医療応用を目指した蛍光半導体ナノ粒子の開発

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半導体ナノ粒子（QD）は、蛍光が高輝度かつ長寿命であるという点において、現行の有機蛍光色素よりも優れている。この特性を生かして、生化学・医学研究用の蛍光ラベルとして使用されており、微小量物質の検出や、腫瘍部位の非侵襲性検出（画像診断）への応用が期待されている。

しかしながら、現在使用されているQDの多くは、カドミウムを含有しており、人体や環境への影響を考慮した場合、直接的な医療応用は難しい。我々は、mercapto-undacanoic acidで表面修飾したQDの毒性検討試験を行なった結果、100 μg/mL以上の濃度において、細胞の生存率に影響を与える可能性を示唆している。

材料毒性の影響を取り除くため、より安心・安全な材料への移行が必要だと考えられる。候補としては、シリコンが挙げられ研究も進んでいるが、酸化による安定性の問題や、多量に製造することが難しいという点から、生化学応用への移行が進んでいない。最近の研究で、共同研究者である東京電機大学・平栗教授らは、数十ミリグラム単位での蛍光シリコンナノ粒子の製造に成功している。我々は、応用への礎として、シリコンナノ粒子を用いて、物性の検討、細胞レベルでの毒性試験、及び共培養後の検出実験を行なった。

本発表では、（1）蛍光ナノ粒子ＱＤの生化学的応用、（2）ＱＤの毒性について（3）新規蛍光シリコンナノ粒子の安全性について報告をする。

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MicroRNAs as biomarkers and therapeutic drugs in human cancer

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MicroRNAs as biomarkers and therapeutic drugs in human cancer

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Abstract
MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, noncoding small RNAs that act as post-transcriptional gene regulators. Experimental evidence has shown that miRNAs can play roles as oncogenes or tumor suppressor genes, suggesting their contribution to cancer development and progression. Expression profiles of human miRNAs demonstrated that many miRNAs are deregulated in cancers and are differentially expressed in normal tissues and cancers. Therefore, miRNA profiling is used to create signatures for a variety of cancers, indicating that the profile will help further establish molecular diagnosis, prognosis and therapy using miRNAs. This paper introduces the aberrant expression of miRNAs in human cancer, and discusses the potential of these miRNAs as biomarkers and targets/molecules for molecular therapy.

Keywords: *Biomarker, cancer, microRNA, therapeutic drugs*

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Introduction

MicroRNAs (miRNAs, miRs) are endogenous noncoding ~22 base pair (bp) RNAs that suppress gene expression in a sequence-specific manner and are important in a wide range of physiologic and pathologic processes (Bartel 2004, Stefani & Slack 2008), although a few miRNAs (e.g., miR-369-3) are known to directly activate translation of target genes on cell cycle (Vasudevan et al. 2007). In the first miRNA, described in 1993, the *Caenorhabditis elegans* heterochronic gene *lin-4* encoded small RNAs with antisense complementarity to *lin-14* (Lee et al. 1993). Based on miRBase release 11.0, >600 human miRNAs have been registered, a number that is expected to increase up to 1,000 (Bentwich et al. 2005), with a large number being evolutionarily conserved (Pasquinelli et al. 2000). These miRNAs are predicted to regulate expression of ~90% of all human genes (Miranda et al. 2006). Moreover, Calin et al. reported that a single cluster of microRNAs (namely miR-15a/16-1

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cluster) has been found to regulate, directly or indirectly, ~14% of all human genome (Calin et al. 2008).

In mammalian cells, the primary transcript for miRNAs (pri-miRNA) is generated in the form of long, polycistronic messages by a type II polymerase (Cai et al. 2004, Lee et al. 2004). The pri-miRNAs have short and imperfectly base-paired stem loop structures that are processed in the nucleus by Drosha, an RNase III enzyme, into 60 – 110 bp called precursor miRNAs (pre-miRNA). Exportin 5 transports the pre-miRNA into the cytoplasm, where Dicer processes the molecule into double-stranded, 18 – 24 bp mature miRNAs (Ketting et al. 2001, Yi et al. 2003). The double-strand mature miRNA is composed of two complementary single-strand molecules, known as guide strand, which is integrated in the miRNA-protein complex (miRNP), and antiguide or passenger strand (marked as *), which is degraded as a miRNP complex substrate (Gregory et al. 2005). Perfect-to-near-perfect base pairing leads to degradation of the targeted mRNA. Alternatively, the miRNA can bind to the complementary sequence in the 3′ untranslated region (3′UTR) of target mRNAs (Bartel 2004, Liu et al. 2005, Saxena et al. 2003, Sen & Blau 2005). This base pairing subsequently causes degradation of the mRNA and/or inhibition of protein translation. The potential mechanisms underlying this process were recently reviewed (Pillai et al. 2007). Protein levels of the target gene are consequently reduced, whereas mRNA levels may or may not be decreased. In humans, miRNAs mainly inhibit protein translation of their target genes and only infrequently cause degradation or cleavage of the mRNA (Bartel 2004).

Expression of miRNA is highly specific for tissues and developmental stages, and its functions have been appreciated in various fundamental biological processes such as cell proliferation (Hayashi et al. 2005), stem cell division (Zhang et al. 2006) and apoptosis (Jovanovic & Hangartner 2006). Recently, it has been revealed that altered expression of specific miRNA genes contributes to the initiation and progression of cancer (Calin et al. 2002, Calin et al. 2004a, He et al. 2005, Lu et al. 2005).

In this review, we introduce aspects of aberrant expression of miRNAs in human cancer tissues, and describe the potential for miRNAs as diagnostic markers and molecules for therapy in human cancers.

miRNAs as biomarkers to diagnose human cancer

New approaches that can complement and improve on current strategies for cancer detection are urgently needed. Many independent studies on different tissues have demonstrated that cancer cells have different miRNA profiles compared with normal cells (Table I), suggesting that miRNA expression could be used for the diagnosis of cancer. In a report by Chan et al. (2005), expression of miR-21 was evaluated by northern blotting and membrane array in human brain tumors. The expression level was shown to have increased 5- to 100-fold in human glioblastoma multiforme tissue compared with control non-neoplastic brains. Furthermore, they found a similarly robust increase in miR-21 expression in six commonly used model cell lines derived from human glioblastomata. Takamizawa et al. showed by northern blotting the frequent occurrence (43.8%) of a significant reduction (>80.0%) in let-7 miRNA expression in lung cancers when compared with that in corresponding normal lung tissues (Takamizawa et al. 2004). As shown in Table I, upregulation of miR-21 and downregulation of let-7 were reported not only in glioblastoma or lung cancer,

Table I. Cancer-associated microRNAs and their potential diagnostic markers.

