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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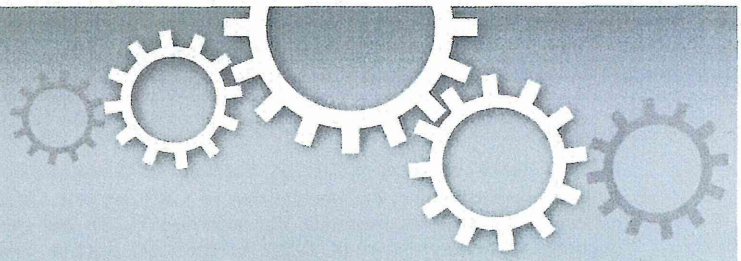

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Ribophorin II regulates breast tumor initiation and metastasis through the functional suppression of GSK3β

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Mutant p53 (mtp53) gain of function (GOF) contributes to various aspects of tumor progression including cancer stem cell (CSC) property acquisition. A key factor of GOF is stabilization and accumulation of mtp53. However, the precise molecular mechanism of the mtp53 oncogenic activity remains unclear. Here, we show that ribophorin II (RPN2) regulates CSC properties through the stabilization of mtp53 (R280K and del126-133) in breast cancer. RPN2 stabilized mtp53 by inactivation of glycogen synthase kinase-3β (GSK3β) which suppresses Snail, a master regulator of epithelial to mesenchymal transition. RPN2 knockdown promoted GSK3β-mediated suppression of heat shock proteins that are essential for mtp53 stabilization. Furthermore, our study reveals that high expression of RPN2 and concomitant accumulation of mtp53 were associated with cancer tissues in a small cohort of metastatic breast cancer patients. These findings elucidate a molecular mechanism for mtp53 stabilization and suggest that RPN2 could be a promising target for anti-CSC therapy.

Recent studies show that some p53 mutations result in the loss of tumor-suppressing function (LOF) by the mutant allele and *trans*-dominant inactivation of the remaining wtp53[1]. Importantly, the mutant p53 (mtp53) contributes to tumor progression. This mechanism is referred to as mtp53 gain of function (GOF). A key property of GOF is the stabilization and accumulation of mtp53[2]. The mtp53 protein is rescued from degradation and contributes to malignant phenotypes such as invasion and metastasis or genomic instability by binding and inactivating p63 and Mre11, respectively[3–5]. In contrast to wild-type p53 (wtp53), the mtp53 protein adopts an aberrant conformation. Mtp53 forms stable complexes with heat shock proteins HSP90 and HSP70, MDM2, and the carboxyl terminus of HSP70-interacting protein (CHIP), which prevent mtp53 misfolding and aggregation[6,7]. In malignant cancer cells, the HSP90 and HSP70 chaperone machinery is upregulated and activated to protect mutated and overexpressed oncoproteins from degradation[8,9]. While several studies succeeded in identifying the molecular mechanisms that regulate mtp53 stability[6,7] and the small molecules that induce mtp53 destabilization[6,10], the mechanism that leads to mtp53 stabilization is not yet fully understood.

In breast cancer, p53 LOF or mutation induces epithelial to mesenchymal transition (EMT)[11], which contributes to cancer progression and metastasis[12,13]. Several studies show a link between EMT and the acquisition of CSC properties[11,14]. Ectopic expression of EMT regulators, such as Twist and Snail, or shRNA-mediated knockdown of E-cadherin confer cancer stem cell (CSC) properties to mammary epithelial cells[12]. Snail expression is suppressed by glycogen synthase kinase-3β (GSK3β) at transcriptional and post-transcriptional levels[15,16], and the inhibition of GSK3β by small molecules induces EMT and promotes CSC phenotypes in breast cancer[17]. Despite the critical role of GSK3β in the regulation of CSCs phenotypes, the physiological and molecular mechanisms underlying its function remain unclear.

Breast CSCs exhibit a $CD44^{+}CD24^{-/low}$ antigenic phenotype with low expression of epithelial markers such as E-cadherin, and are characterized by high tumorigenicity and drug resistance[14,18]. Previously, we showed that ribophorin II (RPN2) is a novel regulator of drug resistance in breast cancer and affects docetaxel resistance by modulating the *N*-linked glycosylation of P-glycoprotein (ABCB1)[19]. To gain further insight into the regulation of mtp53 stability in CSCs, we screened for possible interactions between RPN2 and GSK3β. The present study suggests that the stabilization of mtp53 (R280K and del126-133) in breast cancer depends on RPN2 inhibition of GSK3β-mediated inactivation of HSP70 and HSP90 that are essential factors for the stabilization and oncogenic activities of mtp53.

