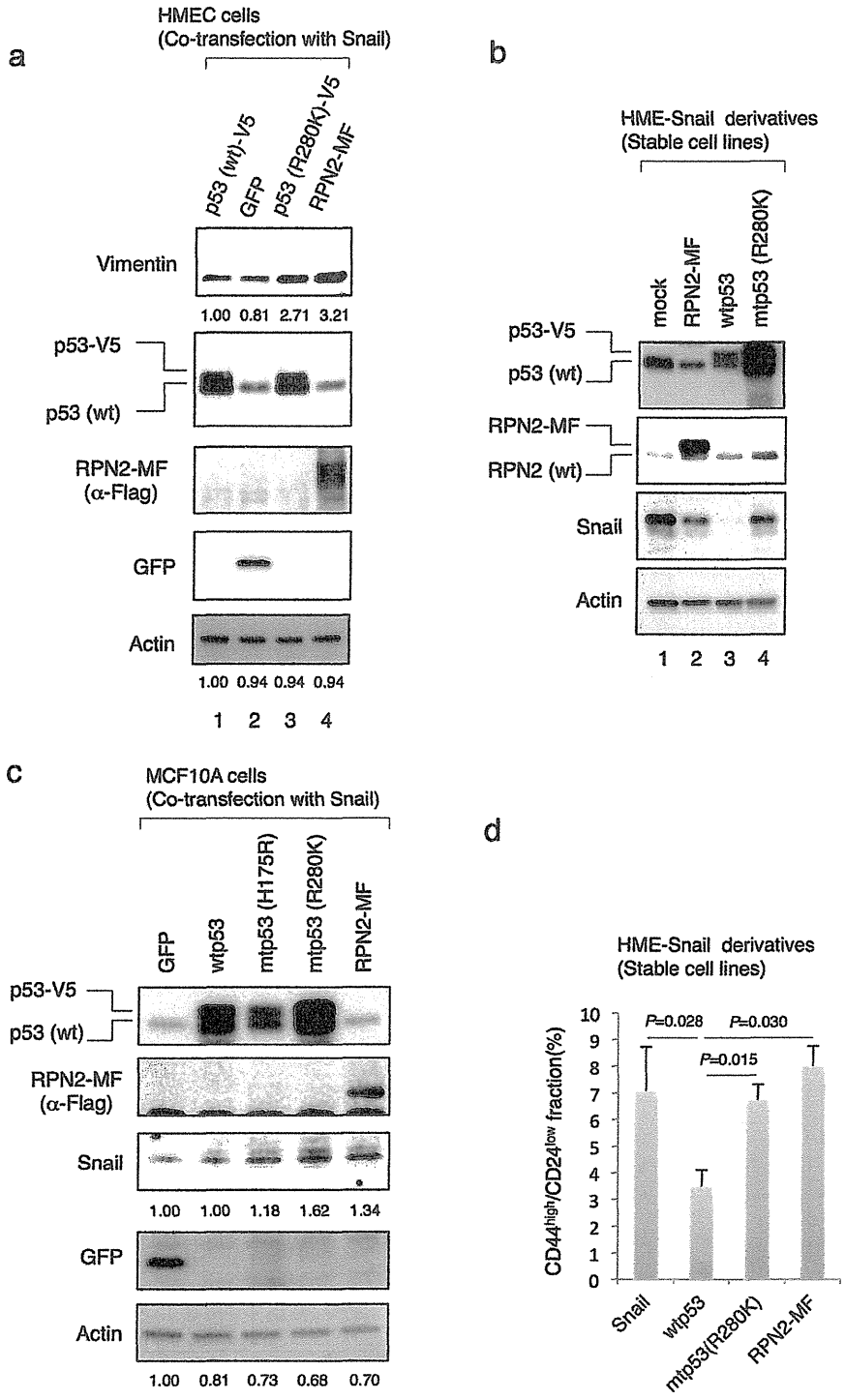


Figure 2 | RPN2 plays the important roles in the generation of CSC fraction in breast cancer cells. (a)–(c) Western blot analysis. Cell lysates were subjected to western blotting with anti-p53, anti-RPN2, anti-Snail, anti-Vimentin, anti-Flag, anti-GFP and anti-actin antibodies. (d) p53 status and RPN2 affected the population of CSCs in breast cancer cells. Flow cytometric analysis of CD44 and CD24 expression in HME-Snail cell line and its derivatives. Full-length gels and blots are shown in supplementary figure 10-12.

molecular mechanism by which E-cadherin negative fraction acquires drug resistance.

RPN2 knockdown promoted GSK3β−mediated inactivation of heat shock proteins. Recent studies show that different types of mtp53 form stable complexes with MDM2, CHIP, HSP90 and HSP70[25–27] and that HSP70 is transcriptionally regulated by heat shock transcription factor1 (HSF1)[28,29]. HSF1 transcriptional activity is negatively regulated by GSK3β[30], which suggests that RPN2 knockdown may promote GSK3β−induced downregulation of HSP70

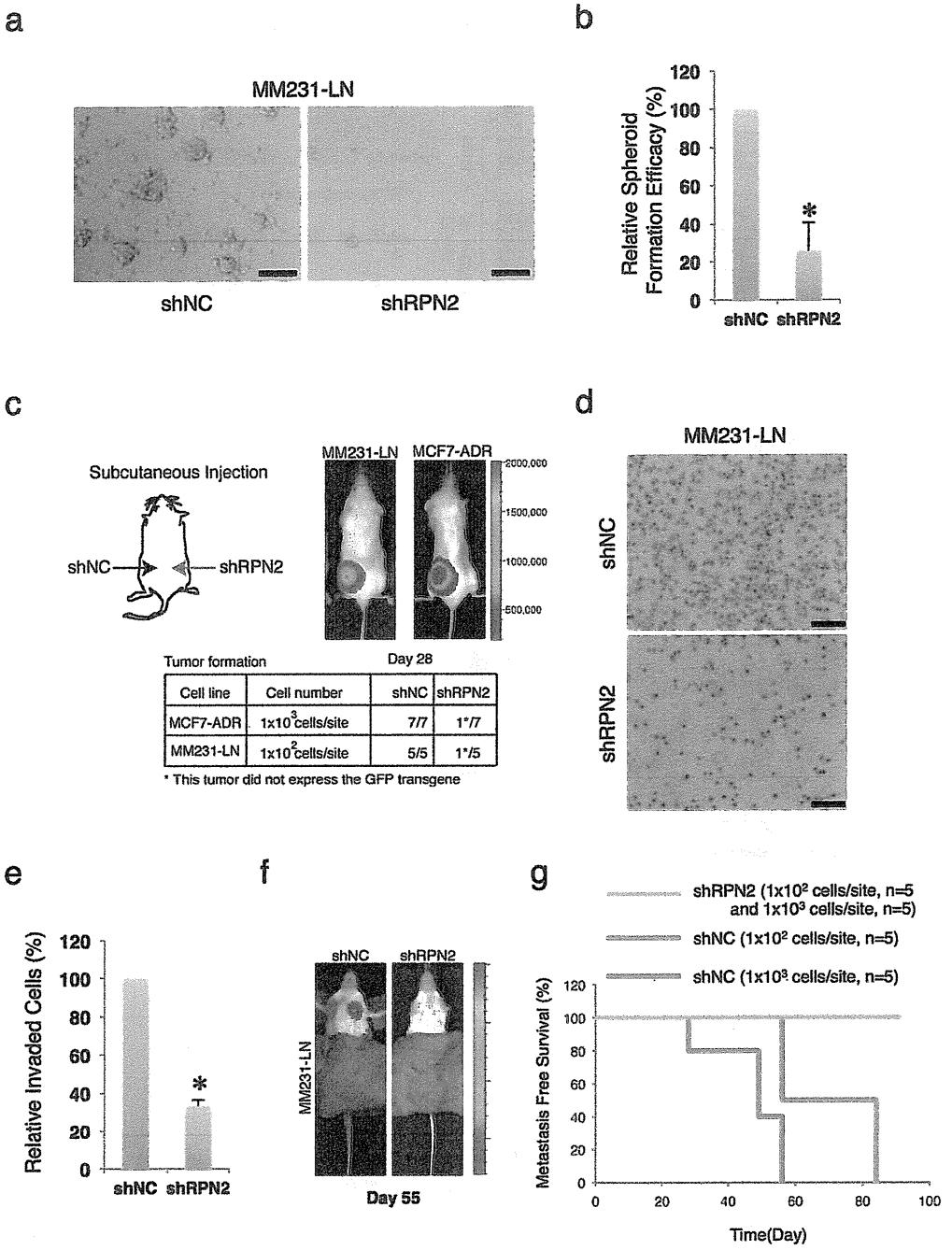


Figure 3 | RPN2 regulates the tumorigenicity and metastatic activity of CSCs. (a) Phase-contrast images of spheroids seeded by MM231-LN shNC (right) and MM231-LN shRPN2 (left) cells. Scale bar, 50 μm. (b) Quantification of spheroid formation in MM231-LN shNC and MM231-LN shRPN2 cells. The data in b) represent three independent experiments, and values are means ± s.d. (n = 3, $*P < 0.05$). (c) CSC ($CD44^{high}/CD24^{low}/GFP^{high}$) tumor formation in MM231-LN shNC (left) and MM231-LN shRPN2 (right) cells after subcutaneous injection. (d) and (e) Matrix invasion in MM231-LN shNC and MM231-LN shRPN2 cells (error bars = s.d., $n = 3$, $*P < 0.05$). (f) and (g) CSC ($CD44^{high}/CD24^{low}/GFP^{high}$) tumor metastasis in MM231-LN shNC and MM231-LN shRPN2 cells after mammary fat pad injection and monitoring of survival (shNC, $n = 5$; shRPN2, $n = 5$).

(Suppl. Fig. S5A). Immunoblot analysis revealed that RPN2 knockdown reduced the expression of HSP70 (Fig. 5a, lanes 1 and 6). We also confirmed the downregulation of HSP27 that is a transcriptional target of HSF1[31,32] and contributes the maintenance of breast CSCs through the regulation of EMT and NF-κB activity[33] (Fig. 5a, lane 1 and 6). Importantly, HSP70 protein also functions as co-chaperones for HSP90[9]. Therefore, we examined the effect of HSP70 downregulation on the destabilization of HSP90 by blocking protein synthesis with cycloheximide (CHX) treatment (Fig. 5a). While the effect on HSP70 protein stability was minimal, RPN2 knockdown reduced the stability and half-life of HSP90 (Fig. 5a and Suppl. Fig. S6). We also confirmed that the expression of N-terminal Flag-tagged HSF1 (Flag-HSF1) restored HSP27 expression in MM231-LN shRPN2 cells (Fig. 5b).