Cancer type	miRNAs		Ref.
	upregulated	downregulated	
Brain, GBM	miR-21, 221	miR-128, 181	Chan et al 2005, Takeshita et al. 2005
Breast Ca	miR-9-1, 10b, 17-5p21, 21, 29b-2, 34, 146, 155, 181b-1, 213	let-7, miR-15a, 16, 125a, 125b, 127, 145, 204	Calin & Croce 2006, Saito et al. 2006, Takamizawa et al. 2004, Volinia et al. 2006, Zhang et al. 2007a
Lung Ca	miR-17-5p, 17-92, 21, 24-2, 106a, 128b, 146, 150, 155, 191, 192, 197, 199a-1, 203, 205, 210, 212, 214	let-7, miR-9, 26a-1-p, 27b, 29b-2, 32, 33, 30a-5p, 95, 101-1, 124, 124a-3, 125a, 125a-p, 126, 140, 143, 145, 181c-p, 198, 192-p, 199b-p, 216-p, 218-2, 219-1, 220, 224	Takamizawa et al. 2004, Volinia et al. 2006, Yanaihara et al. 2006
Esophageal Ca	miR-21, 93	miR-203, 205	Feber et al. 2008
Gastric Ca	miR-21, 24-1, 24-2, 25, 92-2, 107, 191, 214, 221, 223	let-7	Volinia et al. 2006, Zhang et al. 2007b
Colorectal Ca	miR-17-5p, 20a, 21, 24-1, 24-2, 29b-2, 30c, 31, 32, 96, 106a, 107, 128b, 135b, 155, 183, 191, 221, 223	let-7, miR-34, 127, 133b, 143, 145	Bandres et al. 2006, Gramantieri et al. 2007, Michael et al. 2003, Volinia et al. 2006
Hepatocellular Ca	miR-15b, 18a, 21, 106b, 221, 222, 224	let-7, miR-101, 122a, 125a, 195, 199a, 200a	Gramantieri et al. 2007, Meng et al. 2007, Murakami et al. 2006
Pancreatic Ca	miR-17-5p, 20a, 21, 24-1, 24-2, 25, 29b-2, 30c, 32, 92-2, 100, 106a, 107, 125b, 128b, 146, 155, 181a, 181b-1, 191, 196a, 196b, 199a-1, 212, 214, 221, 223, 301, 376a	miR-139, 142-p, 345, 375	Volinia et al. 2006,32,65
Prostate Ca	miR-17-5p, 20a, 21, 25, 30c, 32, 92-2, 106a, 146, 181b-1, 191, 199a-1, 214,223	miR-15a, 16, 143, 145, 218-2	Volinia et al. 2006
Cervical Ca	miR-21, 199a	miR-143, 145	Esquela-kerscher & Slack 2006, Lui et al. 2007
CLL	miR-17-92, 155	miR-15a, 16, 143, 145, 192, 213, 220	Akao et al. 2007, Cailin et al. 2002, Calin et al. 2005

Ref, references; GBM, glioblastoma maltiforme; Ca, cancer; B-CLL, B cell chronic lymphocytic leukemia.

respectively, but also in a variety of human cancers, suggesting that overexpression of miR-21 and/or underexpression of let-7 might be common markers in cancer diagnosis.

Large profiling studies using tumor tissues have proven the utility of miRNA profiling for diagnosis. Microarray analysis is the most comprehensive method for

disclosing expression levels of miRNAs and has been invaluable in evaluating human cancers. Iorio et al. (2005) demonstrated the existence of a breast-cancer-specific miRNA signature with a genome-wide miRNA expression profiling in a large set of normal and tumor breast tissues, in which 29 miRNAs were differentially expressed in breast cancer versus normal tissues. Among them, confirmed by northern blot, miR-21 and miR-155 were upregulated, whereas miR-10b, miR-125b and miR-145 were downregulated, suggesting that these miRNAs may potentially be diagnostic markers. The same group further examined the miRNA expression signature of human solid tumors including breast cancer. The authors identified 27 miRNAs that were differentially expressed in breast cancer versus adjacent normal tissues, that is, 15 miRNAs were upregulated and 12 were downregulated in breast cancer versus normal tissues (Volinia et al. 2006).

In lung cancer, Yanaihara et al. confirmed a unique miRNA expression profile using microarray (Yanaihara et al. 2006). Forty-three miRNA were found to be up- or downregulated and could accurately distinguish non-tumoral versus cancer tissues (Table I), even in Stage I cancer. Moreover, they also found a molecular signature for subsets of lung cancer.

Murakami et al. reported that three miRNAs were upregulated and five were downregulated in hepatocellular carcinomas (HCCs) compared to non-tumoral adjacent regions using microarray technology (Murakami et al. 2006). Hepatocellular carcinoma was distinguishable with high accuracy by the miRNA expression pattern from non-tumoral tissue including liver cirrhosis. Moreover, they also reported that the expression level was correlated with differentiation of the tumor. However, they were unable to find differences between hepatitis-B-positive and hepatitis-C-positive samples. Subsequently, Li et al. reported that eight miRNAs were differentially expressed in HCC compared to adjacent non-tumoral tissues (Li et al. 2007). Among them, however, only one in each report was reproducible. This discrepancy may reflect the application of different miRNA platforms, various techniques and the varieties of clinical backgrounds of the resected samples.

Bloomston et al. identified global expression patterns in 65 pancreatic ductal adenocarcinomas with matched benign adjacent pancreas and 42 chronic pancreatitis tissues (Bloomston et al. 2008). In pancreatic cancers, 21 miRNAs were differentially overexpressed and 4 were underexpressed relative to chronic pancreatitis and adjacent benign pancreas tissue. Szafranska et al. also reported miR-196a and miR-196b were overexpressed in pancreatic ductal adenocarcinomas but not in non-tumoral tissues including pancreatitis (Szafranska et al. 2007).

Calin et al. reported genome-wide expression profiling of miRNAs in 38 human chronic lymphocyte leukemia (CLL) samples (Calin et al. 2004b). Expression levels of miR-15a and miR-16, encoded within the 13q14 region, a deletion found in $>65\%$ of CLL cases, were downregulated in 75% of CLL cases that harbored this chromosomal abnormality. Inversely, He et al. reported that the miR-17-92 polycistron, which is located in 13q31–32, a region commonly amplified in B cell lymphoma, was upregulated in 65% of the B cell lymphoma patients (Calin et al. 2004a). These findings highlighted the potential of miRNA profiling in cancer diagnosis.

Detection of miRNA has also been applied to early detection of cancer or monitoring of cancer recurrence using peripheral blood. Mitchell et al. identified

miR-141 from serum samples as a specific marker of human prostate cancer (Mitchell et al. 2008). They found that serum levels of miR-141 were, in general, substantially higher in cancer patients ($n = 25$) compared with the healthy control group ($n = 25$). Furthermore, serum levels of miR-141 were able to detect individuals with cancer with 60% sensitivity at 100% specificity. Another study, reported by Taylor et al., demonstrated that 8 miRNAs (miR-21, -141, -200a, -200b, -200c, -203, -205 and -214) detected from peripheral blood were also identified as markers of ovarian cancer (Taylor et al. 2008). In addition, they revealed that these circulating miRNAs in the blood were incorporated in tumor-derived exosomes, which were small (50 – 100 nm) membrane vesicles of endocytic origin. Chen et al. recently reported that the expression levels of two miRNAs (miR-25 and miR-223) in serum are significantly higher in 152 non-small cell lung cancer patients than in 75 healthy volunteers (Chen et al. 2008). Interestingly, the authors revealed that serum miRNAs are resistant to RNase A digestion, treatment of HCl (pH = 1) and NaOH (pH = 13) and 10-cycle repeats of freeze–thaw, indicating that serum miRNAs are stable even after drawing blood, which is very important for a reliable biomarker.

Taken together, it is suggested that detection of cancer-associated specific miRNAs in each malignant tumor from tissue sample and/or serum will contribute to early detection and accurate diagnosis for cancer. Therefore, the miRNAs shown in Table I should be robust targets in the development of a molecular diagnosis of cancer, although further studies are needed to evaluate which miRNAs would be valid diagnostic markers in each cancer.

miRNAs in cancer classification and prognosis

Lu et al. asked the question whether global miRNA expression profiles could classify human cancer. In reply to this question, they used a bead-based flow cytometric miRNA expression profiling method to present a systematic expression analysis of miRNAs from human cancer tissue specimens (Lu et al. 2005). The authors established and applied miRNA expression profiles to an independent series of 17 poorly differentiated tumors of unknown origin. Based on the differential expression of 217 miRNAs, a correct diagnosis could be established in 12 out of 17 of the tumors. In contrast, when the same samples were profiled by expression of 16,000 mRNAs, they did not accurately classify the tumors. It is suggested that a small number of miRNAs define a cancer better than expression data from a large number of mRNAs. Volinia et al. also described a large-scale microarray analysis in 540 samples from 6 solid types of the most frequent human cancers (lung, breast, stomach, prostate, colon and pancreatic cancers) and found a common signature composed of 57 miRNAs (Volinia et al. 2006). In this study, out of the 228 miRNAs analyzed, 36 were overexpressed and 21 were underexpressed in cancer versus normal cells. Hierarchical clustering analysis showed that such an miRNA signature enabled the tumor samples to be classified based on their tissue of origin.

