleomorphic types. Depending on the size and location of the primary tumor, most cases are treated with a combination of chemotherapy, radiation therapy, and surgery. Adult patients with a complete response to chemotherapy had a 5-year survival rate of 57% compared to only 7% for poor responders [57].

Miyachi et al. [31] were the first to suggest use of circulating miRNAs for sarcoma diagnosis. They focused on muscle-specific miRNAs (miR-1, miR-133a, miR-133b, and miR-206) that were shown to be more abundantly expressed in myogenic tumors. Expression levels of these muscle-specific miRNAs were confirmed to be higher in RMS cell lines and culture supernatants than in other cell lines. In their analysis of muscle-specific miRNA serum levels in RMS patients, normalized serum miR-206 showed the highest sensitivity and specificity among muscle-specific miRNAs [31]. Importantly, miR-206 expression decreased after treatment of RMS [31]. In the analysis of miR-206 expression levels with RMS cells, Missiaglia et al. [58] found that muscle-specific miRNA levels were lower in RMS than in skeletal muscles, but generally higher than that in other normal tissues [58]. Moreover, low miR-206 expression correlated with poor overall survival in patients with RMS, and increased miR-206 expression in cell lines inhibited cell growth and migration and induced apoptosis [58]. Similar results were reported by Tauli et al. [59] who showed that increased miR-206 expression caused a major switch in the global expression profile toward mature muscle, rescued differentiation of both eRMS and aRMS, and blocked tumor growth [59]. Therefore, serum miR-206 expression may be used as a predictive biomarker of tumor aggressiveness and patient prognosis, but further studies with larger patient cohorts are needed to confirm this supposition.

Malignant Peripheral Nerve Sheath Tumor

Malignant peripheral nerve sheath tumors (MPNSTs) are highly aggressive soft tissue sarcomas that account for 3%–10% of all soft tissue sarcomas [60]. These tumors typically originate from cells constituting the nerve sheath, such as Schwann and perineural cells.

Approximately half of MPNSTs occur sporadically, with the remaining originating in patients with the autosomal dominant genetic disorder neurofibromatosis type 1 (NF1). Individuals with NF1 have high lifetime risk of developing MPNST. However, screening for malignant transformation in patients with NF1 is difficult because of the large number and diverse anatomical sites of neurofibromas that occur in these patients as well as the lack of useful biomarkers for differential diagnosis.

Weng et al. [61] investigated the role of serum miRNAs to distinguish MPNST patients with and without NF1. They applied Solexa sequencing to screen for differentially expressed miRNA in pooled serum from 10 patients with NF1, 10 patients with sporadic MPNST, and 10 patients with NF1 MPNST patients [61]. As a result, miR-801 and miR-214 showed higher expression levels in sporadic MPNST patients and NF1 MPNST patients than NF1 patients [61]. Moreover, miR-24 was significantly upregulated in NF1 MPNST patients. Therefore, they concluded that the combination of the three miRNAs (miR-801, miR-214, and miR-24) could be used to distinguish NF1 MPNST patients from NF1 patients [61]. A previous report from Subramanian et al. [62] also demonstrated that miR-214 was relatively upregulated in MPNSTs compared to benign tumors[62]. They considered that high expression of *TWIST1* in the majority of MPNSTs might be involved in miR-214 expression in MPNSTs, since *TWIST1* has been known to induce miR-214 expression in mouse neural cells[62].

miRNAs as Potential Treatment Targets

Analysis of miRNA expression in serum and tumor tissue involved in sarcomagenesis may be useful to identify novel targets for miRNA-based therapy. Among the miRNAs discussed as potential biomarkers of sarcoma (Table 1), miR-143 has already been investigated for therapeutic potential *in vivo*. Based on the evidence that miR-143 was downregulated in metastatic 143B osteosarcoma cells compared to non-metastatic HOS cells, Osaki et al. [52] assessed the therapeutic potential of miR-143 against spontaneous lung metastasis mouse model using 143B osteosarcoma cells by systemic administration of a miR-143 mimic and miR-negative control (NC). Experimentally, 50 μg of miR-143 mimic or miR-NC was mixed with atelocollagen and administered intravenously into mice in groups of 10 at 1, 4, 7, 10, 13, 16, and 19 days after inoculation of 143B cells [52]. The results showed that, at 3 weeks after inoculation, six of eight mice exhibited lung metastasis on *in vivo* imaging system and the other two mice died due to lung metastasis following miR-NC/atelocollagen treatment, whereas only two of the 10 mice in the miR-143/atelocollagen-treated group showed lung metastasis. This preclinical trial has shed light on the therapeutic potential of miRNAs against osteosarcoma. However, the toxicity of miRNA therapy should be considered, since miRNA can simultaneously regulate multiple target mRNAs. Thus, a large series to study the safety of miRNA-based therapy is necessary. On the other hand, development of a drug delivery system (DDS) would be an important step toward the clinical application of miRNA-based therapy. While Atelocollagen has been shown to be effective against osteosarcoma in an *in vivo* study; there is little consensus regarding the standard use of DDS. Further investigations for key miRNAs for each type of sarcoma and toxicological testing of miRNA mimics, along with development of DDS, would accelerate the therapeutic possibility of targeting miRNAs as novel treatment options for sarcomas.

Conclusions

There is a growing amount of evidence of miRNA profiling in bone and soft tissue sarcoma not only in tumor cells and tissues but also patient serum and plasma samples. Despite some exceptions, most of these findings have shown that aberrant expression of circulating miRNAs correlated with that of tumor cells and tissues, indicating that serum or plasma miRNA expression could serve as a novel biomarker for sarcoma. To date, there are few useful biomarkers to monitor sarcoma. Although some issues remain unresolved regarding the measurement of circulating miRNA levels, we believe that a novel noninvasive miRNA-based assay with high sensitivity and specificity for and its therapeutic use will be available for clinical applications in the near future.

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RPN2 Gene Confers Osteosarcoma Cell Malignant Phenotypes and Determines Clinical Prognosis

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Drug resistance and metastasis are lethal characteristics of tumors. We previously demonstrated that silencing of ribophorin II (RPN2), which is part of the N-oligosaccharyl transferase complex, efficiently induced apoptosis and reduced resistance to docetaxel in human breast cancer cells. Here, we report the clinical and functional correlations of RPN2 expression in osteosarcoma. Immunohistochemical evaluation of 35 osteosarcoma patient biopsies revealed that RPN2 was moderately to highly expressed in all specimens, and higher RPN2 mRNA expression was significantly correlated with poor prognosis. To investigate whether lethal phenotypes of osteosarcoma could be reduced by regulating the expression of RPN2, we conducted a study of RNAi-induced RPN2 knockdown in highly metastatic human osteosarcoma cells. The results indicated that RPN2 silencing reduced cell proliferation, sphere formation, cell invasion, and sensitized drug response *in vitro*. Mice bearing RPN2-silenced highly metastatic osteosarcoma xenografts showed reduced tumor growth and lung metastasis, and survived longer than mice bearing control tumor xenografts. Taken together, our data suggest that RPN2 silencing contributes to regulation of lethal osteosarcoma phenotypes and could be a novel target for RNAi-based therapeutics against osteosarcoma.