Next, we examined the HSF1 transcriptional activity by using the expression vector of HSP70 promoter-driven secreted cypridina luci-

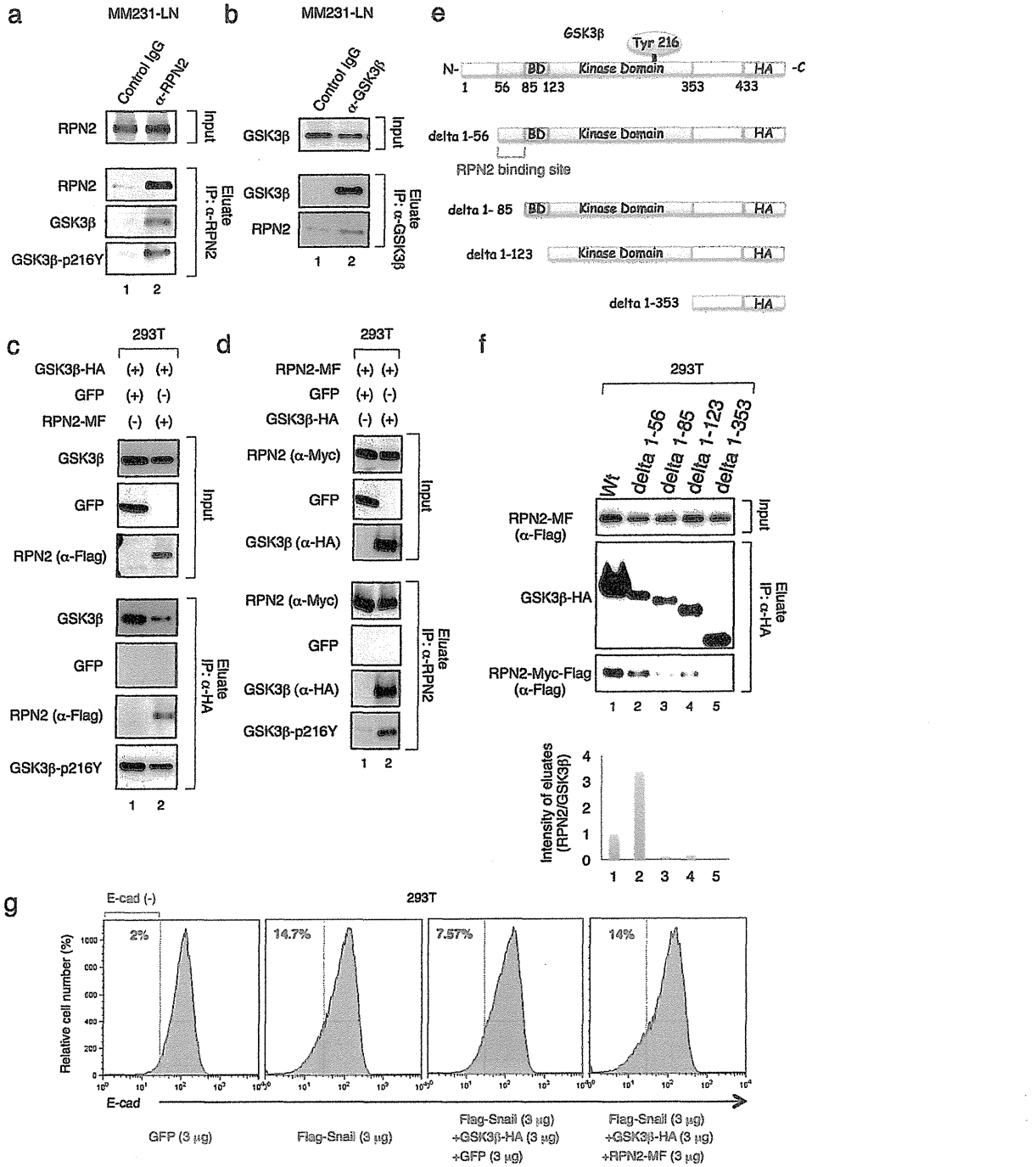


Figure 4 | RPN2 antagonizes GSK3βfunction via physical interaction. (a) and (b) RPN2 associated with GSK3β in MM231-LN cells. In the left panel, cell lysates were subjected to co-immunoprecipitation with an anti-RPN2 antibody and subjected to western blotting with anti-RPN2 (top), anti-GSK3β (middle) or anti-Phospho-GSK3β (Tyr216) antibodies (bottom). In the right panel, cell lysates were subjected to co-immunoprecipitation with an anti-GSK3β antibody and subjected to western blotting with anti-GSK3β (top and middle) and anti-RPN2 antibodies (bottom). (c) GSK3β associated with RPN2 in 293T cells. Cell lysates were subjected to co-immunoprecipitation with anti-HA Agarose and western blotting with anti-GSK3β (top), anti-GFP (middle), anti-RPN2 (bottom) or anti-Phospho-GSK3β (Tyr216) antibodies. (d) RPN2 associated with phosphorylated GSK3β in 293T cells. Cell lysates were subjected to co-immunoprecipitation with an anti-RPN2 antibody and western blotting with anti-RPN2 (top), anti-GFP (middle), anti-GSK3β (bottom) or anti-Phospho-GSK3β (Tyr216) antibodies. (e) Schematic representation of GSK3β and its deletion mutants. The binding domain (BD) designates the GSK3β-specific binding sites for its substrates and protein complexes. (f) RPN2-Myc-Flag, GSK3β-HA and the GSK3β deletion mutants were expressed in 293T cells and immunoprecipitated (IP) with an anti-HA antibody. Lysates (input) and immunoprecipitates were immunoblotted to detect co-immunoprecipitated RPN2. (g) Flow cytometry analysis and quantification of the percentage of E-cadherin-negative cells in 293T cells at 48 h after transfection. The amount of plasmid DNA used in each experiment was indicated in the figure. Full-length gels and blots are shown in supplementary figure 13 and 14.

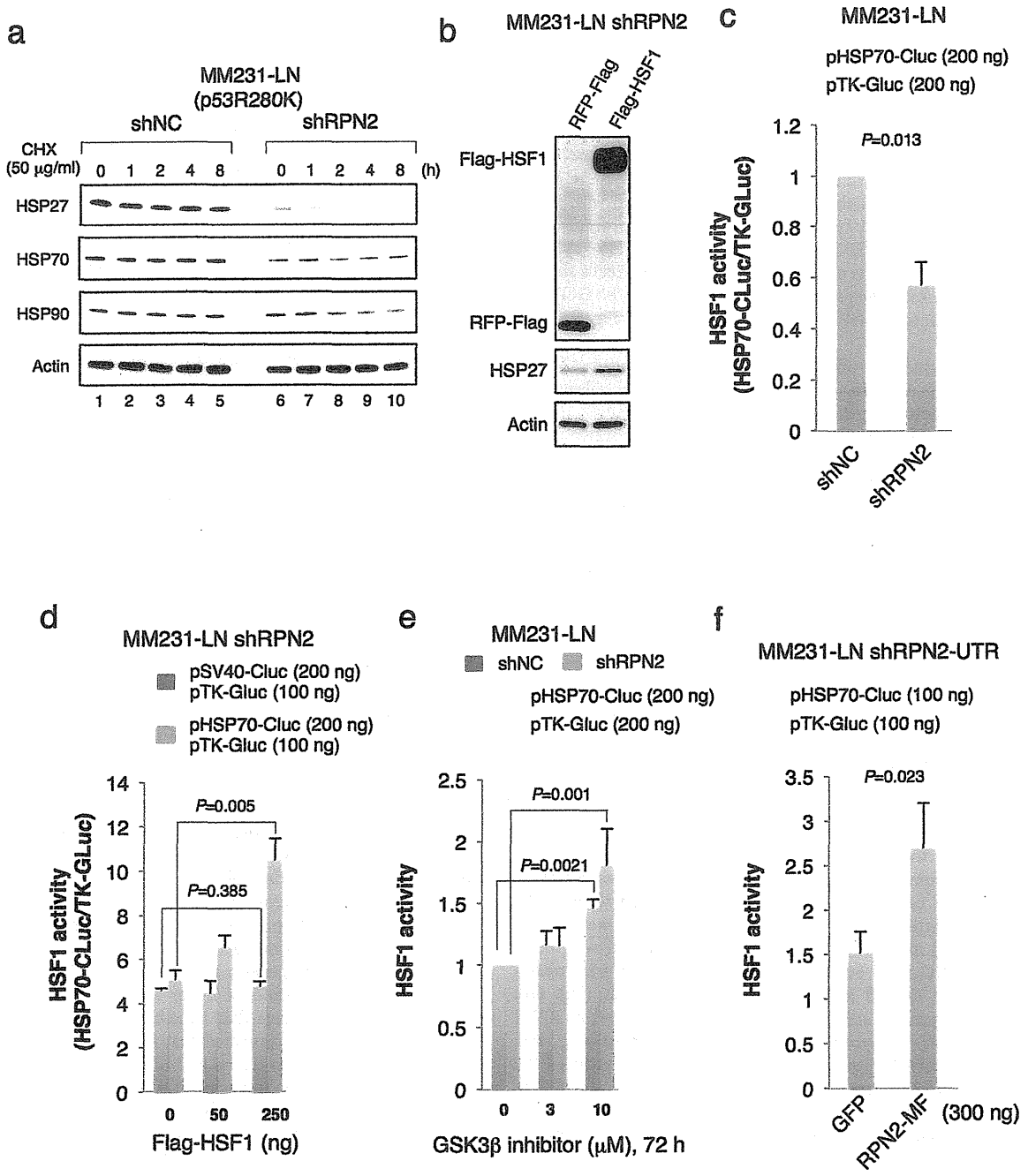


Figure 5 | RPN2 knockdown suppressed the heat shock proteins via GSK3β activation. (a) RPN2 knockdown suppressed the expression of HSP27 and HSP70 and reduced HSP90 protein stability. MM231-LN shNC and MM231-LN shRPN2 cells were treated with cycloheximide (50 μg/ml) for 0–8 h. Cell lysates were subjected to western blotting with anti-HSP27, anti-HSP70, anti-HSP90 and anti-actin antibodies. (b) HSF1 restored the HSP27 expression in MM231-LN shRPN2 cells. After transfection of pFlag-HSF1 into MM231-LN shRPN2 cells, Cell lysates were subjected to western blotting with anti-Flag (top), anti-HSP27 (middle), anti-actin (bottom) antibodies. (c) HSF1 transcriptional activity in RPN2 knockdown cell line. HSP70 promoter-driven cypridina luciferase activity (HSP70-CLuc) was normalized to the thymidine kinase promoter-driven gaussia luciferase activity (TK-GLuc). MM231-LN shNC or MM231-LN shRPN2 cells were transfected with 200 ng of pHSP70-Cluc in combination with 200 ng of pTK-Gluc. Transfection and luciferase assays were performed as described in experimental procedure. (d) HSF1 restored the HSP70 expression in MM231-LN shRPN2 cells. MM231-LN shRPN2 cells were transfected with pFlag-HSF1 with 200 ng of pHSP70-Cluc, or pSV40-Cluc in combination with 100 ng of pTK-Gluc, and luciferase activity was measured at 48 h after transfection. (e) GSK3β inhibitor restored HSF1 transcriptional activity. Six hours after transfection, cells were exposed to CHIR99021 (3, 10 μM) or an equivalent volume of dimethyl sulfoxide as a control added to the growth medium (CHIR99021, 0 μM). Treatments lasted for 72 h, after which the luciferase activity was examined. (f) Rescue experiment using MM231-LN shRPN2-UTR cells. RPN2-MF restored the HSP70 activity in MM231-LN shRPN2-UTR cells. pHSP70-Cluc was transfected into MM231-LN shRPN2-UTR cells with 300 ng of pCMV-RPN2-MF, or pEGFP-N1 in combination with 100 ng of pTK-Gluc, and luciferase activity was measured at 48 h after transfection. Full-length gels and blots are shown in supplementary figure 15.

ferase (HSP70-Cluc, Fig. 5c). Luciferase assays showed that RPN2 knockdown induced a 50% repression of HSF1 activity (Fig. 5c). When Flag-HSF1 was transfected with HSP70-Cluc in MM231-LN shRPN2 cells, luciferase activity was markedly enhanced 1.5- to 3-fold compared with those transfected with SV40 promoter-driven secreted cypridina luciferase (SV40-Cluc, Fig. 5d). We also confirmed that GSK3β inhibitor restored the HSF1 transcriptional activity in MM231-LN shRPN2 cells (Fig. 5e).