There is emerging evidence that miRNAs can be used for cancer prognosis (Table II). In the majority of CLL patients, the prognosis is relatively good and the treatment after diagnosis is started only if poor prognostic markers are evident. By performing an miRNA profiling screen on 144 CLL patients, a unique signature of 13 miRNAs was

Table II. Cancer-associated microRNAs and their poor prognostic markers

Cancer type	miRNAs		Ref.
	upregulated	downregulated	
Brain, GBM	miR-10b, 21, 221	miR-128, 181	Ciafre et al. 2005, Yanaihara et al. 2005
Breast Ca	miR-21, 155	miR-125,145	Bandres et al. 2006a, Iorio et al. 2005, Volinia et al. 2006
Lung Ca	miR-17-3p, 93, 106a, 155	let-7, miR-145,	Takamizawa et al. 2004, Yanaihara et al. 2006
Colorectal Ca	miR-21, 31, 96, 135b, 183	miR-133b, 143, 145	Akao et al. 2006, Bandres et al. 2006b, Cummins et al. 2006, Schetter et al. 2008
Hepatocellular Ca	miR-18, 224	miR-122, 125a, 195, 199a, 200a	Kutay et al. 2006, Murakami et al. 2006
Pancreatic Ca	miR-21, 103, 107, 196a	miR-204	Bloomston et al. 2007, Roldo et al. 2006
CLL	miR-21, 23a, 23b, 24-2, 146, 150, 155, 181a, 221	miR-15a, 16, 29c, 192, 222	Calin et al. 2002, Calin et al. 2005, Eis et al. 2004, Fulci et al. 2007

Ref, references; GBM, glioblastoma maltiforme; Ca, cancer; CLL, B cell chronic lymphocytic leukemia.

shown to differentiate cases on the basis of a good or bad prognosis or on the presence or absence of disease progression (Calin et al. 2005). Among them, miR-16 and miR-15a were expressed at lower levels in patients with a good prognosis, in agreement with early reports that 13q14.3 genomic deletions at the locus harboring these genes are related with a favorable course of the disease. Iorio et al. reported that miRNA expression profiling in breast cancer has also demonstrated prognostic value (Iorio et al. 2005). Analysis of 76 primary breast cancer samples demonstrated a consistently altered expression, including decreased miR-125b and -145, and increased miR-21 and -155. More significantly, most differentially expressed miRNAs were associated with invasive breast cancer biopathological features, including estrogen and progesterone receptor expression, tumor stage, vascular invasion or proliferation index.

Yanaihara et al. determined miRNA expression with 352 miRNA probes in 144 lung cancer tumor–normal pairs. Malignant lung tissues were consistently differentiated from normal tissues, according to the expression profile of 43 miRNAs. Further, univariant regression analysis identified 8 miRNAs (miRs-17-3p, -21, -93, -106a, -145, -155, let-7a-2 and let-7b) as independent survival prognosticators in lung adenocarcinoma (Yanaihara et al. 2006). In particular, reduced expression of let-7 and high expression of miR-155 in human lung cancers were associated with significant poor survival. Thus, these miRNAs may be used to identify those patients for closer monitoring and adjunct therapy.

In pancreatic cancer, a subset of six miRNAs was shown to be predictive of long-term survival in lymphnode-positive patients who show a poor prognosis. On the other hand, overexpression of miR-196a was associated with a poorer prognosis (Bloomston et al. 2007). Further correlations with existing biomarkers and miRNA expression are likely to improve our understanding of the prognostic relevance of miRNA profiling in human cancers.

If miRNAs prove useful for pathological diagnosis, their key advantage might be their high stability. In contrast to most mRNAs, miRNAs are long-lived *in vivo* (Lim et al. 2005) and very stable *in vitro* (Tang et al. 2006), which might allow an analysis of paraffin-embedded samples for routine diagnostic applications. In fact, Rosenfeld et al. reported that miRNAs, which were extracted from 333 formalin-fixed paraffin-embedded (FFPE) archival human cancer tissue samples, were used to identify cancer tissue origin by measuring the miRNA expression levels using an miRNA microarray (Rosenfeld et al. 2008). Two-thirds of the samples were classified with high confidence, with >90% accuracy. These data strongly suggested that identification of miRNAs expression profiling might be useful for diagnosis as biomarkers, even if miRNAs are from FFPE samples. Identification of miRNAs expression profiling using FFPE samples is considered to be effective, especially in histological diagnosis of metastatic cancer of an unknown primary lesion because a delayed diagnosis fails to prompt appropriate curatives and leads to a poor prognosis.

Application of miRNA for cancer therapy

It has been reported that miRNAs play a crucial role in the initiation and progression of human cancer. Deregulation of miRNAs is associated with genetic or epigenetic alterations, including deletion, amplification, point mutation and aberrant DNA methylation (Calin & Croce 2006). Calin et al. first made the connection between microRNAs and cancer by showing that miR-15 and miR-16 are located at 13q14 within a 30kb region of loss in CLL, and that both genes were deleted or downregulated in ~68% of CLL cases (Calin et al. 2002). More than 50% of miRNAs are located in cancer-associated genomic regions or in fragile sites (Calin et al. 2004a). Toyota et al. reported that miR-34b and miR-34c, two components of the p53 network, are epigenetically silenced in colorectal cancer, whereas 5-aza-2′-deoxycytidine treatment rapidly restores miR-34b/c expression (Toyota et al. 2008). Moreover, Lujambio et al. revealed that reintroduction of miR-34b/c in human cancer cells with epigenetic inactivation reduced tumor growth and inhibited cell motility and metastasis formation in xenograft model (Lujambio et al. 2008). Therefore, inhibitors of DNA methylation could induce expression of some miRNAs that may act as tumor suppressors. These results suggested that aberrant expression of miRNAs might be important in human cancer pathogenesis and these miRNAs might be targets for cancer therapy.

The most direct way for molecules to correct altered miRNA–mRNA interactions is RNA oligonucleotides. Therapeutic potentials using these RNA oligonucleotides have been proposed, although our understanding of the miRNAs' role in cancer is still very limited. There are two possible approaches: blocking oncogenic miRNAs (oncomiRs) or overexpressing miRNAs with tumor suppressor activity. Theoretically, antimiRNA oligonucleotides (AMOs) can be used to suppress miRNA activity if the AMOs can bind strongly to the miRNA and are stable enough in physiological conditions (Krutzfeldt et al. 2007). AntimiRNA oligonucleotides have complementary sequences to miRNAs and contain several chemical modifications to achieve that goal. Two types of modifications were developed to attain strong

binding: 2′-O-methylation of RNA nucleotides (Krutzfeldt et al. 2005, Hutvagner et al. 2004) and locked nucleic acid (LNA) DNA nucleotides (Vester et al. 2004). These modifications are also used in the opposite approach where modified oligonucleotides are delivered into cells that underexpress miRNAs with tumor suppressor activity. This approach showed promising results in cell culture (Johnson et al. 2005) and needs to be tested in animal models. It is important for *in vivo* trials that these oligonucleotides be chemically modified to allow for stability in serum and cellular uptake. Modified antisense oligonucleotides are already being developed to utilize the intrinsic RNAi pathway for delivery of a gene therapy. Krutzfeldt et al. demonstrated that modified cholesterol-conjugated antisense RNAs designated 'antagomiRs' could effectively inhibit miRNA function *in vivo* in the adult mouse (Krutzfeldt et al. 2005). The authors applied three daily i.v. injections of antagomiRs and achieved effective inhibition of four miRNAs over a period of weeks in most tissues.