Results

RPN2 is highly expressed in the CSC fraction. RPN2 was initially considered essential for the maintenance of CSCs because CSCs exhibit resistance to conventional chemotherapy[18,20]. To test this hypothesis, the expression of RPN2 in the CSC fraction was examined using two breast cancer cell lines, MCF7-ADR drug resistant human breast cancer cells and MDA-MB-231-D3H2-LN highly metastatic human breast cancer cells (MM231-LN). Since CSC fraction in breast cancer cells is reported to show the resistance to chemotherapy and the metastatic ability[21,22], we selected these two cell lines. Flow cytometry analysis showed that MCF7-ADR and MM231-LN cells comprise approximately 15% and 50% of CSCs, respectively (Fig. 1a). In breast CSCs, the $CD44^{high}/CD24^{low}$ fraction shows higher tumorigenicity than the $CD44^{high}/CD24^{high}$ fraction[23]. Flow cytometry and quantitative reverse transcription PCR (qRT-PCR) of MCF7-ADR and MM231-LN cells showed that RPN2 was more highly expressed in the CSC fraction than in the non-CSC fraction (Fig. 1b). In addition, the CSC fraction of MM231-LN showed high tumorigenicity in an animal model (Suppl. Fig. S1). Immunostaining analysis showed inverse correlation between RPN2 and E-cadherin expression in MM231-LN xenograft tumors (Fig. 1c).

To further investigate the role of RPN2 in CSCs, RPN2 knockdown experiments were performed in the two breast cancer cell lines, MCF7-ADR and MM231-LN, using lentivirus vectors expressing GFP and a small hairpin RNA against *RPN2* (shRPN2-site2) (Suppl. Fig. S2A and S2B). RPN2 knockdown reduced the E-cadherin negative fraction in MM231-LN cells as detected by flow cytometry analysis (Suppl. Fig. S3A). We also found that RPN2 knockdown induced Snail suppression in MM231-LN cells (Fig. 1d). Compared with MM231-LN shNC, a 40% decrease in *Snail* expression was observed in MM231-LN shRPN2 (Fig. 1d). Moreover, the GSK3β inhibitor CH99021 caused *Snail* upregulation in MM231-LN shRPN2 (Fig. 1d).

Consistent with the previous studies showing loss of epithelial phenotype by inactivation of p53[11,14], we confirmed that the ectopic expression of Snail and a point mutant p53 (R280K) in human mammary epithelial cells (HME cells) promoted the expression of Vimentin which is one of the mesenchymal cell markers (Fig. 2a, lane 3). Co-expression of Snail and C-terminal Myc-Flag tagged RPN2 (RPN2-MF) also promoted the expression of Vimentin in HME cells (Fig. 2a, lane 4). Next we established HME-Snail cell line that shows predominant mesenchymal phenotype and contains the $CD44^{high}/CD24^{low}$ fraction[11] (Fig. 2b and 2d). Flow cytometry and western blot analysis revealed that while the expression of wtp53 suppressed Snail expression and reduced CSC fraction in HME-Snail cells, the expression of mtp53 or RPN2-MF did not alter the population of CSCs and Snail expression (Fig. 2b and 2d). More importantly, we also found that mtp53 (R280K) promoted the expression of RPN2 in HME-Snail cells (Fig. 2b lane 4). We also observed that mtp53 (R280K and R175H) promoted the protein stability of Snail and co-expression of RPN2-MF and mtp53 (R280K) induced the expression of N-cadherin in other human mammary epithelial cells (MCF10A cells) (Fig. 2c and Suppl. Fig. S3A). These results suggest that RPN2 plays an important role in the generation of CSC with EMT phenotype in breast cancer cells.