We also examined whether HSF1 activity correlated with RPN2 expression using MM231-LN cells expressing HSP70 promoter-driven GFP (MM231-LN HSP70-GFP) (Suppl. Fig. S5B). Flow cytometry and qRT-PCR analysis of MM231-LN HSP70-GFP cells showed that HSP70 and RPN2 were more highly expressed in the GFP high cell fraction than in the GFP low cell fraction (Suppl. Fig. S5C and 5D).

Finally, to exclude the off-target effects of shRNA against RPN2, we performed the rescue experiment using MM231-LN cells expressing shRNA against the 3′-untranslated region of RPN2 (Suppl. Fig.5E, MM231-LN shRPN2-UTR) to replace endogenous RPN2 with ectopically expressed RPN2-MF. Compared to the co-expression of shRPN2-UTR with GFP, co-expression of shRPN2-UTR with RPN2-MF restored the HSF1 activity in MM231-LN shRPN2-UTR cells (fig. 5f). These results strongly suggest that the formation of RPN2/GSK3β protein complexes inhibits the function of GSK3β, and that RPN2 knockdown induces the destabilization of mtp53 by GSK3β−mediated inactivation of heat shock proteins, leading to CSC phenotypic suppression.

RPN2 knockdown induces mtp53 degradation. Since RPN2 knockdown promoted GSK3β−mediated inactivation of heat shock proteins that are essential for mtp53 stabilization[6,25], we examined the expression and stability of mtp53 in MCF7-ADR and MM231-LN cells that express a p53 deletion mutant (p53 deletion 126–133)[34] and a p53 point mutant (p53R280K)[5], respectively (Fig. 6a and b). Immunoblot analysis with MCF7-ADR and MM231-LN cells showed that RPN2 knockdown induced mtp53 downregulation (Fig. 6b). As the lentivirus vector expressing shRPN2 also expressed the reporter gene GFP, mtp53 expression was examined in GFP-positive cells. Immunofluorescence staining with anti-p53 and anti-GFP antibodies revealed that mtp53 levels were considerably reduced in cells expressing shRPN2 compared with those expressing shNC (Fig. 6c). QRT-PCR revealed that *p53* mRNA levels were not altered in MM231-LN shRPN2 cells (Fig. 6d). Therefore, the protein stability of mtp53 was examined in RPN2 knockdown cells by cycloheximide (CHX) treatment (Fig. 6e). Immunoblot analysis showed that RPN2 knockdown reduced the half-life of mtp53 compared to the control (Fig. 6e and Suppl. Fig. S6). These findings were confirmed by RPN2 and mtp53 immunohistochemical staining in mouse MM231-LN tumors. Accumulation of mtp53 was observed in primary tumors with strong RPN2 expression but was reduced in primary tumors with low RPN2 expression (Fig. 6f). Immunohistochemical staining for RPN2 and mtp53 expression in breast cancer tissues yielded similar results to those obtained using tissues from subjects with a high incidence of lymph node metastasis (Fig. 6g and Suppl. Fig. S7).

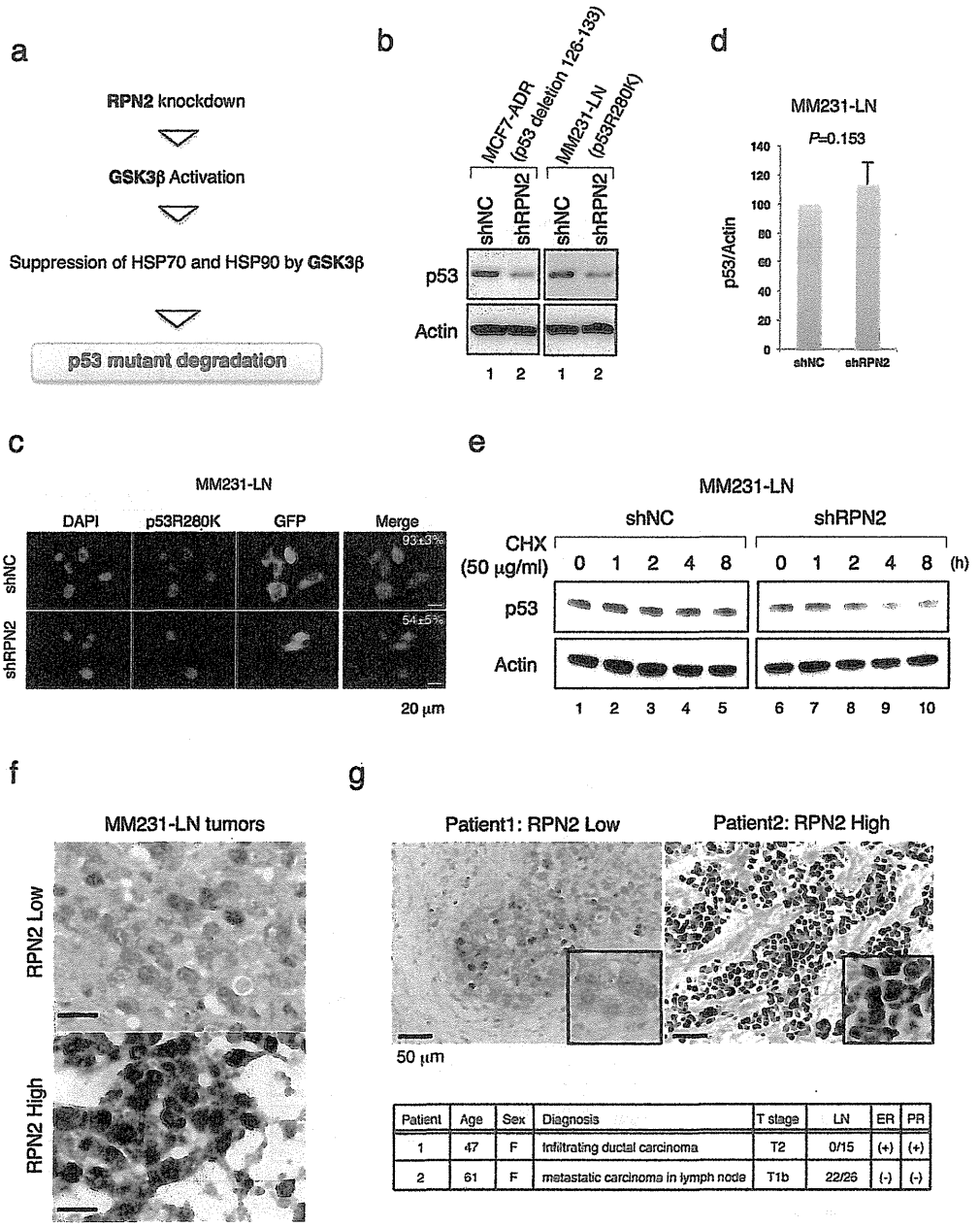
To further elucidate the role of RPN2 in tumor initiation and metastasis, we performed label-free proteomic analysis, two-dimensional image-converted analysis of liquid chromatography and mass spectrometry (2DICAL)[35], and identified proteins differentially expressed between MM231-LN shRPN2 and MM231-LN shNC cells (Fig. 7a–c and Suppl. Table S1). Although several proteins were differentially expressed, we focused on 14-3-3zeta upregulation, which has been implicated in breast cancer pathogenesis[36,37]. Despite the suppression of CSC phenotypes by RPN2 knockdown (Fig. 1–6), 14-3-3zeta was upregulated in RPN2 knockdown cells, which is a typical feature of the early stages of breast cancer wherein 14-3-3zeta upregulation promotes wtp53 degradation via phosphorylation of the MDM2 E3 ligase, and thus, cancer progression[36,37]. Although the peptide specificity was very low, 2DICAL also showed that RPN2 knockdown induced the downregulation of mtp53 (Suppl. Table S1). Consistent with previous reports[36,37], we confirmed that N-terminal Halo-tagged 14-3-3zeta (H-14-3-3zeta) induced downregulation of wtp53 in MCF7 cells (Fig. 7d). Determination of the effect of 14-3-3zeta upregulation on the mtp53 protein stability revealed that H-14-3-3zeta expression partially induced RPN2 expression in MM231-LN cells (Fig. 7e). We also observed that RPN2 overexpression induced mtp53 upregulation in MM231-LN cells (Fig. 7f) and that mtp53 knockdown strongly reduced the expression of 14-3-3zeta in MM231-LN cells (Fig. 7g). Moreover, 14-3-3zeta knockdown reduced the expression of RPN2 in MM231-LN cells (Fig. 7h and Suppl. Fig. S18). These results suggest that 14-3-3zeta-mediated RPN2 upregulation contributes to the stability and accumulation of mtp53 and that RPN2 knockdown inhibits the 14-3-3zeta-dependent feedback regulation of mtp53 protein stability (Suppl. Fig S8).

Discussion

LOF or mtp53 accumulation is associated with acquisition of the EMT phenotype and development of high-grade tumors in breast cancers[11]. The half-life of wtp53 is < 30 min, whereas mtp53 is more stable, with a half-life of several hours[34,38]. Although the molecular mechanism of mtp53 stabilization is not completely understood, recent studies demonstrated that the contribution of mtp53 to tumor progression and its promotion of EMT onset account for the development of CSC properties[11,14]. Several pathways such as Wnt/β-catenin, TGF-β, Notch and Hedgehog signaling are critical for the acquisition of CSC properties[20]. In the present study, we provide evidence that the expression of RPN2 is regulated by p53 mutant (R280K and del126-133) which is associated with the acquisition of EMT phenotype in breast cancer and that RPN2 regulates CSC phenotypes via stabilization of mtp53 (R280K and del126-133). We demonstrated that shRNA-mediated knockdown of RPN2 significantly suppresses the CSC phenotype *in vitro* and *in vivo* and promotes GSK3β-mediated destabilization of mtp53.

While GSK3β promotes ubiquitin-proteasome pathway-mediated β-catenin degradation[39] and suppresses Snail expression at transcriptional and post-transcriptional levels[15,16], it is unclear how GSK3β suppresses CSC tumorigenesis and metastasis. Our results demonstrate, for the first time, a novel role for GSK3β in mtp53 destabilization and indicate that its activity is regulated by RPN2 (Fig. 4–6). We also demonstrated that RPN2 antagonized GSK3β function via physical interaction, and identified N-terminal amino acids 56–85 of GSK3β as important for its interaction with RPN2 (Fig. 4). Our previous study showed that RPN2 affects docetaxel resistance by modulating the *N*-linked glycosylation of P-glycoprotein[19]. Ribophorin-1 (RPN1) is also a component of the oligosaccharide transferase (OST) complex[40,41]. However, a recent study showed that RPN1 regulates the cell surface localization of μ-opioid receptor (MOR) via direct interaction with MOR[42]. Therefore, our data suggest that, in addition to being a component of the OST complex, RPN2 may play an important role in tumor onset and metastasis.