Not only RNA oligonucleotides' modification but also a drug delivery system (DDS) of nucleic acid including siRNA using atelocollagen has been reported by our group (Ochiya et al. 1999, Ochiya et al. 2001). Atelocollagen was the first biomaterial with the potential for use as a carrier for gene delivery. Atelocollagen is liquid at low temperature, making admixing of nucleic acid solutions easy. Because the surface of atelocollagen molecules is positively charged, the molecules can bond electrostatically with negatively charged nucleic acid molecules. Tazawa et al. demonstrated in a mice model that direct intratumoral injection of an miR-34a/atelocollagen complex successfully suppressed the growth of tumors derived from human colon cancer cells (HCT116 and RKO) (Tazawa et al. 2007). Furthermore, significant reduction of the tumor volume was observed until day 6 after miR-34a administration. Interestingly, the authors showed that expression of miR-34a was downregulated in more than one-third of human colon cancers compared with counterpart normal colon mucosae. Therefore, these data suggested that restoring decreased miRNAs into cancer cells could suppress progression of cancer *in vivo*.

One problem for systemic treatment *in vivo* is that some serum RNases can degrade RNA oligonucleotide. However, siRNA, probably as well as miRNA, complexed with atelocollagen is resistant to nucleases and is transduced efficiently into cells, thereby allowing long-term gene silencing (Minakuchi et al. 2004). In fact, we previously reported the usefulness of atelocollagen DDS in systemic delivery of synthetic siRNA for cancer treatment (Takeshita et al. 2005). An siRNA/atelocollagen complex was administered through the tail vein of mice with bone metastasis of prostate cancer cells to examine the delivery of the siRNA to the systemic metastasis foci. The siRNA/atelocollagen was able to be efficiently delivered to tumors 24 h after injection and was able to exist intact for ≥ 3 days. Furthermore, atelocollagen-mediated systemic administration of siRNAs such as enhancer of zeste homolog 2 and phosphoinositide 3′-hydroxykinase p110-α-subunit, which were selected as candidate targets for inhibition of bone metastasis, resulted in an efficient inhibition of metastatic tumor growth in bone tissues. These results suggest that miRNA/atelocollagen could also be available for systemic delivery to suppress tumor growth and to inhibit cancer metastasis.

The great challenge for these approaches is the specific delivery of functional oligonucleotides into a tumor tissue. As these molecules are equally active in both healthy and cancer cells their side effects must be minimized before they can be considered for clinical trials.

Conclusion

miRNA has emerged in the field of cancer research in recent years. Many aberrant expressions of miRNA have been reported in a variety of human malignancies. Although the potential of miRNA as a diagnostic and/or prognostic marker in cancer has been identified, further studies are needed to identify and validate useful miRNAs in each cancer. Evaluation of the potential for miRNAs as diagnostic and prognostic

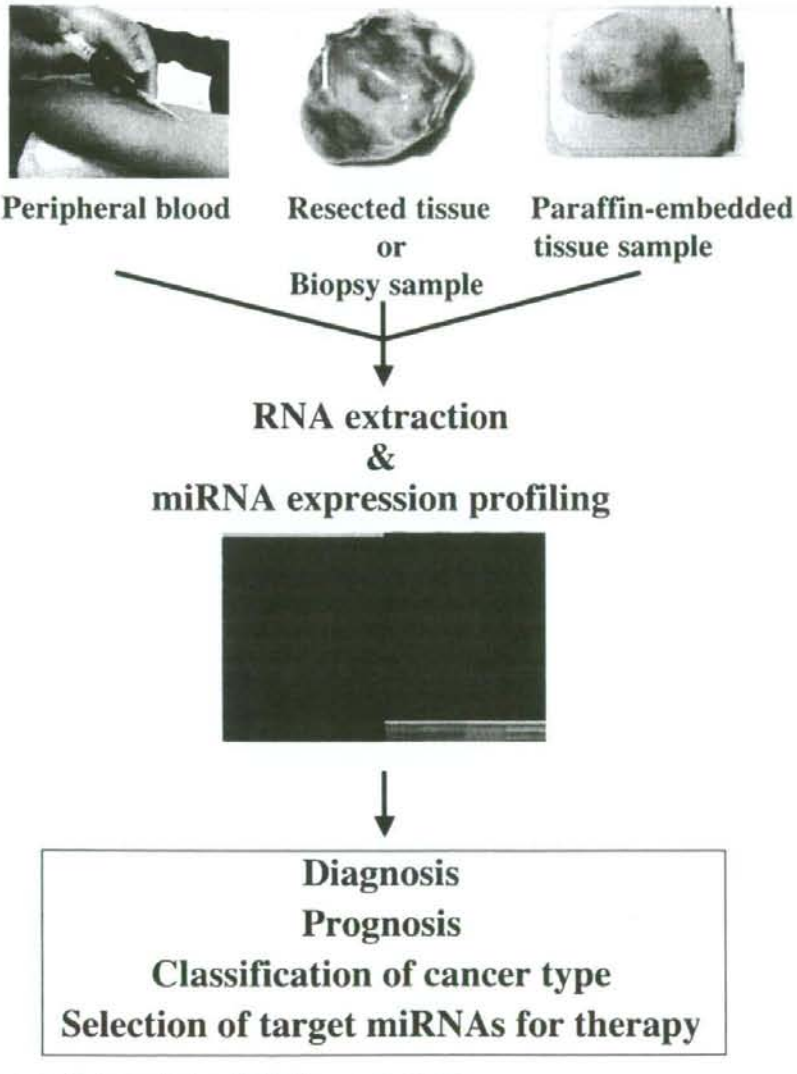

Figure 1. Clinical application of miRNA expression profiling.

markers or therapeutic molecules or targets has just begun. Therefore, not only retrospective studies but also prospective ones using clinical samples will serve to identify relevant markers. Moreover, the development of animal models will be useful for *in vivo* evaluation of miRNA molecules as anticancer drugs.

Discussion

In recent years, much evidence on miRNAs has accumulated in the field of cancer research. How will miRNAs in cancer diagnosis and therapy be applied in the future? Our prediction is illustrated in Figure 1. A rapid and accurate cancer diagnosis and prognosis will be possible by examining miRNA expression profiling using tissue specimens, even if they are biopsy samples, formalin-fixed samples and various fluids. Moreover, abnormal expression of miRNA might be detected from a peripheral blood sample as well as conventional tumor markers such as CEA (carcinoembryonic antigen), CA19-9 and PSA (prostate-specific antigen). From the point of cancer therapy, it might be possible to correct the abnormal expression of miRNAs associated with cancer by injecting tumor suppressor miRNAs or molecules inhibiting oncomiRs. The combination of miRNAs to be injected will depend on the miRNA expression profiling of each cancer patient, which indicates the possibility for ultimate personalized medicine. It will be also possible to use miRNAs in combination with chemotherapy and radiotherapy to attenuate drug resistance of cancer cells and/or to relieve side effect to normal tissue.

In this review, although we described the possibilities of miRNAs on diagnosis, prognosis and therapy in cancer, there might be another possibility of using miRNA expression data for cancer prevention. miRNAs will bring about many revolutionary changes in cancer diagnosis, therapy and prevention.