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Introduction

The most lethal characteristics of tumors include drug resistance and metastasis.[1–5] Osteosarcoma is no exception, and various cohort studies have shown that both response to chemotherapy and metastasis are independent prognostic factors.[6–13] Osteosarcoma is the most common primary bone malignancy arising in children and young adults.[6,14,15] Along with the development of multi-agent chemotherapy and surgical techniques including the concepts of surgical margins and reconstruction,[16,17] patient prognosis has gradually improved over the past 30 years. Current chemotherapeutic regimens including pre- and postoperative doxorubicin, cisplatin, methotrexate, and/or ifosfamide have maintained 5-year overall survival rates at approximately 60–80%.[11,12] However, osteosarcoma patients who show a poor response to chemotherapy or who have multiple pulmonary metastases have a poor prognosis, with an overall survival rate of <50% and <30%, respectively.[10,18] The molecular background supports these data, as the presence of increased levels of P-glycoprotein[9] or metastasis-related genes such as ezrin[19] in tumor cells has been associated with a significantly poor prognosis of osteosarcoma patients. Therefore, the development of a novel approach targeting these key molecules would provide new hope for patients.

Our previous study showed that downregulation of ribophorin II (RPN2), which is part of the N-oligosaccharyl transferase complex, efficiently induced apoptosis in docetaxel-resistant human breast cancer cells in the presence of docetaxel.[20] Silencing of RPN2 decreased membrane localization of P-glycoprotein through a reduction of glycosylation status, and restored sensitivity to docetaxel. These results indicated that regulation of RPN2 expression contributes to a more effective response to docetaxel-based chemotherapy. However, it has been unclear whether these mechanisms would be effective in other cancers, including neoplasms of mesenchymal origin. In this study, we examined RPN2 expression using immunohistochemical staining and quantitative real-time polymerase chain reaction (qRT-PCR) of pretreatment biopsy samples from patients with osteosarcoma, and evaluated the correlation between RPN2 expression and clinicopathological features. In addition, we investigated whether the level of RPN2 expression affected cell proliferation, drug sensitivity, sphere formation ability, and cell invasion in osteosarcoma *in vitro*, as well as tumor growth and metastatic ability *in vivo*.

Results

High expression of RPN2 in osteosarcoma biopsies is significantly correlated with poor patient survival

We evaluated tissue samples from 35 osteosarcomas obtained by diagnostic incisional biopsy of primary osteosarcoma at the National Cancer Center Hospital, Japan, between 1997 and 2010. Immunohistochemically, RPN2 protein was moderately to strongly expressed in all of these specimens, and localized in the cytoplasm (**Figure 1a**). RPN2 protein expression was negative to weakly positive in normal tissues, including mesenchymal tissues such as adipose or fibrous tissues, which was consistent with the findings of our previous study.[20] We next performed qRT-PCR using cDNA obtained from these osteosarcoma patients and evaluated the clinicopathological features according to the expression of RPN2 in the same cohort set. We determined

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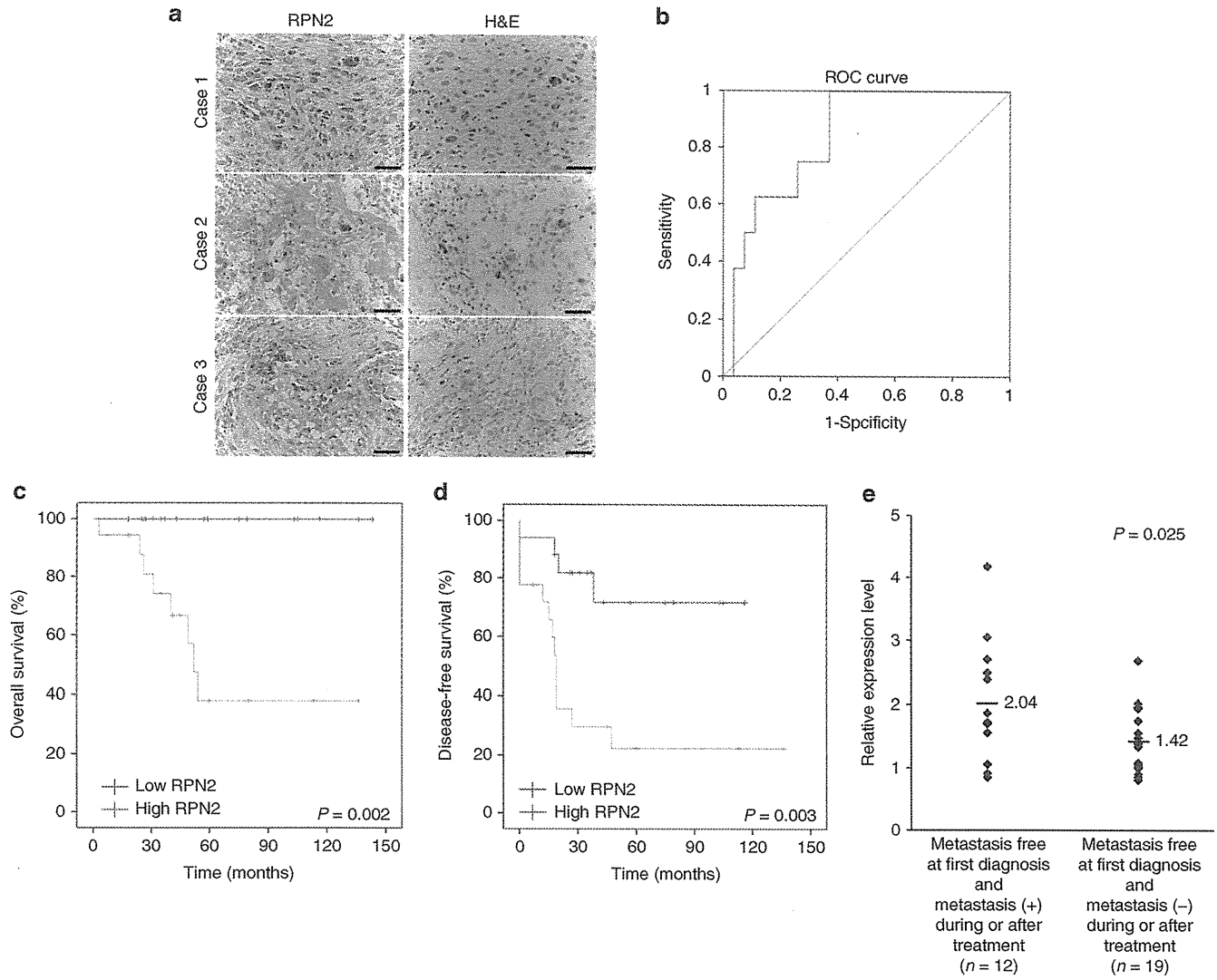