HSP90 is a promising therapeutic target for cancer therapy because the HSP90 chaperone machinery is upregulated and activated in malignant cancers and inhibition of this single protein causes the simultaneous degradation of multiple oncoproteins[8,9]. The stabilization and accumulation of mtp53 is associated with the formation of stable protein complexes between HSP90 and various types of mtp53, and the formation of such complexes inhibits the constitutive E3 ligase activity of MDM2 and CHIP[6,25]. Therefore, the identification and development of HSP90 inhibitors has been a focus of several studies[8,43]. One of main problems associated with the inhibition of HSP90 is the HSF1-dependent induction of HSP70



Patient	Age	Sex	Diagnosis	T stage	LN	ER	PR
1	47	F	Infiltrating ductal carcinoma	T2	0/15	(+)	(+)
2	61	F	metastatic carcinoma in lymph node	T1b	22/26	(-)	(-)

Figure 6 | RPN2 knockdown induces mtp53 degradation. (a) Working model for GSK3β-mediated downregulation of mtp53 in RPN2 knockdown cells. (b) and (d) Expression of mutant p53 (mtp53) in MCF7-ADR and MM231-LN cells. (c) Expression of mtp53 in MM231-LN shNC and MM231-LN shRPN2 cells. Immunofluorescence staining of mtp53 (Red) and GFP (Green) and merged images are shown. Nuclei are shown in blue (DAPI). Scale bar, 20 μm. (e) RPN2 knockdown reduced mtp53 protein stability. MM231-LN shNC and MM231-LN shRPN2 cells were treated with cycloheximide (50 μg/ml) for 0–8 h. Cell lysates were subjected to western blotting with anti-p53 and anti-actin antibodies. (f) Expression of RPN2 and mtp53 in MM231-LN tumors in mice. Panels show representative immunohistochemistry results for RPN2 (Brown) and mtp53 (Blue) in MM231-LN tumors. Scale bar, 20 μm. (g) The status of RPN2 (Brown) and mtp53 (Blue) in breast cancer tissues. The tissue sections obtained from patients were classified by the extent of lymph node metastasis (LN). ER, Estrogen Receptor; PR, Progesterone Receptor. Scale bar, 50 μm. Full-length gels and blots are shown in supplementary figure 16.

and HSP27 in response to HSP90 inhibitor treatment[8,9]. Considering that HSP70 chaperones also act as co-chaperones for HSP90 and have a well-documented antiapoptotic function that is independent of their interaction with HSP90[9,44], our finding that RPN2 knockdown suppresses HSP70 expression (Fig. 6) is important not only for understanding the regulation of mtp53 stability, but also for developing ways to overcome the side effects of HSP90 inhibitors.

We also found that 14-3-3zeta involved in the stabilization and accumulation of mtp53 (Fig. 7 and Suppl. Fig S8). Since forced expression of 14-3-3zeta showed only a modest effect on RPN2 expression in MM231-LN (Fig. 7e), it might be necessary to consider the other molecules and pathways that regulate RPN2 expression. In a clinical study, the upregulation of *14-3-3zeta* via amplification of the chromosome region where it is located (8q22) promoted chemoresistance to anthracyclines and metastatic recurrence of breast

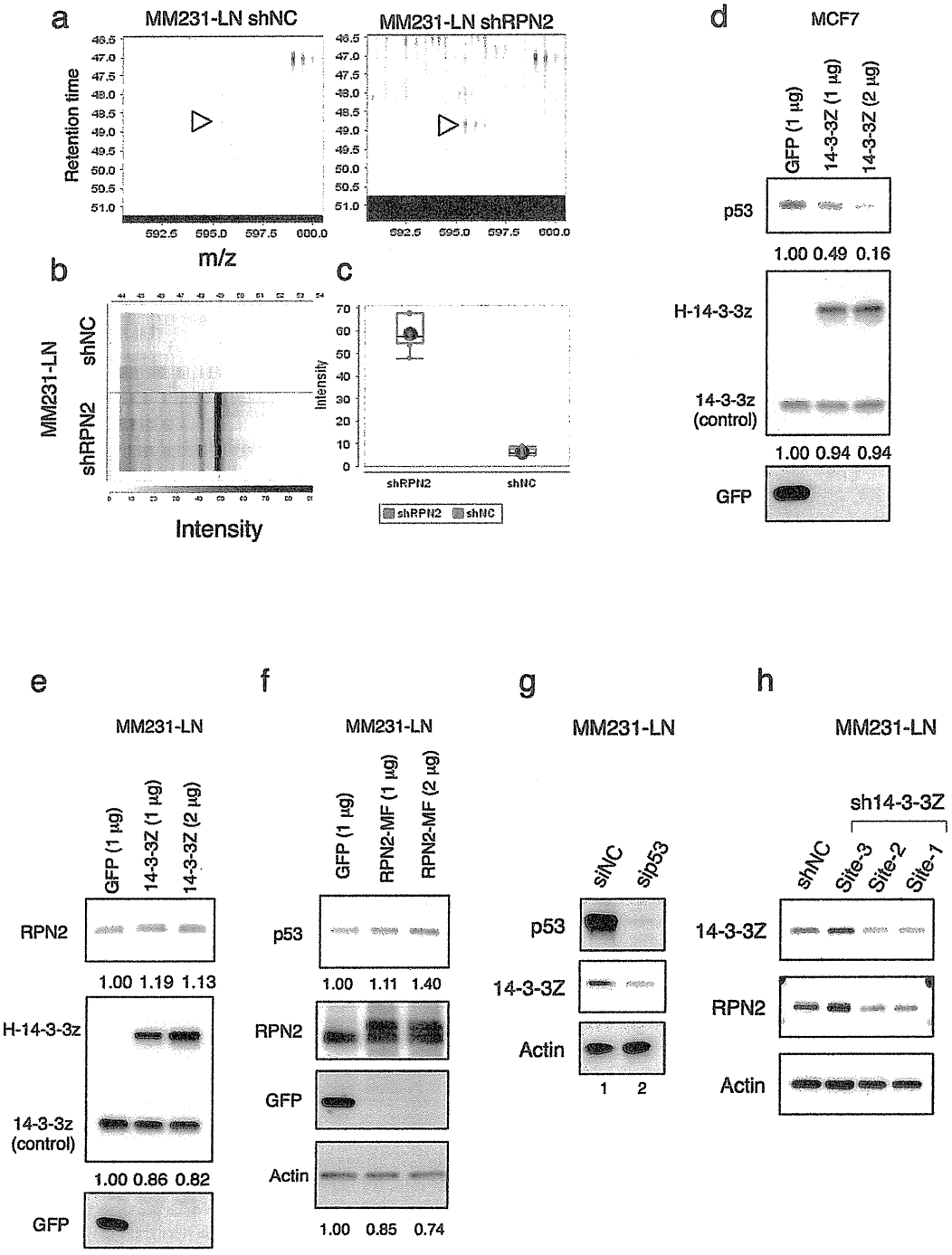


Figure 7 | 14-3-3zeta-mediated RPN2 upregulation is required for the mtp53 stability. (a) Detected peptides that differed between RPN2 knockdown cell line (MM231-LN shRPN2) and control (MM231-LN shNC). MS peak of 14-3-3Zeta (1433Z) in MM231-LN shNC (Right) and MM231-LN shRPN2 (Left) (indicated by arrows). (b) Gel-like views of MS peaks with retention time (RT) along the vertical axes (top) and distribution of the mean peak intensity of duplicates (bottom) across 6 samples. (c) Spot intensity of 14-3-3Z in MM231-LN shNC and MM231-LN shRPN2. (d) 14-3-3zeta reduced wtp53 expression. Lysates from MCF7 cells, transfected as indicated, were immunoblotted as shown. (e) and (f) RPN2 stabilized mtp53 in MM231-LN cells. Lysates from MM231-LN cells, transfected as indicated, were immunoblotted as shown. (g) and (h) 14-3-3zeta-dependent feedback regulation of mtp53 protein stability. Full-length gels and blots are shown in supplementary figure 17 and 18.

cancer[45]. However, the molecular mechanism underlying the chemoresistance and tumor recurrence has not been addressed. A recent study also showed that anthracyclines increase the amount of mtp53 mRNA and protein[46]. Therefore, the present study provides further insight into these mechanisms by showing that 14-3-3zeta promotes breast cancer malignancy.

TP53 mutations are present in 80% of triple-negative breast cancers (TNBC), which are characterized by the lack of estrogen and

progesterone receptors and the absence of human epidermal growth factor receptor 2[47]. Patients with TNBC show resistance to conventional chemotherapies and are in need of effective therapeutic agents to prevent recurrence and improve survival[48]. As shown in Figures 6g and S7, high expression of RPN2 and accumulation of mtp53 were observed in clinical samples of breast cancer tissues associated with lymph node metastasis. Moreover, our results are supported by a recent clinical study that showed that HSP70 expression correlates significantly with metastasis in TNBC patients[49]. Therefore, the concomitant expression of RPN2 and mtp53 could be a novel diagnostic marker for malignant progression and poor prognosis in TNBC. This is currently being investigated in a large cohort of TNBC patients at our NCC hospital and it would reveal that which types of p53 mutants are associated with RPN2 expression.

In summary, the present results indicate that inhibition of the RPN2/mtp53 regulatory network is a promising approach for overcoming the progression of malignant breast tumors and suppressing the CSC phenotype. Furthermore, our data suggest a novel mechanism for the modulation of nucleocytoplasmic proteins in cancer biology. Given the diverse biological roles of RPN2, further investigation aimed at understanding the role of RPN2 in processes associated with tumor progression is warranted.

Methods

Plasmids. N-terminal Flag-tagged Snail (pFlag-Snail), N-terminal Flag-tagged HSF1 (pFlag-HSF1), pBabe-puro-Snail, C-terminal V5-tagged wtp53 (pLenti6/V5-p53), C-terminal V5-tagged mtp53 (R280K, pLenti6/V5-p53R280K) and C-terminal HA-tagged GSK3β (pcDNA3-GSK3β-HA) were purchased from Addgene. To obtain C-terminal Myc- and Flag-tagged RPN2 constructs (pLenti-RPN2-Myc-Flag), cDNA encoding full-length human RPN2 (GenBank accession number Y00282) was amplified by polymerase chain reaction (PCR) using cDNA pools from MDA-MB231-D3H2-LN cells (MM231-LN). PCR fragments were inserted into EcoRI- and NotI-treated pLenti-C-Myc-DDK (Origene: PS100064). To obtain a HSP70 promoter-driven GFP (pHSP70-GFP), HSP70 promoter region was amplified by PCR using genomic DNA from MM231-LN cells (GenBank accession number M11717). The PCR fragment was inserted into BglII- and SalI-treated pEGFP-1 (Clontech). To obtain a HSP70 promoter-driven secreted cypridina luciferase (pHSP70-CLuc), HSP70-GFP was digested with KpnI and NotI and the fragment containing GFP was replaced with cypridina luciferase gene derived from pCLuc-Basic2 (NEB). All constructs were verified by DNA sequencing.

Cell culture. MCF7, MCF7-ADR, MCF7-ADR-Luc, MM231-LN, 293T, MCF10A and HME cells have been described previously[14,19]. To establish MM231-LN cells expressing HSP70-GFP, pHSP70-GFP containing the neomycin resistance gene was transfected with lipofectamine LTX (Invitrogen). The transfected cells were cultivated under selective growth medium including G418 (0.6 mg/ml). For spheroid culture, cells were plated on NanoCulture plates (Scivax) and cultured for 3 days.