RPN2 regulates the tumorigenicity and metastasis of CSCs. The tumorigenicity of RPN2 knockdown cell lines was then examined using a 3D spheroid culture system[24]. In several cancer cell lines, CSCs form spheroids, which are essential for tumor onset in immunodeficient mice[14,24]. MM231-LN CSCs exhibited high tumorigenicity in an animal model (Suppl. Fig. S1). Compared with the control CSC fraction (MM231-LN shNC), the RPN2-knockdown CSC fraction (MM231-LN shRPN2) formed very few spheroids (Fig. 3a and b). To evaluate tumor formation by *in vivo* imaging, a limiting-dilution assay was performed using 6-week-old NOD/SCID mice that had been injected in the hind legs with 10^2 cells from a CSC fraction ($CD44^{high}/CD24^{low}/GFP^{high}$) derived from MM231-LN cells expressing firefly luciferase (Fig. 3c). The control CSC fraction formed tumors in all mice, whereas the RPN2-knockdown CSC fraction only formed one tumor from five injections (Fig. 3c and Suppl. Fig. S4A), and in that tumor, low RPN2 knockdown was confirmed by *in vivo* imaging (Suppl. Fig. S4B). Similar results were obtained with MCF7-ADR cells (Fig. 3c). The metastatic ability of RPN2 knockdown cell lines was examined next. In transwell migration assays, RPN2 knockdown reduced the invasiveness of CSCs in MM231-LN cells (Fig. 3d and e). After the transplantation of CSCs derived from MM231-LN shNC into the mammary fat pads of NOD/SCID mice, nodal and lung metastasis was observed in all mice (Fig. 3f). However, after transplantation of CSCs derived from MM231-LN shRPN2, nodal and lung metastasis was no longer observed (Fig. 3f and g), suggesting that RPN2 is essential for tumor formation and metastasis.

RPN2 antagonizes GSK3β function via physical interaction. Several studies show that GSK3β suppresses Snail expression at transcriptional and post-transcriptional levels[15,16], and the inhibition of GSK3β by small molecules induces EMT and promotes CSC phenotypes in breast cancer[17]. Our results also confirmed that the GSK3β inhibitor CH99021 suppressed E-cadherin expression in MCF7 cells (Suppl. Fig. S3B). In the present study, RPN2 knockdown reduced the E-cadherin negative fraction in MM231-LN cells via suppressing *Snail* expression (Fig. 1d and Suppl. Fig. S3C). These results indicate that RPN2 knockdown promotes GSK3β activation, and such activation is associated with the suppression of CSC phenotypes (Fig. 1 and 3).

The results above indicate that RPN2 regulates GSK3β activity in MM231-LN cells (Fig. 1c and d and Suppl. Fig. S3C); however, the *N*-linked glycosylation of GSK3β has not been previously observed. We therefore examined the physical interaction between RPN2 and GSK3β in MM231-LN cells by immunoprecipitation of endogenous RPN2 from MM231-LN cells. Since the phosphorylation of GSK3β at Tyr216 (GSK3β-216Y) is essential for the nuclear localization and activation of GSK3β[16], we examined the association of RPN2 with both Y^{216}-phosphorylated and unphosphorylated GSK3β (Fig. 4a). Immunoprecipitation analysis showed that RPN2 associated with both Y^{216}-phosphorylated and unphosphorylated GSK3β (Fig. 4a, lane 2). The physical interaction between RPN2 and GSK3β was also confirmed by using an anti-GSK3β antibody (Fig. 4b1, lane 2).

To determine the binding site of GSK3β on RPN2, in vitro binding assays were performed using extracts from 293T cells expressing RPN2-MF and C-terminal HA-tagged GSK3β (GSK3β-HA). Co-immunoprecipitation analysis revealed that GSK3β interacted with RPN2 in this extract (Fig. 4c and d, lane 2). Next, the binding region for GSK3β to RPN2 was examined using 293T cell extracts expressing RPN2-MF and GSK3β deletion mutants (Fig. 4e). Co-immunoprecipitation analysis revealed that the N-terminal amino acid region between 56–85 of GSK3β was critical for RPN2 binding (Fig. 4f, lane3).

To further investigate whether RPN2 antagonizes GSK3β activity via physical interaction, we examined the E-cadherin expression in 293T cells. In a control experiment, we confirmed that ectopic expression of N-terminal Flag-tagged Snail (Flag-Snail) induced E-cadherin suppression (Fig. 4g) and that expression of GSK3β-HA inhibited the Snail-mediated suppression of E-cadherin (Fig. 4g). Therefore, we examined whether ectopic expression of RPN2-MF with GSK3β-HA restores the Snail activity. Flow cytometry analysis showed that the expression of RPN2-MF restored the Snail-mediated suppression of E-cadherin (Fig. 4g). These results indicate that RPN2 inhibits the nuclear localization and activation of GSK3β via a physical interaction with its N-terminal region, and partially provide a