Figure 1 Clinical relevance of RPN2 expression in osteosarcoma. (**a**) Immunohistochemical staining of RPN2 protein and hematoxylin and eosin staining in osteosarcoma biopsy specimens of osteosarcoma. RPN2 protein expression was moderately to strongly detected in the cytoplasm in all biopsy samples. Scale bar, 50 μm. (**b**) ROC curve for expression of RPN2. The area under the ROC curve was 0.838. The cutoff was set at the point representing 100% sensitivity and 63.0% specificity. (**c**) The Kaplan–Meier curves for overall survival according to the RPN2 expression (log-rank test; $P = 0.002$). (**d**) The Kaplan–Meier curves for disease-free survival according to the RPN2 expression (log-rank test; $P = 0.003$). (**e**) RPN2 expression in biopsy specimens of primary osteosarcoma. Thirty-one specimens of primary osteosarcoma were divided into two groups: cases remaining metastasis-positive during or after treatment ($n = 12$, left) and cases remaining metastasis-free for at least 3 years after treatment ($n = 19$, right). The average value for each dataset is shown as a horizontal line. *P* values were calculated using Welch's *t*-test ($P = 0.025$).

the cutoff point that yielded optimum sensitivity and specificity using receiver-operating characteristic (ROC) curve analysis. The area under the ROC curve was 0.838 (**Figure 1b**), and Kaplan–Meier analysis showed that high levels of RPN2 expression were associated with significantly worse overall survival rates (log-rank test, $P = 0.002$; **Figure 1c**) and disease-free survival rates (log-rank test, $P = 0.003$; **Figure 1d**). In this statistical analysis, all low-RPN2 patients survived, indicating that the expression of RPN2 had significant prognostic value. The clinicopathological features of the patients in relation to the expression of RPN2 are summarized in **Table 1**. Univariate analysis revealed a significant correlation between high-RPN2 expression and the presence of metastasis at initial diagnosis ($P = 0.039$), and we found that four patients who had metastatic disease at the time of initial diagnosis were all ranked in the high-RPN2 group (**Table 1**). Among 31 patients who showed no metastasis at initial diagnosis, 12 developed lung metastasis during or after treatment, and the other 19 showed no metastasis for at least 3 years after treatment. Expression of RPN2 was significantly higher in the metastasis-positive group ($n = 12$, 2.04 ± 0.97) than in the group with no metastasis ($n = 19$, 1.42 ± 0.48) (**Figure 1e**). Although we found a close correlation between high-RPN2 expression and a poor response to neoadjuvant chemotherapy, it was not statistically significant ($P = 0.063$) (**Table 1**). We found no significant correlations between RPN2 expression and other factors such as patient gender, tumor site, or histological subtype (**Table 1**). These

data suggested that higher expression of RPN2 in osteosarcoma might be associated with the metastatic phenotype and could be of novel prognostic value.

RPN2 regulates drug response and invasiveness of osteosarcoma cells

To evaluate the functional effects of regulating RPN2 expression in osteosarcoma cells, we first confirmed the expression of RPN2 mRNA in several osteosarcoma cell lines. As a result, we found higher expression of RPN2 in 143B, a highly metastatic osteosarcoma cell line, than in SaOS2 or HOS, which are poorly metastatic osteosarcoma cell lines (**Figure 2a**). We then established stable clones of 143B expressing short hairpin RNA (shRNA) against RPN2 (143B-shRPN2) and control shRNA (143B-shNC). The reduced expression of RPN2 was confirmed by RT-PCR (**Figure 2b**), as well as by Western blot analysis (**Figure 2c**). Cell proliferation was slightly inhibited by RPN2 silencing (**Figure 2d**). After 48 hours of doxorubicin treatment, we found substantial cell death in 143B-shRPN2 relative to the control 143B-shNC (**Figure 2e**). We then tested the tumor cell responses to a wide range of drugs that have been used for treatment of osteosarcoma, and found that RPN2 silencing increased the sensitivity to doxorubicin, methotrexate, and docetaxel (**Figure 2f** and **Supplementary Figure S2**). In comparison with control 143B-shNC cells, 143B-shRPN2 formed fewer and smaller spheres in a serum-free, growth factor-supplemented, anchorage-independent environment (**Figure 2g,h**). Additionally, we analyzed the effect of RPN2 silencing on cell invasion, and found that 143B-shRPN2 cells were less invasive than 143B-shNC cells (**Figure 2i,j**).

RPN2 expression in osteosarcoma cells is induced by doxorubicin treatment

Our previous investigation had shown that expression of RPN2 mRNA in docetaxel-sensitive breast cancer cells was markedly and dose-dependently induced by docetaxel. To confirm the effect of RPN2 mRNA expression in osteosarcoma cells by treatment with currently used drugs, we performed qRT-PCR for 143B cells after doxorubicin treatment. We found that expression of mRNA for both RPN2 and multidrug resistance gene 1 (MDR1) in 143B cells was markedly and dose-dependently induced by doxorubicin after 48 hours of treatment (**Figure 3a,b**). These data indicated that the cells surviving after doxorubicin treatment expressed a high amount the MDR1 and RPN2 gene products, suggesting that the development of drug resistance might correlate with induction of their expression in osteosarcoma cells.

RPN2 silencing contributes to the inhibition of tumor growth and lung metastasis formation

To examine the role of RPN2 in primary tumor growth and metastasis, we transplanted 143B-shRPN2 and 143B-shNC cells into mice and evaluated the resulting tumor progression. 143B-shRPN2 ($n = 5$) and 143B-shNC ($n = 5$) were orthotopically implanted into the right proximal tibia of 4- to 6-week-old athymic nude mice at 1.5×10^6 cells/mouse. The growth of the implanted tumors was measured once a week, and the presence of lung metastases was analyzed weekly by luciferase bioluminescence using an *in vivo* imaging system. We found that the primary tumor growth of 143B-shRPN2 was less than that of 143B-shNC (**Figure 4a,b**). After 3 weeks of orthotopic transplantation, there was significantly lesser lung metastasis in 143B-shRPN2-bearing mice than in 143B-shNC-bearing mice: four of five 143B-shRPN2-bearing mice exhibited lung metastases in comparison with only one of five 143B-shNC-bearing mice (**Figure 4c**). We evaluated the intensity of luminescence of chest lesions and identified apparently less signal intensity in 143B-shRPN2-bearing mice (**Figure 4d**). Presented differently, 143B-shRPN2-bearing mice had an 87% lower metastasis index than 143B-shNC-bearing mice (**Supplementary Figure S3**). All the mice were evaluated

Table 1 Clinicopathological correlation of RPN2 expression in osteosarcoma

Variable	Number of cases	Low RPN2	High RPN2	Correlation (*P* value)
Age (years)				0.032
0–10	7	6	1	
11–20	25	11	14	
21+	3	0	3	
Gender				0.903
Male	23	11	12	
Female	12	6	6	
Site				0.629
Femur	21	9	12	
Tibia	9	6	3	
Humerus	2	1	1	
Others (Pelvis, Fibula)	3	1	2	
Histology				0.178
Osteoblastic	16	8	8	
Chondroblastic	6	4	2	
Fibroblastic	2	2	0	
Others, NA	11	3	8	
Metastasis at diagnosis				0.039
Present	4	0	4	
Absent	31	17	14	
Neoadjuvant chemotherapy				0.578
MTX+DOX/CDDP	21	11	10	
IFO+DOX/CDDP	13	6	7	
Others	1	0	1	
Response to neoadjuvant chemotherapy				0.063
Good (necrosis > 90%)	11	8	3	
Poor (necrosis < 90%)	21	8	13	
NA	3	1	2	
Disease status				0.008
CDF	18	13	5	
NED	5	2	3	
AWD	4	2	2	
DOD	8	0	8	
RPN2 mRNA expression				—
High	18	0	18	
Low	17	17	0	

AWD, alive with disease; CDDP, cisplatin; CDF, continuous disease free; DOD, dead of disease; DOX, doxorubicin; IFO, ifosfamide; MTX, methotrexate; NA, not available; NED, no evidence of disease.