Antibodies. The primary antibodies and dilutions were anti-RPN2 (1 : 2000, sc-166421, Santa Cruz Biotechnology), anti-p53 (1 : 2000, sc-126, Santa Cruz Biotechnology), anti-V5 (1 : 1000, V8137, Sigma), anti-Vimentin (1 : 2000, 550513, BD Pharmingen), anti-HA (1 : 2000, #3724, CST), anti-HSP27(1 : 2000, #2402, CST), anti-Myc (1 : 1000, 2278, CST), anti-Flag (1 : 1000, M185-7, MBL), anti-HA agarose (#3956S, CST), anti-HSP70 (1 : 1000, 610607, BD Transduction Laboratories), anti-HSP90 (1 : 1000, 610418, BD Transduction Laboratories), anti-GSK3β (1 : 1000, 610201, BD Transduction Laboratories), anti-Phospho-GSK3β (Tyr216) (1 : 1000, 612312, BD Transduction Laboratories), anti-N-cadherin (1 : 2000, #4061S, CST) and anti-actin antibodies (1 : 5000, MAB1501, Millipore). An anti-p53 antibody (Abcam, Pab240) was used for the detection of mtp53 by IHC. Staining was visualized using Alexa 488 or Alexa 594 (Molecular Probes). Immunofluorescence-stained cells were observed by fluorescence microscopy or confocal fluorescence microscopy (Leica). The signal intensity in immunoblot analysis was quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

Lentiviral shRNA transduction. Cell lines stably expressing *RPN2* shRNA, *14-3-3zeta* shRNA or control non-target shRNA were established using a vector-based shRNA technique. Human *RPN2* shRNA targets 5′-GGAGGAGATTGAGGACCT TGT-3′ (shRPN2-site1), 5′-GCCACTTTGAAGAACCCAATC-3′ (shRPN2-site2), 5′-TCCAGATTGTAGTTATACTTC-3′ (shRPN2-UTR), human *14-3-3zeta* shRNA targets 5′-GCAGAGAGCAAAGTCTTCTAT-3′ (sh14-3-3zeta-site1), 5′-GCAAT TACTGAGAGACAACTT-3′ (sh14-3-3zeta-site2), 5′-GCTCGAGAATACAGAG AGAAA-3′ (sh14-3-3zeta-site3), 5′-GAGAGGAATCTTCTCTCAGTT-3′ (sh14-3-3zeta-site4), 5′-CTCTGTGTTCTATTATGAGAT-3′ (sh14-3-3zeta-site5) and control shRNA targets 5′-GAAATGTACTGCGCGTGGAGAC-3′. Briefly, each fragment was subcloned into pGreenPuro (System Biosciences). Recombinant lentiviruses were produced according to the manufacturer's instructions. In knockdown experiments, MCF7-ADR and MM231-LN cells were infected with recombinant lentiviruses expressing control shRNA (shNC) or shRNA against RPN2 (shRPN2).

Matrigel invasion assay. The matrigel invasion assay was performed using the Matrigel Invasion Chamber (BD Bioscience) according to the manufacturer's protocol. In brief, 5×10^4 cells were plated in the upper chamber in serum-free media. The bottom chamber contained RPMI media with 10% FBS. After 24–48 h, the bottom of the chamber insert was fixed and stained with Diff-Quick stain. Cells on the stained membrane were counted under a dissecting microscope. Each membrane was divided into four quadrants and an average from all four quadrants was calculated. The matrigel invasion assays were performed with biological triplicates.

Dual luciferase assay. MM231-LN shNC or MM231-LNshRPN2 cells (2×10^4 cells) were seeded in a 24-well plate and cotransfected with HSF1 expression vector (pFlag-HSF1) in combination with reporter constructs of HSP70 promoter (pHSP70-Cluc), and SV40 promoter (pSV40-Cluc) using Lipofectamine 2000 (Invitrogen). The amount of plasmid DNA used in each experiment is indicated in the figure legends. A thymidine kinase promoter-driven secreted gaussia luciferase (pTK-Gluc, NEB) was mixed in a DNA–liposome complex as an internal control. Luciferase activity was quantified by a dual-luciferase assay system (NEB) and relative transactivation was calculated according to the manufacturer's instructions. All experiments were repeated at least 3 times.

Fluorescence-activated cell sorting. FITC or APC-conjugated anti-CD44 antibody (BD Bioscience, clone G44-26), PE-conjugated anti-CD24 antibody (Biolegend, clone LM5), APC-conjugated anti-E cadherin antibody (Biolegend, clone 67A4) and propidium iodide (5 μg/ml) were used for fluorescence-activated cell sorting (FACS) analysis using JSAN in accordance with the manufacturer's protocols. Data were processed by FlowJo software.

Immunoprecipitation. MM231-LN cells were lysed using immunoprecipitation buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM DTT, protease inhibitor cocktail, phosphatase inhibitor). After brief sonication, the lysates were cleared by centrifugation at 4°C. Supernatants were incubated with anti-RPN2 or anti- anti-GSK3β antibodies for 4 h and protein A/G-Sepharose beads (Invitrogen) for 4 h at 4°C. The immunocomplexes were washed four times, boiled in sample buffer and immunoblotted with anti-GSK3β, anti-Phospho-GSK3β (Tyr216) and anti-RPN2 antibodies.

Co-immunoprecipitation analysis. Extracts of 293T cells were obtained using immunoprecipitation buffer as described above. Supernatants were incubated with anti-HA Agarose (CST) under rotation for 4 h at 4°C. After washing four times with immunoprecipitation buffer, the immunoprecipitated protein complex bound to the beads was eluted with the HA peptide (Wako). The eluates from the immunoprecipitation and cell lysates were immunoblotted with anti-GSK3β, anti-Phospho-GSK3β (Tyr216) and anti-GFP antibodies.

Real-time reverse transcription PCR. Total RNA was isolated from cells and tumor tissues with an RNeasy Mini Kit and an RNase-Free DNase Set (Qiagen), and cDNA was produced with an ExScript RT reagent Kit (Takara). The cDNA samples were subjected to real-time PCR with SYBR Premix Ex Taq (Invitrogen) and specific primers (Supplementary Methods). All reactions were performed in a Light Cycler (Applied Biosystems). Gene expression levels were normalized to those of β-actin.

Bioluminescence imaging. Animal experiments were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prdkc^{scid}/J, CLEA Japan, Shizuoka, Japan) aged 4–6 weeks were anaesthetized by exposure to 3% isoflurane on day zero and subsequent days. Images were analyzed with Living Image software (Xenogen, part of Caliper Life Sciences). Bioluminescent flux (photonss^{-1} sr^{-1} cm^{-2}) was determined for the primary tumors, lungs or lymph nodes (upper abdomen, region of interest).

Mammary fat pad xenografts. MCF7-ADR or MM231-LN cells were suspended in a PBS/Matrigel (Sigma) mixture (1 : 1) and injected into the mammary fat pad in a 50 μl volume (n = 5 each and 10^2–10^6 cells per animal).

Tissue arrays. The tissue arrays of breast cancer samples were purchased from Super Bio CHIP. Immunohistochemical staining of RPN2 and mutant p53 was performed with DAB peroxidase and alkaline phosphatase substrate kits (Vector Laboratories).

Statistical analysis. Data are presented as mean ± standard error of the mean (s.e.m.) or mean ± standard deviation (s.d.). Statistical significance was determined by Student's two-tailed *t*-test unless otherwise noted. A P value < 0.05 was considered statistically significant.

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Author contributions

R.T. and K.H. designed the experiments and analysed the data. R.T. and F.T. performed the experiments. K.K. provided human breast cancer pathology information. M.O. performed proteome analysis by 2DICAL. R.T. and T.O. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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RNAi Therapeutics and Applications of MicroRNAs in Cancer Treatment

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RNA interference-based therapies are proving to be powerful tools for combating various diseases, including cancer. Scientists are researching the development of safe and efficient systems for the delivery of small RNA molecules, which are extremely fragile in serum, to target organs and cells in the human body. A dozen pre-clinical and clinical trials have been under way over the past few years involving biodegradable nanoparticles, lipids, chemical modification and conjugation. On the other hand, microRNAs, which control the balance of cellular biological processes, have been studied as attractive therapeutic targets in cancer treatment. In this review, we provide an overview of RNA interference-based therapeutics in clinical trials and discuss the latest technology for the systemic delivery of nucleic acid drugs. Furthermore, we focus on dysregulated microRNAs in human cancer, which have progressed in pre-clinical trials as therapeutic targets, and describe a wide range of strategies to control the expression levels of endogenous microRNAs. Further development of RNA interference technologies and progression of clinical trials will contribute to the achievement of practical applications of nucleic acid drugs.

Key words: RNA interference – microRNA – DDS – Cancer

INTRODUCTION

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants initiated by double-stranded RNA (dsRNA). It is the most significant recent contribution to the field of cell biology, and Fire and Mello who discovered it were awarded the Nobel Prize for Medicine in 2006 (1). The silencing technology to suppress the expression of pathologically or physiologically important genes by using small interfering RNA (siRNA) is applicable to many kinds of research or therapeutics for human diseases caused by specific genes, which are difficult to regulate through traditional approaches. Indeed, as the initial description of RNAi in animals, the development of RNAi-based therapies has provided a powerful new arsenal against various human diseases, such as age-related macular degeneration (AMD) (2,3), respiratory syncytial virus (RSV) infection (4), neurodegenerative disorders (5) and cancers (6–8).

On the other hand, in recent years, microRNAs (miRNAs) have been studied as regulators of gene expression in crucial biological processes, including cell development, differentiation, apoptosis and proliferation (9,10). miRNAs are non-coding small RNAs (~22 nt) which are processed from endogenously expressed transcripts and induce translational suppression and mRNA degradation in animals, plants and viruses (11,12). miRNAs are first transcribed as primary miRNA (pri-miRNA) transcripts by RNA polymerase II and then processed by Drosha in the nucleus to generate

~60–100 nt precursor-miRNA (pre-miRNA) with a hairpin-like structure. After pre-miRNAs are transported to the cytoplasm by Exportin-5, they are processed into mature miRNA duplexes by Dicer assembled with transactivating response RNA-binding protein and protein activator of PKR (13,14). Finally, one strand of the mature miRNA duplex, a guide strand, is incorporated into the Argonaut-containing RNA-induced silencing complex, which induces either cleavage or translational repression of targeted mRNAs based on their sequences (Fig. 1). Once the miRNAs are unbalanced or the functions are disordered, they can be involved in the initiation and development of fatal human ailments, including cancer (15). Indeed, many reports have shown that the widespread disruption of miRNAs was correlated with the initiation and progression of human cancer and demonstrated that an injection with synthetic RNA molecules mimics tumor suppressor miRNAs or the inhibitors of oncogenic miRNA (onco-miR) can switch dozens of cancer-related signals on or off (16). In other words, miRNAs are potential therapeutic tools for cancer treatment, representing a superior molecular target approach to the traditional low-molecular compound approach. However, for the realization of RNAi-based therapies using siRNAs, synthetic miRNAs and miRNA inhibitors, more continuous improvements will be required. For example, the technology to avoid unwanted innate immune responses, instability of nucleic acid *in vivo* and off-target side effects strikingly decreases the levels of potency and efficacy of RNAi effector molecules (17–19). Thus, the development of drug delivery systems (DDS) for RNAi therapeutic strategies that are safer, more stable and more effective is a paramount consideration.

Although clinical applications of RNAi-based therapies have not been fully realized, numerous pre-clinical studies in animal models of human disease are providing opportunities for practical use. In this review, we provide an overview of the current clinical and pre-clinical trials of RNAi therapies and discuss strategies toward a pathway of miRNA to practical applications for cancer therapy from the viewpoint of RNAi DDS.