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RPN2 gene confers docetaxel resistance in breast cancer

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Drug resistance acquired by cancer cells has led to treatment failure. To understand the regulatory network underlying docetaxel resistance in breast cancer cells and to identify molecular targets for therapy, we tested small interfering RNAs (siRNAs) against 36 genes whose expression was elevated in human nonresponders to docetaxel for the ability to promote apoptosis of docetaxel-resistant human breast cancer cells (MCF7-ADR cells). The results indicate that the downregulation of the gene encoding ribophorin II (RPN2), which is part of an *N*-oligosaccharyl transferase complex, most efficiently induces apoptosis of MCF7-ADR cells in the presence of docetaxel. RPN2 silencing induced reduced glycosylation of the P-glycoprotein, as well as decreased membrane localization, thereby sensitizing MCF7-ADR cells to docetaxel. Moreover, *in vivo* delivery of siRNA specific for *RPN2* markedly reduced tumor growth in two types of models for drug resistance. Thus, RPN2 silencing makes cancer cells hypersensitive response to docetaxel, and RPN2 might be a new target for RNA interference–based therapeutics against drug resistance.

Breast cancer is the most common malignancy in women. Either neoadjuvant or adjuvant chemotherapy administered to subjects with stage I–3 breast cancers can improve their survival rates[1–3]. Among chemotherapeutic agents, docetaxel, which belongs to the group of taxanes (mitotic inhibitors and antimicrotubule agents), has been shown to have well-established benefits in breast cancer[4]. The response rate to docetaxel, however, is 50% even in first-line chemotherapy, and it decreases to 20–30% in second- or third-line chemotherapy[5–7]; nearly half of the treated subjects do not respond to it and suffer side effects. There is currently no method to reliably predict tumor responses to docetaxel before therapy or to detect when resistance or hypersensitivity develops. Therefore, the identification of molecular biomarkers in docetaxel-resistant breast cancer that could help in a more accurate assessment of individual treatment and the development of molecular-target therapies that could lead to better tumor reduction are of considerable interest.

It has been reported that the expression of the multidrug transporter P-glycoprotein, encoded by the *MDR1* gene (official gene symbol *ABCB1*), is one of the causes of clinical drug resistance to taxanes[8,9]. Other molecules, such as the multidrug resistance–associated protein MRP1[10,11], breast cancer resistance protein (*ABCG2*) and other transporters[12], which act as energy-dependent efflux pumps capable of expelling a large range of xenobiotics, and GSTpi, which is one of the isoenzymes of the glutathione-*S*-transferase (GST)[13–15], have been extensively reported to be overexpressed in tumor cells showing the multidrug-resistant phenotype. It was recently shown that high thioredoxin expression is associated with resistance to docetaxel in breast cancer[16,17]. These molecules might be clinically useful in the prediction of a response to anticancer drugs. Currently, however, none have proven to be specific target molecules for increasing the efficacy of chemotherapy in breast cancer.

To better understand the regulatory network underlying docetaxel resistance in breast cancer cells and to identify molecular targets for therapy, we initiated gene expression profiling of 44 subjects with breast tumors (22 responders and 22 nonresponders) by adaptor-tagged competitive PCR[18] to identify the genes capable of predicting a docetaxel response in human breast cancer and reported the preliminary results of 85 genes whose expression potentially correlated with docetaxel resistance[16]. In the current study, we used an atelocollagen-based siRNA cell transfection array[19,20] to identify the genes responsible for conferring drug resistance. Among the siRNAs targeting genes that were elevated in nonresponders to docetaxel, siRNA designed for *RPN2* (RNP2 siRNA) significantly promoted docetaxel-dependent apoptosis and cell growth inhibition of MCF7-ADR human breast cancer cells that are resistant to docetaxel. Furthermore, atelocollagen-mediated *in vivo* delivery of RPN2 siRNA significantly reduced drug-resistant tumor growth in mice given docetaxel. RPN2 confers drug resistance via the glycosylation of P-glycoproteins and regulates antiapoptotic genes. Thus, RPN2 siRNA introduction hypersensitizes cancer cell response to chemotherapeutic agents, making RPN2 a potential key target for future RNA interference (RNAi)-based therapeutics against a drug-resistant tumor.

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RESULTS

RNAi-based screening for identification of molecular target

As an extension of our previous strategy of analyzing docetaxel resistance in breast cancer cells and of identifying molecular targets for therapy[16], we conducted a study of RNAi-induced gene knockdown in docetaxel-resistant MCF7-ADR human breast cancer cells. Among the 85 genes listed[16], 61 genes that are potentially targets for siRNA strategy were upregulated in human nonresponders. We selected 36 genes with more than a 0.365 signal-to-noise ratio and successfully designed and synthesized siRNAs specific to these genes (**Table 1**). The siRNAs were conjugated to atelocollagen and arrayed on a 96-well microplate. Then, MCF7-ADR cells expressing the luciferase gene (MCF7-ADR-Luc) were seeded into the microplate (the target validation process by cell transfection array is schematically shown in **Supplementary Fig. 1** online.). To evaluate the efficiency of the atelocollagen-mediated cell transfection array, we used GL3 siRNA against the gene encoding luciferase. Atelocollagen-mediated GL3 siRNA delivery caused an approximate 75% reduction of the luciferase activity in MCF7-ADR-Luc cells relative to the control nontargeting siRNA (data not shown). To identify the genes responsible for docetaxel resistance, we assessed siRNAs for their ability to inhibit cell growth and induce apoptosis in the presence of docetaxel compared with the control nontargeting siRNA. We measured cell growth by luciferase activity and examined apoptosis by caspase-7 activation. The results indicated that the downregulation of eight genes (*PTPLB, GSTP1, TUBB, RPN2, SQRDL, NDUFS3, PDCD5* and *MRPL17*) resulted in marked inhibition of cell growth ($P < 0.05$, **Fig. 1a**). Induction of apoptosis was evidenced in cells by downregulation of six genes (*PTPLB, APRT, CFL1, RPN2, SQRDL* and *MRPL17*; $P < 0.05$, **Fig. 1b**). In particular, RPN2 siRNA strongly enhanced caspase-7 activity in the presence of docetaxel ($P < 0.001$, **Supplementary Fig. 2a** online). We validated these results by counting Hoechst-stained cells showing apoptotic nuclear condensation and fragmentation (**Fig. 2a**) and found that there was a significantly higher apoptotic cell death rate in cells given RPN2 siRNA and docetaxel relative to that in cells given RPN2 siRNA alone ($P < 0.02$, **Fig. 2b**). No significant difference was observed in cells with nontargeting control siRNA (**Fig. 2b**). At 72 h after treatment with siRNA and docetaxel, there was substantial cell death induced by RPN2 siRNA compared with the control nontargeting siRNA (**Fig. 2c**). At 96 h after the transfection, almost all RPN2 siRNA–treated cells were detached and disappeared from the culture dishes.