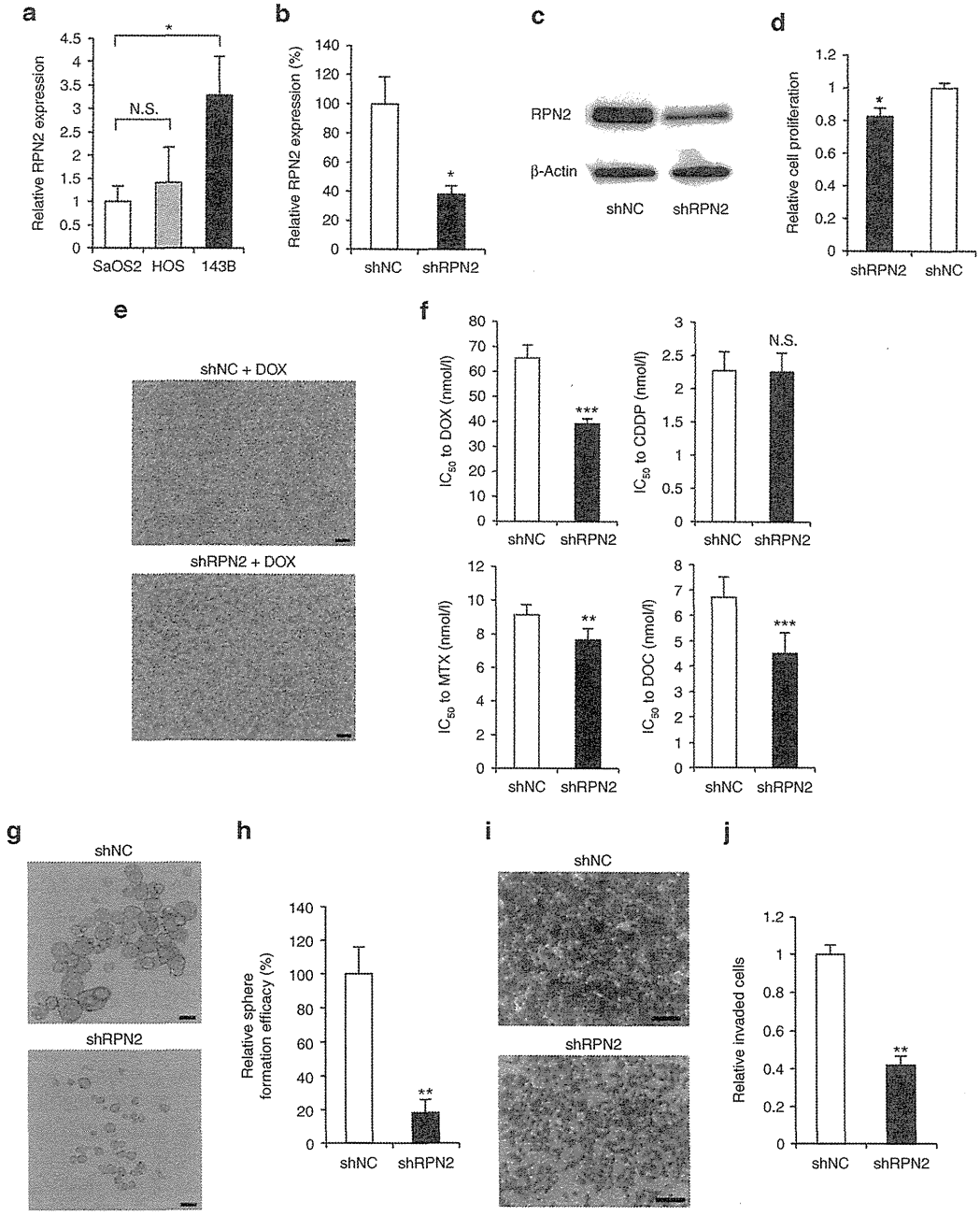


Figure 2 RPN2 regulates malignant phenotypes of osteosarcoma cells. (**a**) The relative expression levels of RPN2 mRNA in osteosarcoma cell lines. Data are presented as mean ± SD ($n = 3$ per group). N.S., not significant, $^{*}P < 0.05$; Student's *t*-test. (**b**) Knockdown of RPN2 mRNA by shRNA, as confirmed using real-time RT-PCR. Data are presented as mean ± SD ($n = 3$ per group). $^{*}P < 0.05$; Student's *t*-test. (**c**) Western blot analysis of RPN2 protein in 143B-shRPN2 and 143B-shNC cells. (**d**) Relative proliferation rates of 143B-shRPN2 and 143B-shNC cells on day 3. Data are presented as mean ± SD ($n = 3$ per group). $^{*}P < 0.05$; Student's *t*-test. (**e**) Phase-contrast micrograph of 143B-shRPN2 and 143B-shNC cells in the presence of 400 nmol/l doxorubicin (DOX). Scale bar, 200 μm. (**f**) Drug sensitivity of 143B-shRPN2 and 143B-shNC cells in the presence of doxorubicin (DOX), cisplatin (CDDP), methotrexate (MTX), and docetaxel (DOC). Data are presented as mean ± SD ($n = 6$ per group). N.S., not significant, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test. (**g**) Phase-contrast micrograph of the 143B-shRPN2 and 143B-shNC cells in a serum-free, growth factor-supplemented, anchorage-independent environment. Scale bar, 100 μm. (**h**) Relative sphere formation efficacy of 143B-shRPN2 and 143B-shNC cells observed in **g**. Spheroids with a diameter beyond 100 μm were counted. Data are presented as mean ± SD ($n = 3$ per group). $^{**}P < 0.01$; Student's *t*-test. (**i**) Phase-contrast micrograph of the invaded cells of 143B-shRPN2 and 143B-shNC cells. (**j**) Relative invasiveness of 143B-shRPN2 and 143B-shNC cells observed in **i**. Data are presented as mean ± SD ($n = 3$ per group). $^{**}P < 0.01$; Student's *t*-test.