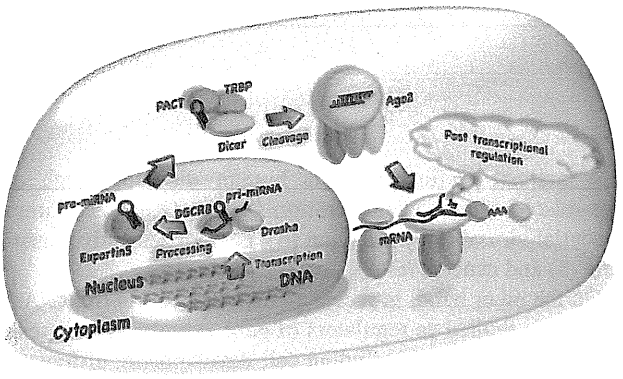

Figure 1. Cellular mechanisms of RNA interference pathway in mammals. First, primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and are cleaved by the enzyme Drosha into ~70 nucleotides as precursor-miRNAs (pre-miRNAs). Next, these pre-miRNAs are exported to the cytoplasm with Exportin-5 and are cleaved to double-strand RNAs that do not contain a loop by Dicer. These duplexes are then associated with Argonote2 (Ago2), and one strand is removed. This RNAi-induced silencing complex (RISC) containing the guide strand triggers post-transcriptional regulation of target mRNA depending on the seed sequence of miRNAs.

RNAi THERAPEUTICS DEVELOPMENT PIPELINE

In the development of RNAi technology for therapeutic medication, since the first demonstration of RNAi triggered by siRNA in mammalian cells in 2001 (20,21), some risk-taking biotechnology companies, such as Sirna Therapeutics, Silence Therapeutics and Tekmira, started to establish a platform to develop the new technology using primarily siRNA. At first, some pharmaceutical companies ascribed the RNAi to research for directed gene silencing; however, after the first exploration of *in vivo* gene knockdown (22), major pharmaceutical firms, such as Medtronic, Novartis and Merck, became involved in clinical applications. Observers were surprised by the acquisition by Merck and Roche of Sirna Therapeutics for more than one billion USD. RNAi was considered an exceptional technology for the knockdown of therapeutic target genes, and scientists anticipated that it would significantly shorten the drug development timeline. However, as a consequence of the global economic turmoil that began in 2008 and the slump in development of DDS for RNAi medicine, companies such as Merck, Pfizer, Abbott Labs and Roche were forced to curtail research in these fields. In particular, the fact that Roche halted its development of RNAi technology in 2010 was a shock throughout the industry. The Roche decision resulted in a loss of confidence in the company's ability to innovate, and their withdrawal from RNAi research was followed by other companies. However, the clinical pipeline of RNAi therapies using siRNA has been gradually growing since approximately 2011 as the RNAi technology has matured.

As shown in Table 1, there are many candidates for clinical development in 2012. In particular, there are a number of sites for topical or local administration, such as the skin, retina and airways, which permit safe and efficient delivery without unwanted side effects. For example, according to some recent animal experiments, transtympanic administration of siRNA targeting NOX3 is significantly useful for the attenuation of cisplatin ototoxicity (23). Furthermore, Paller *et al.* (24) at Northwestern University showed that spherical nucleic acid nanoparticle conjugates gold cores surrounded by immobilized siRNA directed against EGFR can be topically delivered more stably into mouse and human skin without undesirable toxicity. Thus, accessibility is a key requirement for successful RNAi *in vivo* to be delivered tissue or cell specifically. Since around 2008, however, the development pipeline has shifted from local to systemic delivery because more advanced delivery vehicles for systemic

Table 1. Current states of clinical candidate pipeline for RNAi therapy

ClinicalTrials.gov identifier	Drug	Route	Delivery	Disease	Target	Phase	States	Company
NCT00499590	Bevasiranib	IVT	Naked siRNA	Wet AMD	VEGF	III	Terminated	Opko Health
NCT00363714, NCT00395057	AGN211745/ Sirna–027	IVT	Naked siRNA	AMD	VEGF-R1	II	Terminated	Allergan/ Sirna
NCT01065935, NCT00658086	ALN-RSV01	Nebulization	Naked siRNA	RSV infection after lung transplantation	RSV Nucleocapsid	II	Completed	Alnylam
NCT00306904	Bevasiranib	IVT	Naked siRNA	DME	VEGF	II	Completed	Opko Health, Inc.
NCT01445899	PF-04523655	IVT	Naked siRNA	DME	RTP801	II	Recruiting	Quark Pharma
NCT01200420	miravirsen	SC	Naked LNA	HCV	miR-122	II	Completed	Santaris Pharma
NCT01551745 NCT01505166	FANG vaccine	*Ex vivo*, Intradermal	Electroporation	Ovarian cancer, colon cancer	Bi-shRNA-Furin and GM-CSF	II, II	Recruiting, Recruiting	Gradalis, Inc.
NCT00802347	I5NP	IV	Naked siRNA	DGF in kidney transplantation	P53	I/II	Recruiting	Quark Pharma
NCT01227291	SYL040012	Ophthalmic drops	Naked siRNA in ophthalmic drops	Glaucoma and ocular hypertension	Adrenergic receptor beta-2 siRNA	I/II	Completed	Sylentis
NCT00725686, NCT00713518	PF-04523655	IVT	Naked siRNA	Wet AMD	RTP801	I, II	Completed	Pfizer/Quark
NCT00716014	TD101	Intralesional	Naked siRNA	Pachyonychia congenita	Keratin 6a N171K mutant mRNA	Ib	Completed	TransDerm/ IPCC
NCT00882180, NCT01158079	ALN-VSP02	IV	SNALP	Liver cancer, solid tumors	KSP and VEGF	I, I	Completed	Alnylam
NCT00554359, NCT00683553	I5NP	IV	Naked siRNA	AKI for major cardiovascular surgery	P53	I, I	Completed, terminated	Quark Pharma
NCT01148953	ALN-TTR01	IV	SNALP	TTR-mediated amyloidosis	Transthyretin	I	Completed	Alnylam
NCT00689065	CALAA-01	IV	RONDEL	Solid cancer	RRM2	I	Active	Calando Pharma
NCT00466583	EZN-2968	IV	Naked LNA	Advanced solid tumor, lymphoma	HIF-1a	I	Completed	Santaris Pharma
NCT01120288	EZN-2968	IV	Naked LNA	Liver metastases	HIF-1a	I	Recruiting	NCI
NCT00672542	siRNA in dendritic cells	*Ex vivo*, Intradermal	Electroporation	Metastatic melanoma	Immunoproteasome subunits LMP2, LMP7, MECL1	I	Active	Duke University
NCT01061840	FANG vaccine	*Ex vivo*, Intradermal	Electroporation	Solid tumors	Bi-shRNA-Furin and GM-CSF	I	Recruiting	Gradalis, Inc.
NCT01064505	QPI-1007	IVT	Naked siRNA	Optic atrophy	Caspase-2	I	Active	Quark Pharma
NCT00938574	Atu027	IV	AtuPLEX	Advanced solid cancer	PKN3	I	Completed	Silence Therapeutics
NCT01188785	siG12D LODER	EUS biopsy needle	LODER polymer	Pancreatic ductal adenocarcinoma	KRASG12D	I	Recruiting	Silenseed Ltd
NCT01262235	TKM-080301	IV	SNALP	Cancer	PLK1	I	Recruiting	Tekmira
NCT00927459	PRO-040201	IV	SNALP	Hypercholesterolemia	Apo B	I	Terminated	Tekmira

AKI, acute kidney injury; AMD, age-related macular edema; DGF, delayed graft function; DME, diabetic molecular edema; HCV, Hepatitis C Virus; IV, intravenous; IVT, intravitreal; KSP, Kinesin Spindle Protein; LNA, locked nucleic acids; NCI, National Cancer Institute; PEG, polyethylene glycol; PLK1, Polo-like Kinase I; RRM2, Ribonucleotide Reductase M2; RSV, respiratory syncytial virus; SNALP, stable nucleic acid lipid particle; TF, transferrin; TTR, transthyretin; VEGF, vascular endothelial growth factor; SC, subcutaneous.
*From ClinicalTrials.gov.

application, such as stable nucleic acid lipid particles (SNALPs) and RNAi/oligonucleotide nanoparticle delivery (RONDEL), are available. These technologies have been shown to be effective *in vivo* (25–27), and progress is being achieved in some clinical trials (ALN-VSP02, ALN-TTR01, CALAA-01, TKM-080301, PRO-040201). In cancer treatment, siRNAs targeting polo-like kinase I (PLK1), kinesin spindle protein (KSP) and vascular endothelial growth factor, which are formulated with SNALP or RONDEL, have been developed as candidate pipelines in Phase I (Table 1).

DRUG DELIVERY SYSTEM FOR SYNTHETIC OLIGONUCLEOTIDE

Nucleic acid medicines, including siRNA, miRNA and anti-miRNA, work only after they penetrate hydrophobic cellular membranes. However, it is not easy for them to go through the lipid bilayer membrane without their carrier because synthetic oligonucleotides are negatively charged. In addition to this, RNAs are very easily degraded by RNase *in vivo*. Accordingly, assisting carriers or chemical modifications for the progression of the transmembrane transport and for the inhibition of the degradation by serum RNases are required. Historically, viral and non-viral delivery has been utilized (Table 2). In a viral delivery system, it was reported that an adenovirus carried short hairpin RNA (shRNA) expression vector targeting angiotensin type 1 (AT1) delivered into the brain intracerebroventricularly (ICV) (28) and that the miR-23b expression vector and miR-23b sponge worked in inflammatory autoimmune diseases *via* intra-articular (IA) infection (29). An adeno-associated virus (AAV) was also successful at carrying a miRNA cluster into the muscle and shRNA vectors targeting mutant huntingtin into the brain by topical administration (30,31). Furthermore, miR-34a treatment prevented lung cancer initiation and progression *via* transtracheal infection, and shRNA targeting superoxide dismutase 1 (SOD1) inhibited amyotrophic lateral sclerosis progression by lentiviral-mediated RNAi (32,33). The herpes simplex virus, which commonly causes an eruption of fluid-containing vesicles on the mouth, lips or face, also has potential for cancer treatment and therapeutic pain relief (34,35). Thus, viral-mediated gene silencing is very useful for local infection, particularly at sites that make frequent administration difficult. Although viral delivery has frequently shown higher efficiency than that by non-viral systems, preliminary clinical studies have shown that it triggered strong inflammatory reactions (36), and these delivery vectors have caused the death of several patients in the clinic (37,38). Thus, understanding the details of the inflammatory mechanism and developing safer viral vehicles are important tasks ahead.

On the other hand, the focus has recently been on the non-viral approach because of its advantages over viral vectors, such as non-immunogenicity, low production cost and easy quality control. This approach requires an optimized delivery reagent, such as a cationic lipid, polymers, nanoparticles, carbon nanotubes and atelocollagen (Table 2). In cancer treatment, atelocollagen or cationic liposome- or polymer-mediated transfection reagents have commonly been used to deliver siRNA or miRNA to cells *in vitro* and *in vivo*. In particular, a number of reports have demonstrated a significant anti-cancer effect caused by systemic delivery of siRNA with cationic liposome (39–41). Similarly, a cationic polymer, polyethyleneimine, was commercialized as *in vivo*-jetPEI™, provided by Life Technologies, and was used to successfully deliver siRNAs to cancer cells in animals (42,43). In addition, atelocollagen can be obtained from type I collagen of calf dermis and has also been expected to be a useful carrier because of its low immunogenicity and efficiency (8,44–46). In case of miRNA therapy, a tumor-suppressive miR-16 mimic was successfully delivered by the systemic approach using atelocollagen, and it dramatically inhibited the growth of metastatic prostate cancer (47). Furthermore, chemically functionalized carbon nanotubes also show potential for novel biological applications for the delivery of Caspase-3 siRNA into the brain by topical injection into the cerebral cortex and reduced neurodegeneration without toxic side effects (48).