Table 1 The list of 36 genes whose expression is elevated in nonresponders to docetaxel in subjects with breast cancer

No	Gene	Description	Accession number
1	*UFM1*	Ubiquitin-fold modifier 1	BC005193
2	*PTPLB*	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b	AF052159
3	*S100A10*	S100 calcium binding protein A10	M38591
4	*APRT*	Adenine phosphoribosyltransferase	Y00486
5	*CFL1*	Cofilin-1 (non-muscle)	X95404
6	*GSTP1*	Glutathione *S*-transferase pi 1	M24485
7	*HSPA5*	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	M19645
8	*GNB2L1*	Guanine nucleotide binding protein (G protein), β polypeptide 2 like 1	M24194
9	*TUBB*	Tubulin, β	BC001002
10	*MX1*	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	M33882
11	*COX7C*	Cytochrome *c* oxidase subunit VIIc	BC001005
12	*RPN2*	Ribophorin II	Y00282
13	*DYNLL1*	Dynein, light chain, LC8-type 1	U32944
14	*FXR1*	Fragile X mental retardation, autosomal homolog 1	U25165
15	*SQRDL*	Sulfide quinone reductase-like (yeast)	AF151802
16	*NDUFS3*	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30 kDa (NADH-coenzyme Q reductase)	AL135819
17	*EST*	ESTs	AL358933
18	*C19orf10*	Chromosome 19 open reading frame 10	BC003639
19	*ATP5E*	ATP synthase, H^+ transporting, mitochondrial F1 complex, ε subunit	AF052955
20	*PDCD5*	Programmed cell death 5	AF014955
21	*CLPTM1L*	CLPTM1-like	AL137440
22	*PPP1R14B*	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	X91195
23	*MRPL17*	Mitochondrial ribosomal protein L17	AK026857
24	*TUBA1B*	Tubulin, α1b	BC006481
25	*IFI6*	Interferon, α-inducible protein 6	X02492
26	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	AF261085
27	*SLC25A3*	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	BC006455
28	*MAD2L2*	MAD2 mitotic arrest deficient-like 2 (yeast)	AF157482
29	*CTNNB1*	Catenin (cadherin-associated protein), β1, 88 kDa	X87838
30	*CALR*	Calreticulin	M84739
31	*MRPS6*	Mitochondrial ribosomal protein S6	BC000547
32	*ANGPTL2*	Angiopoietin-like 2	AF007150
33	*RPL38*	Ribosomal protein L38	Z26876
34	*ANAPC7*	Anaphase promoting complex subunit 7	AY007104
35	*ENO1*	Enolase 1, (α)	BC004325
36	*ALDH2*	Aldehyde dehydrogenase 2 family (mitochondrial)	M20456

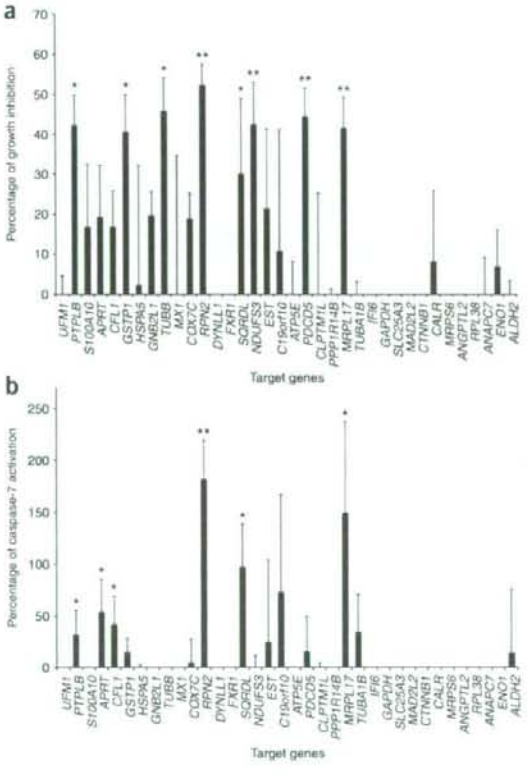

Figure 1 RNAi cell transfection array analysis in cultured breast cancer cells. **(a)** Inhibition of cell growth by 36 siRNAs on atelocollagen-based cell transfection arrays in the presence of docetaxel (1 nM) 72 h after transfection. The cell growth was estimated by luciferase activity in MCF7-ADR-Luc cells, which stably express luciferase ($n = 4$ per group; $^*P < 0.05$, $^{**}P < 0.01$). **(b)** Activation of caspase-7 by 36 siRNAs on atelocollagen-based cell transfection arrays in the presence of docetaxel (1 nM) 72 h after transfection. After the detection of luciferase activity, the same cell transfection arrays were assigned to the measurement of caspase-7 activity, which is elevated in apoptotic MCF7-ADR-Luc cells ($n = 4$ per group; $^*P < 0.05$, $^{**}P < 0.01$). Values are means ± s.d.

However, the expression of other genes was also found to correlate with docetaxel resistance by RNAi-based screening, and they could have some possible additive or synergistic effects with RPN2. Thus, we examined the induction of apoptosis after cotransfection of RPN2 siRNA and siRNAs against other genes that caused cell growth inhibition, apoptosis induction or both in docetaxel-resistant MC7-ADR cells. Knockdown of *PTPLB*, *APRT*, *CFL1*, *GSTP1*, *TUBB*, *SQRDL*, *NDUFS3*, *PDCD5* or *MRPL17* genes with simultaneous knockdown of *RPN2* did not significantly enhance caspase-7 activity relative to the knockdown of *RPN2* alone (**Supplementary Fig. 2b**). This result shows that knockdown of the other genes does not have an additive effect when used together with knockdown of RPN2.

We confirmed the efficacy of RPN2 siRNA for the knockdown of RPN2 messenger RNA by cell-direct real-time RT-PCR analysis. This analysis revealed that RPN2 siRNA inhibited 80% of the mRNA level relative to the control nontargeting siRNA (**Fig. 2d**). Immunofluorescence staining of the RPN2 protein revealed that the RPN2 protein localized in the cytoplasm and its expression was decreased by RPN2 siRNA (**Fig. 2e**). In further experiments, a liposome-mediated RPN2 siRNA transfection was performed. The western blot analysis showed a 45% reduction in RPN2 protein abundance (90% reduction of mRNA by real-time RT-PCR analysis) by RPN2 siRNA transfection in comparison with the control nontargeting siRNA (**Fig. 2f**). These results suggest that downregulation of RPN2 expression by siRNA inhibits cell growth and induces apoptosis in the presence of docetaxel.

In addition, we established stable clones expressing short hairpin RNA (shRNA) against RPN2 (shRNP2) and examined the effect on apoptosis induction in MCF7-ADR cells. The clone that expressed the lowest RPN2 mRNA level showed a 70% reduction of RPN2 expression relative to the control clone ($P < 0.001$, **Supplementary Fig. 2c**), and this shRPN2 clone showed a high caspase-7 activity as compared to the control clone in the presence of docetaxel ($P < 0.001$, **Supplementary Fig. 2d**). We examined seven independent clones and found that they all showed a similar phenotype of increasing drug sensitivity (data not shown). Therefore, consistent with our results with synthetic RPN2 siRNA, the data from the shRPN2 experiments provide evidence for the involvement of RPN2 in drug resistance.

To evaluate the effect of RPN2 siRNA on the drug response of MCF7-ADR cells, we measured the half-maximal inhibitory concentration (IC_{50}) for taxanes. The IC_{50} values for docetaxel in MCF7 and MCF7-ADR cells were 9.48 ± 1.48 nM and 40.22 ± 5.14 nM, respectively ($P < 0.001$). RPN2 siRNA–transfected MCF7-ADR cells were 3.5-fold more sensitive to docetaxel compared with nontargeting siRNA–transfected cells (IC_{50} of 11.47 ± 1.97 nM versus 39.48 ± 2.98 nM, $P < 0.001$). Thus, RPN2 silencing makes MCF7-ADR cells sensitive to docetaxel to a degree similar to that in drug-sensitive MCF7 cells. For paclitaxel, another taxane, the IC_{50} values in MCF7 and MCF7-ADR cells were 13.00 ± 2.02 nM and 89.74 ± 3.43 nM, respectively ($P < 0.001$). RPN2 siRNA–transfected MCF7-ADR cells were 2.6-fold more sensitive to paclitaxel compared with nontargeting siRNA–transfected cells (IC_{50} of 32.92 ± 3.89 nM versus 84.39 ± 5.48 nM, $P < 0.001$). These results indicate that RPN2 silencing bestows a hypersensitive response to taxanes to drug-resistant breast cancer cells.