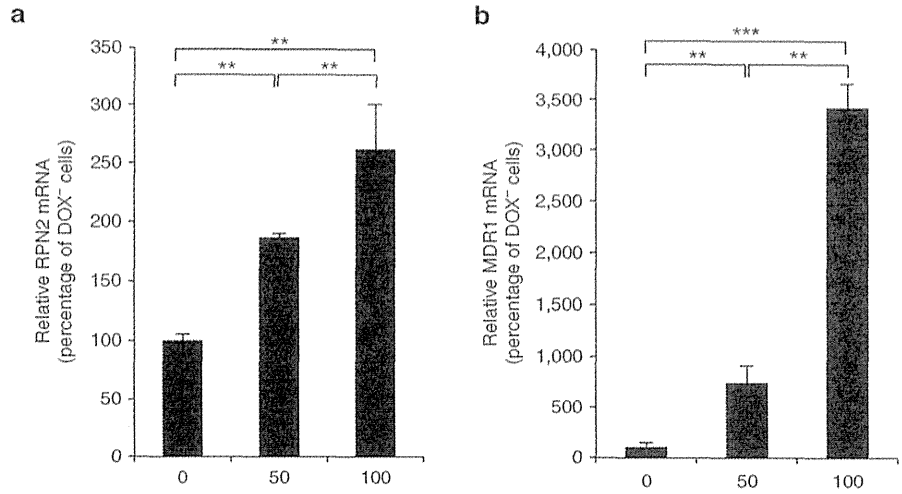


Figure 3 Induction of RPN2 and MDR1 expression by doxorubicin treatment. (**a**) RPN2 expression levels induced by doxorubicin treatment in 143B cells. The data shown are from 48 hours after doxorubicin treatment. Data are presented as mean ± SD ($n = 3$ per group). $^{**}P < 0.01$; Student's *t*-test.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

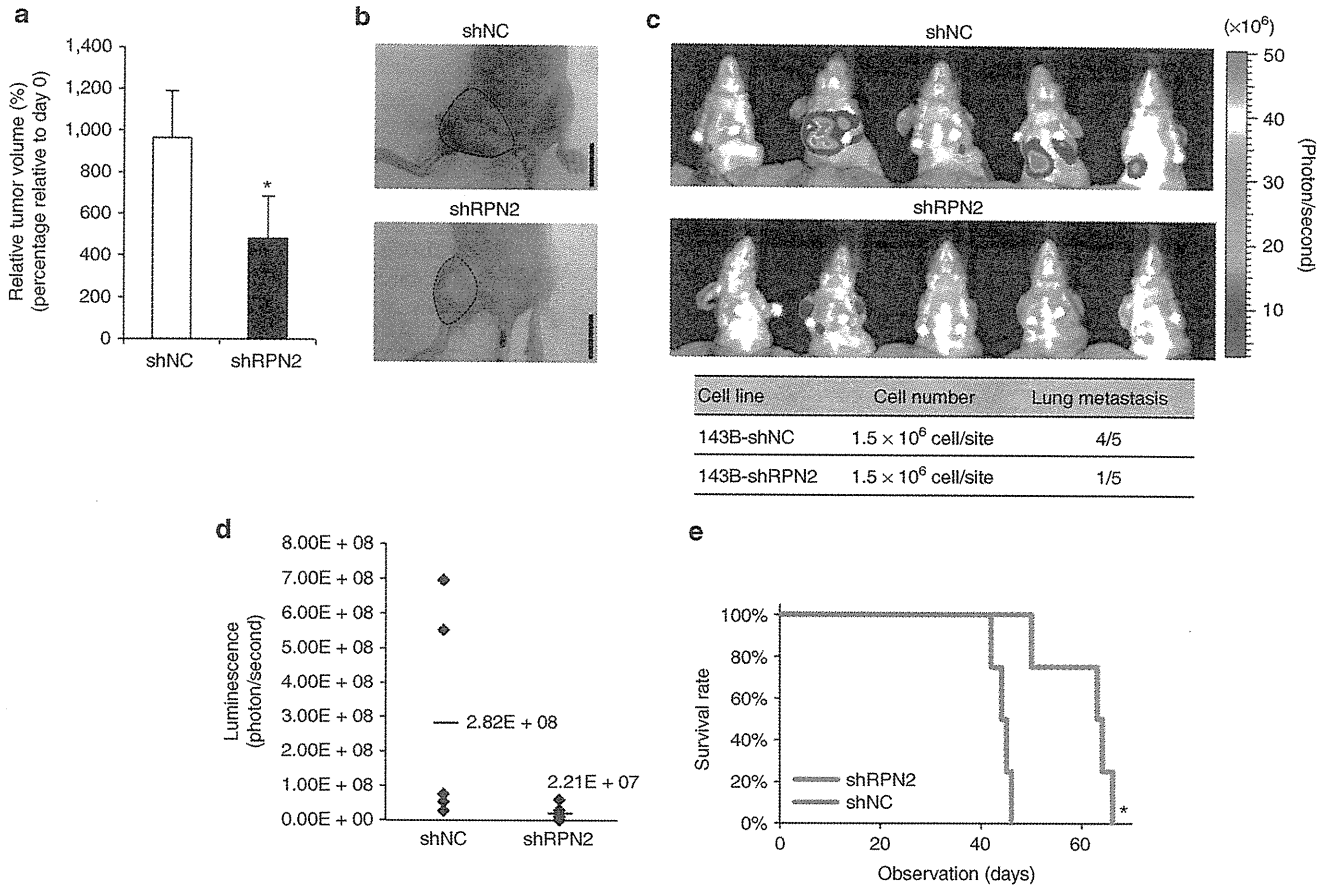
for survival, and 143B-shRPN2-bearing mice showed longer survival than 143B-shNC-bearing mice (log-rank test, $P = 0.020$) (**Figure 4e**), suggesting that decreased RPN2 expression provided a survival advantage on osteosarcoma-bearing mice.

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



Cell line	Cell number	Lung metastasis
143B-shNC	1.5×10^6 cell/site	4/5
143B-shRPN2	1.5×10^6 cell/site	1/5

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.*, $J = \max(\text{sensitivity} + \text{specificity} - 1)$.[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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Review

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Physiological and pathological relevance of secretory microRNAs and a perspective on their clinical application

Abstract: MicroRNAs (miRNAs) have attracted significant attention because of their important roles in a variety of physiological and pathological processes. Recent studies have shown that many cell types secrete miRNAs by packaging them into lipid-bilayered small vesicles called exosomes. Furthermore, exosomal miRNAs travel between cells, exert their RNAi effects in the recipient cells, and play important roles in various biological processes. In this article, we will summarize and describe the latest studies on exosomal miRNAs by focusing on their roles in cancer progression, immune regulation, and tissue repair. We will also provide a perspective on the clinical applications of this research field.

Keywords: cancer; cell-to-cell communication; exosome; microRNA; microvesicle; secretory microRNA.