In a recent study, the focus was on highly stabilized nanoparticles, and these nanoparticles made the systemic delivery system dramatically more efficient (25,49–52). For example, synthetic miR-34a mimic, which was incorporated into cholesterol, and the cationic liposome *N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethyl ammonium propane (DOTAP) (1:1 mol/mol) and polyethylene glycol (PEG)-conjugated CG4-targeting single-chain antibody fragment were efficiently delivered into melanoma and inhibited lung metastasis (53). The nanocarrier 'SNALP' by Tekmira pharmaceuticals is one of the technologies with the most potential in the clinical pipeline. SNALP is a PEG-grafted monolamellar liposome that can easily avoid opsonization and subsequent recognition by the macrophages because the hydrophilic nature of PEG constructs an aqueous coating on its particle surface (54). In the work of Judge *et al.*, SNALP-formulated siRNAs against PLK1 and KSP displayed significant anti-tumor effects in liver tumor model mice (26). Successful results have already been reported in the treatment of transthyretin-mediated amyloidosis, hypercholesterolemia, Ebola virus infection (49) and cancer (50). The clinical trials have been identified as NCT00882180, NCT01158079, NCT01148953, NCT01262235 and NCT00927459 in the ClinicalTrials.gov database (http://clinicaltrials.gov).

The effective systemic delivery of siRNA or miRNA toward target cells or tissues has been enormous challenge for RNAi therapy. Indeed, naked siRNAs are rapidly eliminated by the kidneys, and nanoparticle-formulated siRNAs have a tendency to accumulate in the liver. In particular, their suitability for cancer cells depends on the enhanced permeability and retention effect of nanoparticles. To solve these problems, combined use with orienting molecules, such as a cell-specific ligand, can increase the cell or tumor

Table 2. Technologies for drug delivery systems in RNAi therapy

Delivery	Tissue	Route	RNA	References
Viral vector				
Adenovirus	Articulation	IA	miR-23b	(29)
	Brain	ICV	AT1a, AT1b shRNA	(28)
Adeno-associated virus	Muscles	IM	Anti-VEGF miRNA cluster	(30)
	Brain	Intrastriatal	mHTT shRNA	(31)
Lentivirus	Lung	Transtracheal	miR-34a	(32)
	Spinal cord	Intraspinal	SOD1 shRNA	(33)
Herpes simplex virus	Dorsal root ganglia	Injection into the sciatic nerve	Trpv1 shRNA	(34)
	Glioma	IT	EGFR shRNA	(35)
Non-viral reagent				
Liposome				
Oligofectamine	Colon cancer	IP/IV	B-catenin siRNA	(40)
DOTAP	Liver, spleen	IV	GFP siRNA	(39)
LIC-101	Liver metastasis	IV/SC	BCL-2 siRNA	(41)
PEI	Ovarian canner	IP/SC	HER-2 siRNA	(42)
	Glioblastoma	IP/SC	PTN siRNA	(43)
Nanoparticle				
SNALP	Ebola virus	IP/SC	ZEBOV siRNA	(49)
	Lung cancer	IV	miR-34a/let-7	(50)
RONDEL	Melanoma	IV	RRM2 siRNA	(25)
	Ewing's sarcoma	IV	EWS-FLI 1 siRNA	(51)
AtuPLEX	Prostate/pancreatic cancer	IV	PKN3 siRNA	(52)
DOTAP, cholesterol and PEG	Melanoma	IV	c-Myc/MDM2/VEGF siRNA and miR-34a	(53)
Atelocollagen	Testicular cancer	IT	HST-1/FGF-4 siRNA	(45)
	Osteosarcoma	IV	miR-143	(112)
	Prostate cancer		miR-16	(47)
HDI	Liver	IV	HBV siRNA	(113)
Carbon nanotube	Brain	Into the cerebral cortex	Caspase-3	(48)

ApoB, Apolipoprotein B; AT1, Angiotensin type 1; DDAB, dimethyldioctadecylammonium bromide; DOTAP, (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl-ammonium methylsulfate; HBV, Hepatitis B Virus; HDI, hydrodynamic tail vein injection; HER-2, human epidermal growth factor receptor 2; IA, intra-articular, ICV, intracerebroventricular; IM, intramuscular; IP, intraperitoneal; IT, intratumor; IV, intravenous; mHTT, mutant huntingtin; PBAVE, poly butyl and amino vinyl ethers; PEI, polyethyleneimine; PKN3, Protein Kinase N3; PPARA, peroxisome proliferator-activated receptor alpha; PTN, pleiotrophin; SC, subcutaneously; SLN, solid lipid nanoparticle; SOD1, superoxide dismutase 1; ZEBOV, The Polymerase (L) Gene of the Zaire Species of Ebola Virus.

specificity and delivery efficiency (55–57). Calando's cyclodextrinpolymer-based delivery platform (RONDEL) consists of cyclodextrin-containing polycation, and adamantine-coupled PEG-stabilized some ligands, such as transferrin (TF), and siRNA or miRNA (Fig. 2). This siRNA delivery platform was conceived by Hu-Lieskovan *et al.* in 2005 (51). The TF receptors are known to be upregulated in malignant cells, and TF-stabilized particles are taken up into cancer cells by TF receptor-mediated endocytosis and subsequent release into the cytoplasm in a pH-dependent manner (25). Phase 1b clinical trials of CALAA-01, including the M2 subunit of ribonucleotide reductase (RRM2) targeting siRNA, are being conducted as a novel RNAi therapy for multiple types of solid tumors.

CHEMICAL MODIFICATIONS FOR OLIGONCLEOTIDES

In addition to the nanocarriers mentioned above, others are being sought through chemical modifications. The purpose of such modifications can be permeability into the cells, specificity for specific tissues and stability against nuclease degradation (Fig. 3 and Table 3). For example, as a

permeability-enhancing factor, the covalent conjugation of the lipophilic molecule assists siRNA or miRNA to penetrate into the cellular cytoplasm and trigger gene silencing *in vivo* (58). In particular, high-density lipoprotein (HDL)-conjugated siRNAs are selectivity taken up by the gut, kidney and steroidogenic organs *via* the HDL receptor, scavenger receptor class B, type I (SR-BI) (59–62). In contrast, low-density lipoprotein (LDL)-conjugated siRNAs are efficiently internalized into the hepatocytes after binding to the LDL receptor (59). The Arrowhead Research Corporation demonstrated that the co-injection of cholesterol-siRNA and hepatocyte-targeted endosomolytic polymer achieved high-level target gene knockdown with low doses of cholesterol-siRNA in non-human primates (63). The company is using this strategy and a polymer-based siRNA delivery platform named dynamic polyconjugate polymer in ARC-520, which is a hepatitis B clinical candidate.

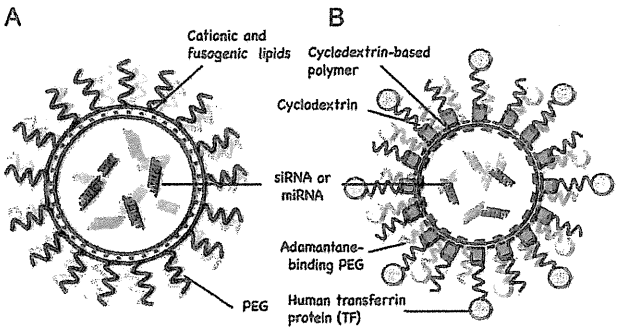

Figure 2. Delivery technology for RNAi therapy. (A) Stable nucleic acid lipid particle (SNALP). The bilayer consists of cationic and neutral lipids and is coated by PEG. The diameter is ~100 nm. (B) RNAi/oligonucleotide nanoparticle delivery (RONDEL). RNAs are protected from degradation in serum by the cyclodextrin-conjugated polymer. The complexes are <100 nm in diameter. In aqueous solution, adamantane easily binds to cyclodextrin as a result of hydrophobic attraction.

In another example, nanoparticles composed of poly (lactic-*co*-glycolic acid) were modified with a cell-penetrating peptide, penetratin, and used for the systemic delivery of the miR-155 inhibitor in the mouse model of lymphoma (64).

On the other hand, cell-specific factors, such as aptamers (65,66), peptide (64,67), antibodies (68,69) and agonists (56), can enhance cell specificity in cases of systemic administration into experimental animals. For example, octaarginine-modified liposomal particles were used to suppress an endogenous gene in the liver at low concentrations of siRNA without any toxicity (67). Usually, targeting proteins were conjugated to cationic bridges, such as polylysine or protamine, which can mediate uptake of nucleic acids, to link targeting proteins to effector oligonucleotide (68,70–72). In contrast, the siRNA-aptamer chimeras have also been of interest because a completely RNA-based approach may have important advantages over other methods for targeted delivery of siRNAs in terms of cost, productivity, safety and flexibility regarding chemical modification. RNA aptamers are single-stranded oligonucleotides and bind with high affinity to specific molecular targets, such as small molecules, proteins and nucleic acids, with their 3D structure (65,66). Here, although antibody-mediated siRNA delivery is required for the biological production of antibodies and antibody-siRNA conjugations by using a linker such as PEG, chimeric aptamer-siRNA can be synthesized as a single unit at once. However, for the utilization of chimeric aptamer-siRNA, more structured RNAs capable of binding with higher affinity and specificity have been required.

Stabilization in serum has been developed for the inhibition of the nuclease activity. Indeed, the backbone linkage introduced phosphorothioate (PS) or the sugar conjugated with protecting groups such as 2′-*O*-methyl (2′-*O*-Me), 2′-fluoro (2′-F), 2′-*O*-(2-methoxyethil) (2′-*O*-MOE), 5′-methylenephosphonate (5′-MP) and 5′-(E)-vinyl-phosphonate (5′-VP)

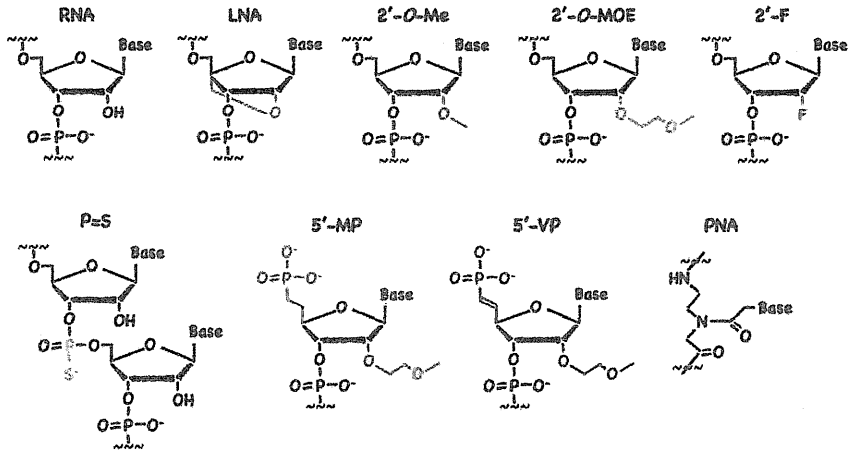

Figure 3. Chemical modifications for stability. Sugar, backbone and base modifications are illustrated. Shown are locked nucleic acid (LNA), phosphorothioate (P = S), 2′-*O*-methyl (2′-*O*-Me), 2′-fluoro (2′-F), 2′-*O*-(2-methoxyethil) (2′-*O*-MOE), 5′-methylenephosphonate (5′-MP), 5′-(*E*)-vinyl-phosphonate (5′-VP) and peptide nucleic acid (PNA).