In addition, we examined docetaxel-resistant EMT6/AR10.0 cells with high expression of the mouse *Rpn2* gene and the *Mdr1* (*Abcb1b*) and *Mdr3* (*Abcb1a*) genes, which reportedly have a similar role in drug resistance to that of the *MDR1* gene in humans, to see whether they have a similar phenotype to MCF7-ADR cells in terms of RPN2 expression status and drug resistance. The siRNA-mediated knockdown of mouse Rpn2 (70% reduction of mRNA by real-time RT-PCR analysis) significantly induced apoptosis of cells in the presence of docetaxel (**Supplementary Fig. 2e–g**). In contrast, nontargeting control siRNA showed no effect (**Supplementary Fig. 2e–g**). Therefore, these results suggest that RPN2 confers docetaxel resistance to both human and mouse cell lines.

Induction of RPN2 expression by docetaxel treatment

Real-time RT-PCR analysis showed that docetaxel-resistant MCF7-ADR cells expressed a slightly increased level of RPN2 mRNA (approximately 20%, $P < 0.01$) relative to parental MCF7 cells (**Fig. 3a**). However, RPN2 mRNA expression in parental MCF7 cells was markedly induced by docetaxel in a dose-dependent manner at

48 h after treatment (**Fig. 3b**). These data indicate that RPN2 mRNA induction may correlate with the observed antiapoptotic phenotype of MCF7-ADR cells.

Furthermore, MCF7-ADR cells expressed abundant MDR1 mRNA, which is a major cause of docetaxel resistance, whereas docetaxel-sensitive MCF7 cells did not (**Fig. 3c**). Additionally, MDR1 mRNA expression in MCF7 cells was strongly induced by docetaxel at 48 h after treatment (**Fig. 3d**). Together, these data provide a new insight into the development of docetaxel resistance in MCF7 cells: when breast cancer cells coordinately express a high amount the of *MDR1* and *RPN2* gene products, the cells become drug-resistant.

RPN2 expression associates with response to docetaxel

In this study, subjects with breast cancer with complete response and partial response were defined as responders, whereas subjects with no change and progressive disease were defined as nonresponders, in accordance with World Health Organization criteria[16] (**Supplementary Note** online).

Of the 44 subjects, 22 showed a pathologic response to docetaxel, and the other 22 showed no response[16]. To understand the clinical importance of the status of RPN2 expression in the subjects, we compared the expression level (signal log ratio) for RPN2 transcript between nonresponder and responder subjects by the Mann-Whitney *U*-test. The subjects with higher RPN2 expression showed a significantly lower response rate to docetaxel than did those with relatively low expression of RPN2 (signal log ratio expressed as mean ± s.e.m. in nonresponders was 0.347 ± 0.062 versus 0.111 ± 0.052 in responders; $P = 0.0052$). Thus, there is a significant association of RPN2 expression with the pathologic response to docetaxel. Although the data are not shown, RPN2 mRNA expression was significantly increased in cancerous tissues compared to that in normal tissues.

Furthermore, we also assessed validated sets of new samples from 26 subjects with breast tumors (12 responders and 14 nonresponders). The expression of RPN2 was higher in nonresponders than in responders (nonresponders, 0.240 ± 0.066, versus responders, 0.025 ± 0.194). Because of the small sample size in the validation set, we have not obtained conclusive results at this time. We are currently seeking larger samples that will be tested in the near future. However, when we combined studies with subjects in the learning and validation sets, RPN2 expression was significantly higher in nonresponders (34 subjects) than in responders (36 subjects) (nonresponders, 0.306 ± 0.046, versus responders, 0.080 ± 0.075; $P = 0.0219$).

Downregulation of RPN2 in orthotopic breast tumors

To extend our *in vitro* findings and to determine whether RPN2 could be an effective therapeutic target for docetaxel-resistant breast cancer, we examined the effect of RPN2 siRNA on an animal model of breast tumors by orthotopically implanting MCF7-ADR cells into mice and using an atelocollagen-mediated *in vivo* siRNA delivery[21,22]. We injected the RPN2 siRNA or nontargeting control siRNA (1 nmol per tumor) with 0.5% atelocollagen in a 200 µl volume into tumors that had reached 4–5 mm in diameter 7 d after inoculation of MCF7-ADR cells. At the time of siRNA administration, docetaxel was intraperitoneally (i.p.) injected into the mice. Subsequent tumor development was monitored by measuring the tumor size for a week. Mice that had been administered the RPN2 siRNA–atelocollagen complex and docetaxel (20 mg kg^{-1} i.p.) showed a significant decrease in tumor size (mean ± s.d.; day 0, 52 ± 8 mm^3; day 7, 21 ± 8 mm^3) relative to mice that had been administered the control nontargeting siRNA–atelocollagen (day 0, 37 ± 7 mm^3; day 7, 35 ± 12 mm^3; $P < 0.01$) (**Fig. 4a**). The tumor size was markedly reduced by administration of RPN2 siRNA with docetaxel at 7 d after treatment (**Fig. 4b**). In the absence of docetaxel, RPN2 siRNA treatment slightly reduced MCF7-ADR tumor size relative to controls; however, there were no statistically significant differences (**Supplementary Fig. 3a** online). We also observed that docetaxel alone had no significant effect on tumor growth (**Supplementary Fig. 3a**). Furthermore, no significant differences were observed in tumor growth between mice treated with control nontargeting siRNA and untreated mice in the presence or in the absence of docetaxel (data not shown). Thus, RPN2 siRNA is useful for reducing the size of orthotopic MCF7-ADR breast tumors in the presence of docetaxel. Additionally, to evaluate the effect of sustained treatment with siRNA, we treated mice with tumors twice by injection of siRNA-atelocollagen complex. RPN2 siRNA or nontargeting control siRNA (1 nmol per tumor) were injected into the tumors (diameter, 4 mm) at days 0

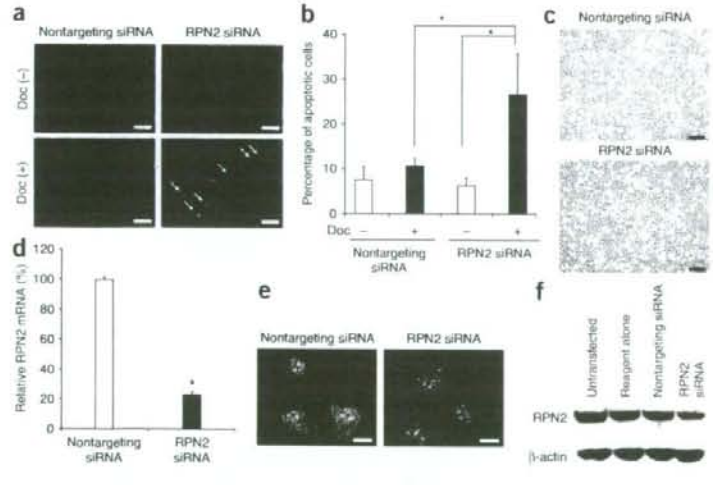

Figure 2 Apoptosis of MCF7-ADR cells transduced with RPN2 siRNA. (**a**) Hoechst staining of cells in the presence or absence of docetaxel (Doc, 1 nM) 72 h after the transfection of RPN2 siRNA. Scale bar, 50 µm. The arrows indicate cells with nuclear condensation and fragmentation. (**b**) Numbers of apoptotic cells from **a**. The data show the percentage of apoptotic cells in the presence or absence of docetaxel (1 nM) 72 h after the transfection of RPN2 siRNA. As a control, nontargeting control siRNA was used ($n = 4$ per group, $^*P < 0.02$). (**c**) Phase-contrast micrograph of MCF7-ADR cells 72 h after treatment with RPN2 siRNAs or control nontargeting siRNAs in the presence of docetaxel. Scale bar, 200 µm. (**d**) Knockdown of RPN2 mRNA by RPN2 siRNA in a cell transfection array, as monitored by cell-direct real-time RT-PCR analysis. As a control, nontargeting siRNA was used ($n = 4$ per group, $^*P < 0.001$). (**e**) Immunofluorescence staining of the RPN2 protein in MCF7-ADR cells 72 h after treatment with RPN2 siRNAs or control nontargeting siRNAs. Scale bar, 5 µm. (**f**) Western blot analysis of RPN2 protein in MCF7-ADR cells treated with RPN2 siRNAs or control nontargeting siRNAs 72 h after the liposome-mediated transfection. Values are means ± s.d.