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Introduction

In the past 20 years, microRNAs (miRNAs) have attracted much attention from various biological fields. miRNAs are small regulatory RNA molecules that modulate the expression of their target genes and play important roles in a variety of physiological and pathological processes, such as development, differentiation, cell proliferation, apoptosis, and stress responses (Bartel, 2009). Since the discovery of miRNAs in *Caenorhabditis elegans* in 1993 (Lee et al., 1993), their presence has been confirmed in many species, including plants (Reinhart et al., 2002) and mammals (Lagos-Quintana et al., 2001). Currently, over 2000 types of miRNAs have been found in the human genome (Table 1) (miRBase ver. 20; http://www.mirbase.org/), and miRNAs are predicted to regulate over one-third of the protein-coding genes (Lewis et al., 2005).

Although miRNAs were first thought to be present and functional exclusively in the cytoplasm, recent studies have shown that they are also present in the extracellular space, for example, in blood, urine, and saliva (Kosaka et al., 2010a). These extracellular miRNAs are protected from RNase degradation, mainly because of encapsulation in lipid-bilayered small vesicles called exosomes. Exosomal miRNAs are not simply cellular byproducts: they are secreted and transported between cells, and they exert their RNAi effects in the recipient cells (Kosaka et al., 2010b; Pegtel et al., 2010; Zhang et al., 2010). Furthermore, exosomal miRNAs play important roles in various biological processes, including cancer progression, immune regulation, and tissue repair.

In this article, we will summarize and describe the latest studies on exosomal miRNAs by focusing on their physiological and pathological roles. We will also provide a perspective on clinical applications of this research field.

miRNAs

miRNAs are non-coding RNAs that are approximately 22 nt in length and that inhibit the expression of various target genes at the post-transcriptional level by binding the 3′ untranslated region of target mRNAs. After transcription from the genome, miRNA genes are processed into mature miRNAs through a two-step incision by Drosha/DGCR8 and Dicer. One of the strands then joins a group of proteins and forms an RNA-induced silencing complex (RISC), which suppresses the expression of target genes (Kwak et al., 2010). The systems are conserved in many species and form important regulators that participate

Table 1 The number of mature miRNAs listed in miRBase.

Species name	miRNA count
Homo sapiens	2578
Mus musculus	1908
Xenopus tropicalis	175
Drosophila melanogaster	426

in multiple biological phenomena, including development, organogenesis, and homeostasis. Because miRNAs can bind to many target mRNAs once their expression is altered, disease can occur through the deregulation of their target gene networks, particularly networks that lead to stress and diseases, such as cancer.

Intercellular transport of exosomal miRNAs and their functions in recipient cells

Exosomes are small vesicles that are released when multivesicular endosomes fuse with the plasma membrane (Théry, 2011) (Figure 1). These vesicles have long been regarded as cellular 'garbage cans' for discarding unwanted molecular components (Théry, 2011). However, exosome research has dramatically changed because of a breakthrough in 1996. Raposo et al. found that exosomes derived from immune cells function as activators of the immune system (Raposo et al., 1996). Subsequently, many groups have reported that exosomes derived from certain cell types contain functional proteins that can activate biological events. These findings have established the novel concept that exosomes serve as a versatile tool for intercellular communication.

The second breakthrough revealed that exosomes shuttle nucleic acids. In 2006, Ratajczak et al. found that exosomes containing mRNA enter target cells, and these mRNAs are translated into the encoded proteins (Ratajczak et al., 2006). The following year, Valadi et al. found that exosomes also contain miRNAs (Valadi et al., 2007). Furthermore, in 2010, three groups independently reported that the miRNAs contained in exosomes travel between cells and suppress the expression of target genes in the recipient cells (Kosaka et al., 2010b; Pegtel et al., 2010; Zhang et al., 2010). Our group reported that

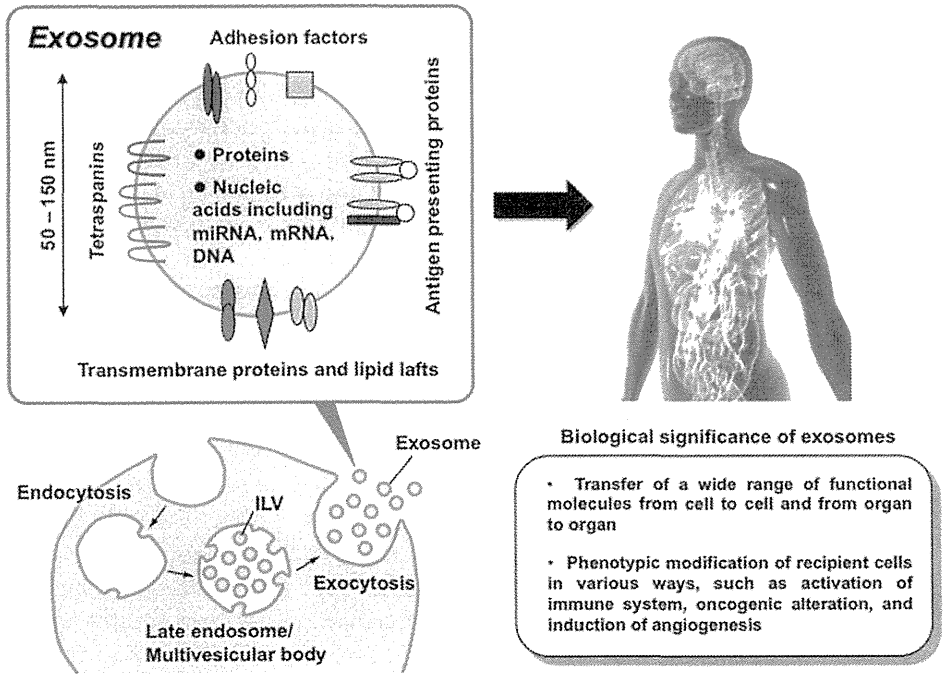

Figure 1 Biosynthesis process, molecular components, and biological significance of exosomes.
During endosomal maturation, intraluminal vesicles (ILVs) are formed through inward budding from the endosomal-limiting membranes. These ILV-containing endosomes are called multivesicular bodies (MVBs) or late endosomes. Some MVBs fuse with the lysosomal compartment and shuttle MVB cargo for degradation, and other MVBs travel to and fuse with the cytoplasmic membrane, where they release cargo ILVs to the extracellular space. These ILVs are secreted via exocytosis and are called exosomes. Exosomes contain a variety of proteins both on the surface membrane and inside the luminal space. Furthermore, exosomes package nucleic acids, including miRNAs, mRNA, and DNA. Recent studies have revealed that exosomes play important roles in various biological events.

a tumor-suppressive miRNA travels between two types of cells and exerts cell growth inhibition. Furthermore, we revealed that the secretion of exosomal miRNAs is dependent on the activity of neutral sphingomyelinase2 (nSMase2), a rate-limiting enzyme of ceramide biosynthesis.

Physiological and pathological relevance of secreted miRNAs

Since the discovery of the functionality of exosomal miRNAs, researchers have investigated the biological significance of secreted miRNAs in a variety of events. miRNAs are well known to be mobile and physiologically functional in plants (Chitwood and Timmermans, 2010), and this has also been found to be the case in mammals. Because miRNAs regulate the expression of various target genes, exosomal miRNAs are likely to serve as a versatile communication tool. Indeed, an increasing number of reports have shown that secreted miRNAs are involved in a wide range of biological processes (Figure 2).