Table 3. Chemical modifications for permeability and specificity

Chemical modification	Tissue	Factor	Route	RNA	References
PEG, PBAVE and ligand	Liver	NAG	IV	ApoB and PPARA siRNA	(114)
Aptamer	PSMA-positive prostate cancer	Anti-PSMA aptamer	IT	PLK-1/BCL-2 siRNA	(65)
	HIV-infected T cells	Anti-gp120 aptamer	IV	tat/rev siRNA	(66)
Cholesterol	Colon adenocarcinoma	Cholesteryl oligo-D-arginine	IT	VEGF siRNA	(58)
	Liver	HDL/LDL	IV	ApoB siRNA	(59)
Antibody	HIV-infected T cells	Anti-HIV Envelope Fab	IV	gag siRNA	(68)
	Hepatocellular carcinoma	Anti-EGFR Fab	IV	Luciferase siRNA	(69)
Peptide	Liver	Octaarginine	IV	SR-B 1 siRNA	(67)
	Lymphoma	Penetratin	IV/IT	Anti-miR-155-PNA	(64)
Agonist	TLR9 + myeloid cells and B cells	Anti-TLR9 agonist	IV/IT	Stat3	(56)

NAG, *N*-acetylgalactosamine; TLR, toll-like receptor.

enhance the resistance against exonuclease or endonuclease activity (73,74) (Fig. 3). Currently, the most consequential modification is the PS inter-nucleotide linkages that have been developed in the history of anti-sense oligonucleotides and have contributed to remarkable stabilization of double-strand RNA as well as the single-strand oligonucleotide (75,76). However, the influence of chirality in the phosphorus atom on the stability and the activity of duplexes is not entirely understood. Therefore, further investigation of the thermodynamic features and physiological activity with regard to the assignment of the absolute configuration will be required for therapeutic applications.

As reported above, a number of chemical modifications have been produced, which have enhanced the potential of siRNA, miRNA, miRNA inhibitors and anti-sense oligonucleotides. However, it has been required that the optimization of the modifications need to be optimized, as their efficiency depends on the position and combination. In 2012, chemical modifications were optimized for single-stranded siRNAs (ss-siRNAs), and the change was an important advancement for the practical application of RNAi therapeutics. It was shown that ss-siRNA with a number of chemical modifications, such as 5′-phosphonate and 2′-MOE-modified 5′-terminal nucleotide, 2′-F and 2′-*O*-Me motifs with contiguous PS modifications and 2′-MOE-modified adenosine dinucleotide at the 3′ terminus and C16 modification, brought about significant and efficient target gene silencing *in vivo via* the Ago2-mediated RNAi pathway (74). Furthermore, chemically modified ss-siRNAs targeting mutant huntingtin mRNAs have been employed as a novel nucleic acid drug for therapeutic application for Huntington's disease (77). Although single-stranded RNAs (ssRNAs) have been shown to have extremely rapid degradation in serum and poor activities so far (78,79), they have advantages, such as the absence of risk of undesirable off-target effects by passenger strand and the potential of systemic delivery without complex lipid formulations that sometimes trigger the inflammatory toxicities (80). Hence, these stabilized ssRNAs are expected to place RNAi therapy in a prominent class of nucleic acid drugs.

DYSREGULATED MIRNA AS THERAPEUTIC TARGET IN CANCER TREATMENT

The alterations of miRNA expression profiling are significantly related with cancer initiation and progression. To identify dysregulated miRNAs in the physiological and pathological pathway of cancer malignancy is the first step for therapeutic applications. Generally, the widespread disruption of miRNAs is caused by at least three different mechanisms: the loss, amplification or mutation of a fragile cancer-related genomic region; the change of epigenetic control; and the abnormality of miRNA-processing steps. The genetic change has the potential to affect radically the abundance of miRNA, and it was reported that >50% of miRNAs locate on the fragile genomic region in cancer (81–83). For instance, a significant downregulation of miR-15 and miR-16, which is caused by deletion or mutation in chromosome 13q14.3, was observed in 70% of patients with chronic lymphocytic leukemia.

On the other hand, CpG-island hypermethylation and histone modification as good markers for functional miRNA have also been investigated by using 5-aza-2′-deoxycytidine and a histone decetylase inhibitor, such as 4-phenylbutyric acid or trichostatin A (84–86). For example, miR-124a that regulates the expression of cyclin D kinase 6 was located in three chromosome loci, 8p23.1, 8q12.3 and 20q13.33, and these regions were hypermethylated in 75% of patients with primary colorectal tumors (87). In addition to genetic and epigenetic validation, alterations of the protein machinery related to the biogenesis of miRNA might impair global

miRNA expression. Indeed, a copy number change of DICER1 and Ago2 is frequently observed in melanoma, breast and brain cancer (88). Especially, TAR RNA-binding protein 2 (TARBP2), in the DICER-containing complex, showed frameshift mutations and caused a destabilization of DICER1 protein, resulting in global downregulation of mature miRNA in colorectal and gastric tumors (89). According to one estimate, the widespread downregulation of the miRNA expression levels is prevalent in several cancer types (90,91). In contrast, a kind of multi-functional polyphenolic compound, resveratrol, which is present in red wine, induced widespread upregulation of miRNAs and inhibited tumor growth through the acceleration of the expression and activity of Ago2 (92). Thus, the observation and management of the total balance of miRNAs are important for cancer diagnosis and treatment.

INHIBITION OF MIRNA EXPRESSION AND FUNCTIONS

For the therapeutic applications of miRNA, the intracellular expression levels of miRNAs have to be artificially controlled. Although it is relatively easy to upregulate miRNAs, the strategy for the downregulation of miRNAs requires a refined miRNA inhibitor such as a chemically modified antisense oligonucleotide. As the inhibitor against endogenous miRNA, locked nucleic acid (LNA), which has a methylene bridge connecting 2′ and 4′ carbons, is one of the most widely used platforms. LNA nucleotide organizes the phosphate backbone in the *N*-type (C3′-endo) conformation, whereas, in general, the conformations of DNA or RNA duplexes are flexible between *N*-type and S-type (C2′-endo). This conformational change contributes to a more efficient stacking of the nucleobases and functional inhibition of target miRNAs (93). In therapeutic applications, LNA against the liver-expressed miR-122, which is a potential therapeutic target in the hepatitis C virus (HCV), accomplished the long-lasting reduction of mature miR-122 and suppression of HCV viremia (94,95). Furthermore, LNA against hypoxia inducible factor 1α, the primary transcription factor activated by hypoxia that allows glycolysis and angiogenesis to progress, provides significant lowering of the expression of HIF1-α and suppression of tumor growth. Clinical trials of these LNA against miR-122 (SPC3649) and HIF1-α (EZN-2968) have progressed to Phases I and II by Santaris Pharma.

In addition to this, as competitive inhibitors of miRNAs, the miRNA sponge (96), the tough decoy (TuD) RNA (97), antagomirs (98), peptide nucleic acids (PNAs) (99) and anti-miRNA oligonucleotides (AMOs) (100) have also been developed toward medical practice targeting onco-miRNA as well as LNA. Antagomirs composed of 2′-*O*-Me, PS and cholesterol modification were the first miRNA inhibitors that provided a significant reduction in mammals (98,101). However, antagomirs were excluded as clinical candidates because they were less effective than other miRNA inhibitors. PNAs are replaced its sugar-phosphate backbone to

Table 4. Programs of clinical/pre-clinical study in miRNA therapeutics

miRNA	Therapy	Disease	Phase	Company
miR-208/499	Inhibitor	Chronic heart failure	Pre-clinical	MiRagen
miR-15/195	Inhibitor	Post-MI remodeling	Pre-clinical	Therapeutics
miR-451	Inhibitor	Polycythemia vera	Pre-clinical	
miR-122	Inhibitor	HCV	Pre-clinical	
miR-21	Inhibitor	HCC, cancer, fibrosis	Pre-clinical	
miR-10b	Inhibitor	Glioblastoma	Pre-clinical	Regulus
miR-33a/b	Inhibitor	Atherosclerosis	Pre-clinical	Therapeutics
miR-155	Inhibitor	Immuno-inflammatory diseases	Pre-clinical	
miR-122	Inhibitor	HCV	Phase II	Santaris Pharma
miR-29	Mimic	Cardiac fibrosis	Pre-clinical	MiRagen Therapeutics
let-7	Mimic	Lung cancer	Pre-clinical	Mirna Therapeutics
miR-34a	Mimic	Solid tumors	Pre-clinical	
miR-16	Mimic	Cancer	Pre-clinical	
miR-34a	Mimic	Hepatocellular carcinoma	Pre-clinical	Regulus
miR-146a	Mimic	Autoimmunity, cancer	Pre-clinical	Therapeutics

From MiRagen Therapeutics (http://www.miragentherapeutics.com), Regulus Therapeutics (http://www.regulusrx.com), Santaris Pharma (http://www.santaris.com), Mirna Therapeutics (http://www.mirnatherapeutics.com).

N-(2-aminoethyl)glycine units, also have a potential to inhibit miRNA activities. Reports indicate that PNA-DNA chimeras have the potential to inhibit miRNA *in vitro* and *in vivo* (99). On the other hand, unlike chemically modified ASOs, a miRNA decoy can be stably integrated into the chromosomes and degrade miRNA targets. The stable suppression of miR-301a by a miRNA decoy was reported to have inhibited tumor growth by the upregulation of NF-κB-repressing factor in pancreatic cancer (102), and TuD-RNA against miR-122a showed a significant suppression of the HCV replication in liver hepatocytes (103).

PIPELINE OF MIRNA IN CANCER TREATMENT

In a recent study, onco-miRs or tumor-suppressive miRs that work as master regulators in cellular processes have been identified, and a number of pre-clinical trials have been conducted by firms such as MiRagen Therapeutics, Regulus Therapeutics, Santaris Pharma and Mirna Therapeutics (Table 4). For example, miR-34a, which is one of the best-studied tumor-suppressive miRNAs, was a therapeutic target in solid tumor treatment by Mirna Therapeutics and Regulus Therapeutics. miR-34a is commonly downregulated in human cancer, such as prostate, breast, lung, kidney, bladder, ovary and skin cancer (104–106), and was identified as a target of the tumor suppressor gene p53. The reduction of miR-34a by CpG methylation is observed in multiple types of cancer. The restoration of miR-34s has the potential to cause cell cycle arrest, senescence and apoptosis (107). Mirna Therapeutics has also been conducting pre-clinical trials with miR-16 and let-7 mimics, which are potent tumor-suppressive miRNAs (47,108,109). Furthermore, pre-clinical trials of miRNA inhibitors against miR-21 and miR-10b, which are targeted as onco-miRs in hepatocellular carcinoma and glioblastoma, are being conducted. In addition to these developments, a number of non-public candidates for miRNA therapy are being considered by Mirna Therapeutics; they include miR-Rx01, 02, 03, 06 and 07. Thus, miRNA therapeutics using miRNA mimics or inhibitors has been growing in pre-clinical studies and might appear in clinical trials over the next several years.

CONCLUSION

RNAi is one of the most versatile knockdown tools in recent biotechnology, and the potential of RNAi therapeutics using miRNA for cancer treatment has been rapidly expanding. In particular, unlike siRNAs as a tool that specifically impairs the function of a target gene, miRNAs work as key regulators that control target genes and establish balanced cellular organization. Indeed, the disruption of such a balance leads to the possibility of a tumor to become malignant (110,111). To utilize these discoveries of cellular biological basic research for clinical investigation, further innovations in the field of the delivery systemic and chemical modifying strategies are desired. Indeed, although chemically modified ASOs and ss-siRNAs are potentially promising nucleic acid drugs that can efficiently manage RNAi in animals, immeasurable synthesis costs and technical difficulties for bulk production remain. In addition, safer and more effective delivery systems, including a viral approach, are needed. However, the progression of RNAi technology over the past decade has been remarkable, and the hope is that ongoing investigations will result in the use of RNAi therapeutics as a prominent cancer treatment.

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Conflict of interest statement
None declared.

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