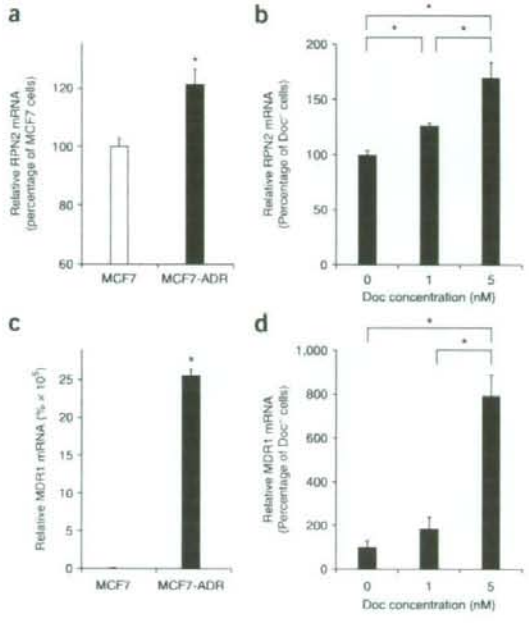

Figure 3 Induction of RPN2 and MDR1 expression by docetaxel treatment. RPN2 mRNA and MDR1 mRNA expression were analyzed by real-time RT-PCR. (**a**) RPN2 expression in drug-resistant MCF7-ADR cells and parental drug-sensitive MCF7 cells ($n = 3$ per group, $^*P < 0.01$). (**b**) Expression of RPN2 induced by docetaxel treatment in parental MCF7 cells. The data shown are from 48 h after the treatment ($n = 3$ per group, $^*P < 0.01$). (**c**) MDR1 expression in drug-resistant MCF7-ADR cells and parental drug-sensitive MCF7 cells ($n = 3$ per group, $^*P < 0.001$). The numbers on the *y* axis represent percentage ($\times 10^5$) of MCF7 cells. (**d**) Expression of MDR1 induced by docetaxel treatment in parental MCF7 cells. The data shown are from 48 h after the treatment ($n = 3$ per group, $^*P < 0.01$). Values are means ± s.d.

and 10. Simultaneously, docetaxel (20 mg kg^{-1} i.p.) was injected into the mice. We observed the mice for 20 d. Mice that had been given RPN2 siRNA and docetaxel showed significantly suppressed tumor growth relative to the mice that were administered control nontargeting siRNA at day 20 after the treatment ($P < 0.05$, **Supplementary Fig. 3b,c**). Mice showed no toxic effect during the observation period.

Furthermore, we examined the effect of RPN2 siRNA on a second animal model of breast tumors by orthotopically implanting MDA-MB-231/MDR1 cells. First, we established an MDA-MB-231/MDR1 cell line, which expresses the *MDR1* gene inducing docetaxel resistance. In this study, MDR1 expression is a key factor, because we are proposing that the coordinate expressions of RPN2 and P-glycoprotein may participate in the mechanism of docetaxel resistance. We injected the RPN2 siRNA or nontargeting control siRNA (2 nmol per tumor) with 0.5% atelocollagen in a 200 µl volume into tumors that were 5–6 mm in diameter 8 d after inoculation of MDA-MB-231/MDR1 cells. At the same time of siRNA administration, we injected docetaxel i.p. into the mice. Because docetaxel at a dose of 20 mg kg^{-1} in mice slightly suppressed MDA-MB-231/MDR1 tumor growth, we reduced the dose of docetaxel to 7 mg kg^{-1}, corresponding to the IC_{50} value of docetaxel in MDA-MB-231/MDR1 cells, which was 35% of that of MCF7-ADR cells. At a dose of 7 mg kg^{-1} docetaxel, mice treated with docetaxel alone showed no significant change in tumor growth. Subsequent tumor development was monitored by measuring the tumor size for a week. Mice that had been administered the RPN2 siRNA–atelocollagen complex and docetaxel (7 mg kg^{-1} i.p.) showed a significant inhibition of tumor growth (day 0, 61 ± 21 mm^3; day 7, 97 ± 24 mm^3) relative to mice that had been administered the control nontargeting siRNA–atelocollagen complex (day 0, 68 ± 9 mm^3; day 7, 154 ± 23 mm^3) (**Fig. 4c,d**). The value was statistically significant, with $P < 0.002$. Tumors treated with RPN2 siRNA in the absence of docetaxel showed no significant inhibition relative to control tumors that had been given nontargeting siRNA or docetaxel alone (data not shown). These results show that the growth of docetaxel-resistant MDA-MB-231/MDR1 tumors was suppressed by administration of RPN2 siRNA and docetaxel. Thus, RPN2 silencing is effective for the suppression of tumor growth in two models for docetaxel-resistant breast cancer in the presence of docetaxel.

RPN2 siRNA delivery augments docetaxel-induced apoptosis

MCF7-ADR tumors treated with RPN2 siRNA were investigated for apoptotic activity after docetaxel treatment for 3 d. TUNEL staining of tumor tissue treated with RPN2 siRNA revealed a significant number of apoptotic cells relative to the number in nontargeting control siRNA–treated tumors ($P < 0.01$, **Fig. 4e,f**). In contrast, RPN2 siRNA–transduced tumors in the absence of docetaxel showed no marked apoptotic cell death (**Fig. 4e,f**). We have also previously shown that atelocollagen alone does not induce any cytotoxic or inflammatory effect when it is injected into mice[23,24]. In a subsequent experiment, the mRNA levels of RPN2 in treated tumors were measured. RPN2 expression was significantly reduced in mouse tumors after combined treatment with RPN2 siRNA and docetaxel ($P < 0.05$, **Fig. 4g**). Furthermore, the RPN2 protein abundance in treated tumors was markedly downregulated by RPN2 siRNA (**Fig. 4h**). Thus, these results altogether indicate that RPN2 siRNA induces tumor inhibition via augmentation of docetaxel-induced apoptotic cell death *in vivo*.

To examine docetaxel retention in the tumors in the *in vivo* experiment, we performed drug disposition analysis. Eleven hours after docetaxel administration, we dissected the tumors and determined the amount of docetaxel incorporated into the tumors by HPLC with ultraviolet detection at 225 nm after solid-liquid extraction. We detected docetaxel in tumors that had received RPN2 siRNA ($n = 4$) at a range of 667 to 1400 ng per wet gram of tissue (**Fig. 4i**). In contrast, the tumors that received control siRNA ($n = 4$) showed a very low amount of docetaxel (~10 ng per wet gram of tissue). Thus, the results clearly indicate that abrogation of RPN2 expression in drug-resistant tumors results in docetaxel accumulation in those tumors.

RPN2 siRNA reduces *N*-linked glycosylation of MDR1

The mammalian *RPN2* gene encodes a type I integral membrane protein found only in the rough endoplasmic reticulum[25,26]. The RPN2 protein is part of an *N*-oligosaccharyl transferase complex that links high mannose oligosaccharides to asparagine residues found in the N-X-S/T consensus motif of nascent polypeptide chains[27]. The expression of the multidrug transporter P-glycoprotein, encoded by *MDR1*, is one of the causes of clinical drug resistance to taxanes. Real-time RT-PCR analysis showed that MCF7-ADR cells expressed abundant MDR1 mRNA, whereas parental cells did not (**Fig. 3c**). In addition, the MDR1 mRNA amount was not significantly decreased