Exosomal miRNAs in cancer biology

The functions of exosomal miRNAs secreted by tumor cells are now of great interest in cancer research (Kosaka and Ochiya, 2011). Since the late 1990s, researchers have explored the involvement of tumor-derived exosomes in cancer development. Early studies identified exosomal proteins that are associated with malignancy. These proteins promote tumor invasion (Higginbotham et al., 2011), promote angiogenesis (Gesierich et al., 2006), and support pre-metastatic and pro-metastatic niche formation (Jung et al., 2009; Peinado et al., 2012). In addition to these proteins, exosomal miRNAs have also been shown to be associated with malignancy in the past few years. In 2008, several groups reported elevated levels of tumor-associated miRNAs in the serum of cancer patients (Lawrie et al., 2008; Mitchell et al., 2008; Taylor and Gercel-Taylor, 2008; Skog et al., 2008). These findings highlighted the relevance of circulating miRNAs in cancer diagnosis. In addition, *in vitro* glioblastoma cell-derived exosomes contain miRNAs, thereby suggesting that they are involved in cancer progression (Skog et al., 2008). Concomitantly, Kogure et al. suggested that a subset of miRNAs enriched in hepatocellular carcinoma (HCC) cell line-derived exosomes can modulate the transforming growth factor β activated kinase-1 (TAK1) pathway (Kogure et al., 2011). Our group recently provided direct evidence that exosomal

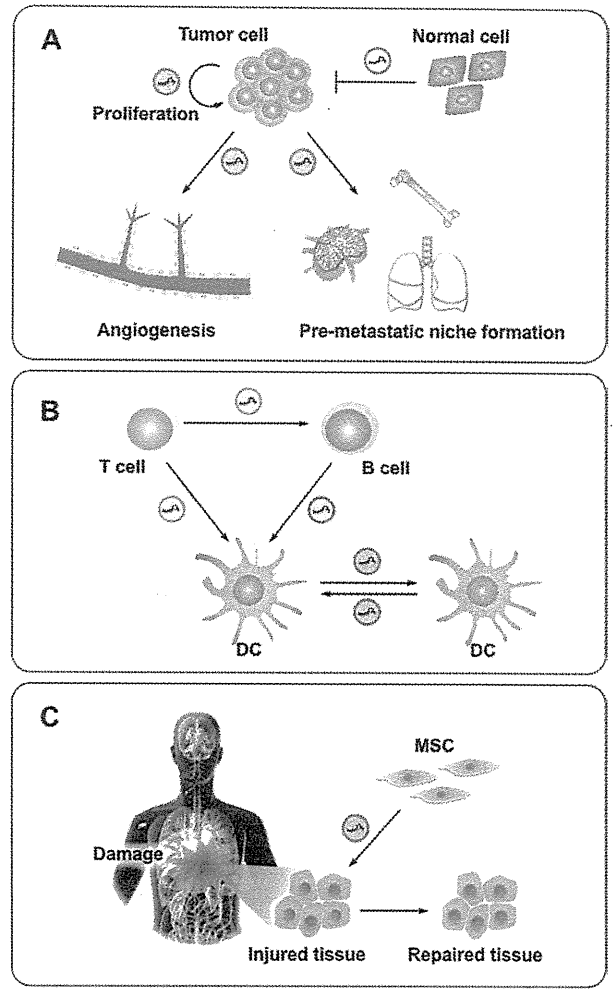

Figure 2 Exosomal miRNAs play important roles in various physiological and pathological events.
(A) Tumor cells produce exosomes containing miRNAs that promote cancer malignancy. These exosomal miRNAs function in several ways. They can promote tumor cell proliferation in an autocrine manner; they can induce angiogenesis, which further supports tumor growth and metastasis; and they can reach distant organs, such as bone marrow, lung, and lymph nodes, thereby promoting pre-metastatic and pro-metastatic niche formation. In contrast, tumor-surrounding normal cells secrete exosomes containing tumor-suppressor miRNAs. (B) Exosomal miRNAs also participate in the immune system. T-cells deliver exosomes containing immune regulatory miRNAs to APCs, including B-cells and DCs. B-cells also transfer exosomal miRNAs to DCs. Meanwhile, DC-to-DC communication, which is essential for amplifying the tolerogenic and immunogenic functions, is partly ascribed to exosomal miRNAs. (C) In response to damage, MSCs produce and deliver exosomes to injured tissue, which promotes tissue repair. Recent reports have shown that these MSC-derived exosomes contain miRNAs that can promote cell proliferation and suppress apoptosis.

transfer of miRNAs promotes cancer cell metastasis *in vitro* and *in vivo* (Kosaka et al., 2013a). miR-210 released by metastatic breast cancer cells enters endothelial cells

and suppresses expression of its target genes, which results in enhanced angiogenesis. Similar *in vitro* observations have also been reported in leukemia and colon cancer studies (Tadokoro et al., 2013; Yamada et al., 2013). Metastatic cancer cells were suggested to transfer oncogenic miRNAs to non-metastatic cells through exosome release, thereby increasing the malignant phenotype of the recipient cells (Camacho et al., 2013). In addition to the local transfer of miRNAs, several reports have shown that exosomal miRNAs are delivered even to distant organs and contribute to pre-metastatic niche formation (Grange et al., 2011; Rana et al., 2013). Grange et al. showed that CD105-positive renal cancer stem cells release exosomes that contain a set of pro-angiogenic mRNAs and miRNAs. Intravenous injection of these exosomes stimulates angiogenesis in the lung and supports lung engraftment of systemically administered renal cancer cells. Rana et al. also showed that highly metastatic rat pancreatic tumor cell (ASML)-derived exosomes are preferentially taken up by lymph node stroma cells and lung fibroblasts and that they support pre-metastatic niche formation. These authors also suggested that abundant miR-494 and miR-542-3p in ASML-derived exosomes target cadherin-17, which results in the upregulation of matrix metalloproteinase in pre-metastatic lung stroma cells.

In addition to tumor cells, the surrounding normal cells secrete exosomal miRNAs and modulate tumor development. Our group reported that exosomes mediate competitive interactions between cancer cells and normal cells (Kosaka et al., 2012). Exosomal miRNAs secreted by normal cells are transferred to cancer cells and inhibit their proliferation. This finding highlights the important role of normal cell-derived exosomes in the homeostatic mechanism and provides insight into a tumor initiation mechanism. Roccaro et al. reported that exosomes secreted by bone marrow mesenchymal stem cells (BM-MSCs) are associated with the pathogenesis of multiple myeloma (MM) (Roccaro et al., 2013). In contrast to the inhibitory effects of normal BM-MSC-derived exosomes on MM cell proliferation and dissemination, exosomes derived from BM-MSCs that were isolated from MM patients promote MM cell proliferation and dissemination. These authors also reported a decreased level of miR-15a in MM BM-MSC-derived exosomes compared with normal BM-MSCs.

Exosomal miRNA-mediated regulation of the immune system

The first evidence of exosome functionality was provided by immunology research, and intensive studies thereafter have been conducted in this field. In 1996, Raposo et al. reported that B-lymphocytes secrete antigen-presenting exosomes, thereby inducing an antigen-specific major histocompatibility complex class II-restricted T-cell response (Raposo et al., 1996). Following their report, other groups established the concept that antigen-presenting cells (APCs) utilize their exosomes to achieve their role (Zitvogel et al., 1998; Hwang et al., 2003). In addition, mast cell-derived exosomes also participate in immune reactions. Skokos et al. showed that mast cell-derived exosomes activate B- and T-lymphocytes (Skokos et al., 2001) and induce the phenotypic and functional maturation of dendritic cells (DCs) (Skokos et al., 2003).

Exosomal miRNAs also play roles in the immune system. Pegtel et al. demonstrated that exosomal miRNAs secreted by Epstein-Barr virus (EBV)-infected B-cells are transferred to uninfected recipient DCs and that the internalized EBV-miRNAs suppress target genes, such as CXCL11 (Pegtel et al., 2010). The suppression of CXCL11 in DCs may result in dysregulation of the host immune system and allow the EBV to circumvent the immunosurveillance. Exosome-mediated transmission of hepatitis C virus (HCV) has also been reported (Ramakrishnaiah et al., 2013). Of note, exosome-mediated transmission is resistant to neutralizing antibodies, which could explain the ineffectiveness of prophylactic neutralizing antibodies and agents that target the entry of HCV into a cell. Exosome-mediated unidirectional transfer of miRNAs from T-cells to APCs has also been reported (Mittelbrunn et al., 2011). Interestingly, immune synapses between these two cell types are required for efficient miRNA transfer. Exosome-mediated miRNA transfer represents a novel mechanism of DC-to-DC communication, in which DCs interact with neighboring DCs to amplify their functions (Montecalvo et al., 2012). Of note, the pattern of exosomal miRNAs varies according to the maturation of the parental DCs. Compared with immature DC-derived exosomes, mature DC-derived exosomes contain higher expression levels of miRNAs that target pro-inflammatory transcripts in myeloid cells and DCs. This finding supports the idea that exosomes can mirror the phenotype of their parent cell. Another recent report demonstrated the important role of the placenta-derived exosomes in protection from the maternal-fetal spread of viruses (Delorme-Axford et al., 2013). miRNAs that are exclusively expressed in placental cells are packaged within exosomes and transferred to non-placental cells; these miRNAs then attenuate viral replication in the recipient cells by inducing autophagy.

Another remarkable finding regarding the involvement of exosomal miRNAs in the immune system is obtained from reports on breast milk-derived exosomes.

Milk is the only nutritional source for newborn mammals and has unique health advantages for infants. However, the mechanisms through which milk regulates the physiology of newborns are not well understood. Our group first reported that miRNAs are contained in human breast milk (Kosaka et al., 2010c). These miRNAs are encapsulated in exosomes, and they are stable in acidic conditions and resistant to RNase digestion. Notably, among these milk miRNAs, we detected high expression levels of immune-related miRNAs in the first 6 months of lactation, which strongly suggests the role of exosomal miRNAs as novel immune regulatory agents in breast milk. We also confirmed that bovine milk contains immune- and development-related miRNAs. Similar observations have been reported by other groups (Weber et al., 2010; Gu et al., 2012; Zhou et al., 2012; Munch et al., 2013). Interestingly, Munch et al. suggested that the miRNA content in milk can be regulated by the maternal diet (Munch et al., 2013). These authors found that the expression of several miRNAs is altered by a perturbed maternal diet, particularly following a high-fat intake. Furthermore, a recent study has shown that colostrum-derived miRNAs are taken up *in vitro* by macrophages and modulate their immune activities, such as migration and cytokine secretion (Sun et al., 2013). Collectively, although direct *in vivo* evidence is still lacking for the physiological relevance of milk miRNAs, accumulating reports strongly suggest their protective roles against early infections in infants. This hypothesis highlights a novel concept that exosomes can mediate not only cell-to-cell or organ-to-organ communication but also individual-to-individual communication.

Beneficial effects of MSC-derived exosomal miRNAs on tissue repair

Exosomal miRNAs secreted by certain cell types may promote tissue repair in damaged tissue. MSCs reside in mesodermal tissue, such as bone marrow and adipose tissue, and they are attracting much attention due to the therapeutic effects of their secretory factors. These secreted factors are mainly thought to be cytokines and growth factors, but recent studies have revealed that exosomes also contribute to the therapeutic effects of MSCs (Katsuda et al., 2013). In particular, several reports have suggested that miRNAs that are involved in tissue repair are transferred from MSCs to damaged tissue and support regeneration. Chen et al. performed a microarray analysis and found that MSC-conditioned medium contains RNAs of <300 nt encapsulated in exosomes (Chen et al., 2010). These authors also found that exosomal miRNAs are present in a high precursor (pre)- to mature miRNA ratio and that these pre-miRNAs are successfully processed by RNase III to mature miRNAs, thereby suggesting that they are functional in recipient cells. Collino et al. also performed miRNA microarray analysis of MSC-derived exosomes, and they predicted that highly expressed miRNAs in the exosomes could be involved in multi-organ development, cell survival, and differentiation (Collino et al., 2010). These authors performed *in vitro* exosome-transfer experiments, and proteins that were targeted by some of the enriched exosomal miRNAs were downregulated in the recipient cells. Xin et al. found that MSC treatment in stroke model rats results in an increased level of miR-133b, a miRNA that is specifically expressed in midbrain dopaminergic neurons and regulates the production of tyrosine hydroxylase and the dopamine transporter (Xin et al., 2012). The increase of miR-133b and subsequent induction of neurite outgrowth depend on exosome-mediated miR-133b transfer from MSCs to neurons and astrocytes *in vitro* and *in vivo* (Xin et al., 2012, 2013). Additionally, MSCs transfer miR-221 to cardiomyocytes via exosomes and enhance cardioprotection by targeting p53-upregulated modulator of apoptosis (PUMA) (Yu et al., 2013).

Summary and perspective

In summary, accumulating evidence has shown that exosomal miRNAs play versatile biological roles. In the next few years, studies on exosomal miRNAs will provide considerable insight into currently undefined mechanisms underlying various biological phenomena. Simultaneously, translation of these research findings into clinical applications is also a critical issue. Here, we discuss the feasibility of the clinical application of exosomal miRNA research by focusing on two specific topics.

First, the findings that tumor cell-derived exosomes contain oncogenic miRNAs suggest a new direction for cancer therapy. Blocking the secretion of exosomal miRNAs from cancer cells is the simplest idea, but there are hurdles to be overcome. Although several proteins, including Rab family GTPases (Hsu et al., 2010; Ostrowski et al., 2010), nSMase2 (Trajkovic et al., 2008; Kosaka et al., 2010b), and heparanase (Thompson et al., 2013), are involved in exosome secretion, the precise molecular mechanisms have not been fully described. Currently, nSMase2 is most widely accepted as a key molecule in the secretion of exosomal miRNAs and thus is