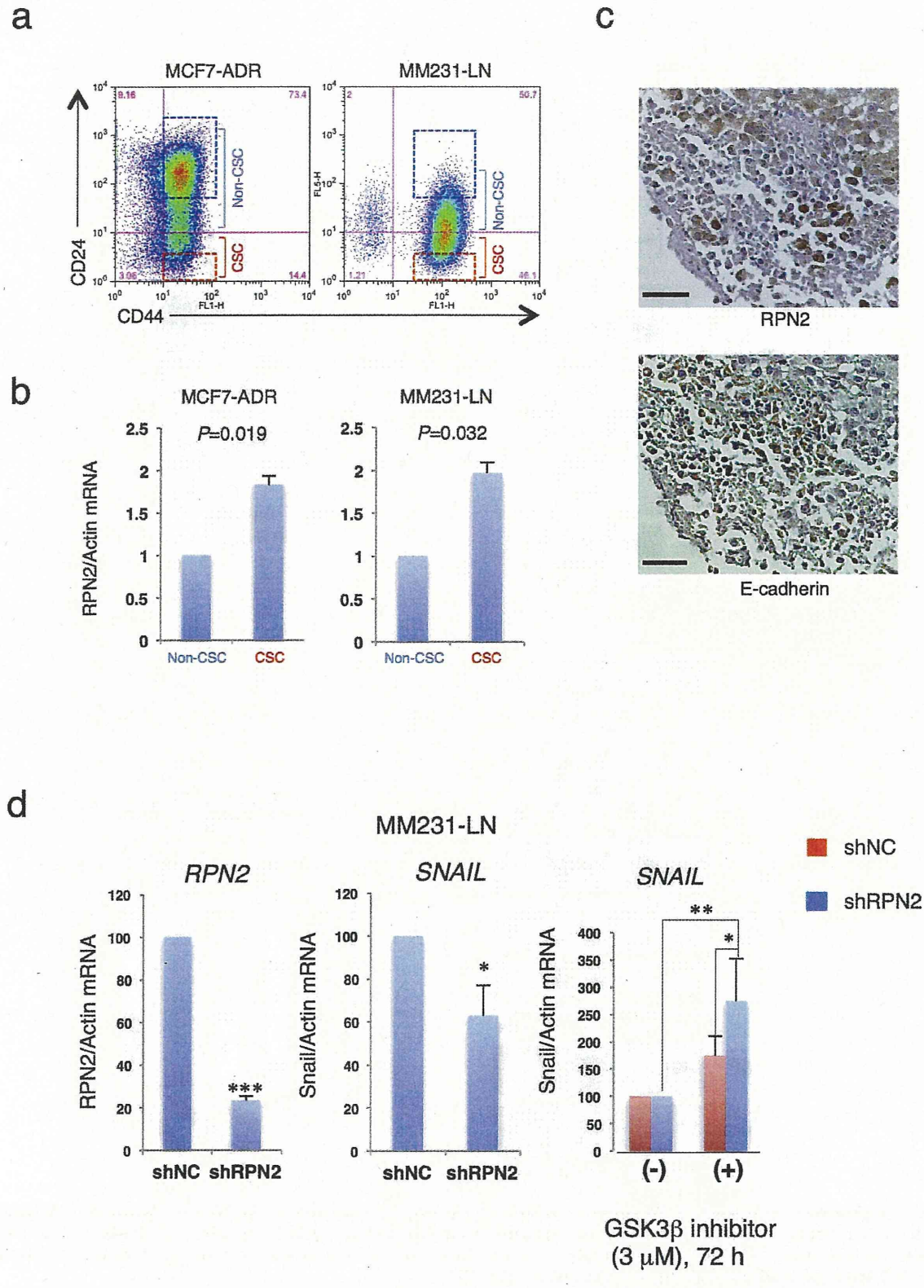

Figure 1 | RPN2 is essential for the maintenance of the CSC fraction. (a) The CSC fraction derived from MCF7-ADR and MM231-LN cells. (b) MCF7-ADR and MM231-LN cells were segregated by fluorescence-activated cell sorting (FACS) into $CD44^{high}/CD24^{low}$ and $CD44^{high}/CD24^{high}$ subsets; sorted subsets were then compared for RPN2 expression by quantitative real-time PCR (qRT-PCR). Each data point is the average of three experiments. (c) Immunohistochemistry (IHC) for RPN2 (Top panel) and E-cadherin (Bottom panel) in MM231-LN tumors. MM231-LN xenografts were grown for 5–6 weeks after fat pad injection. Sections are representative of at least four mice analyzed per group. Scale bar, 200 μm. (d) RPN2 knockdown suppressed *Snail* expression via GSK3β activation. In MM231-LN cells, RPN2 and Snail mRNA expression was monitored by qRT-PCR after 72 h of treatment with a GSK3β inhibitor (CHIR99021, 3 μM). (n = 3, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

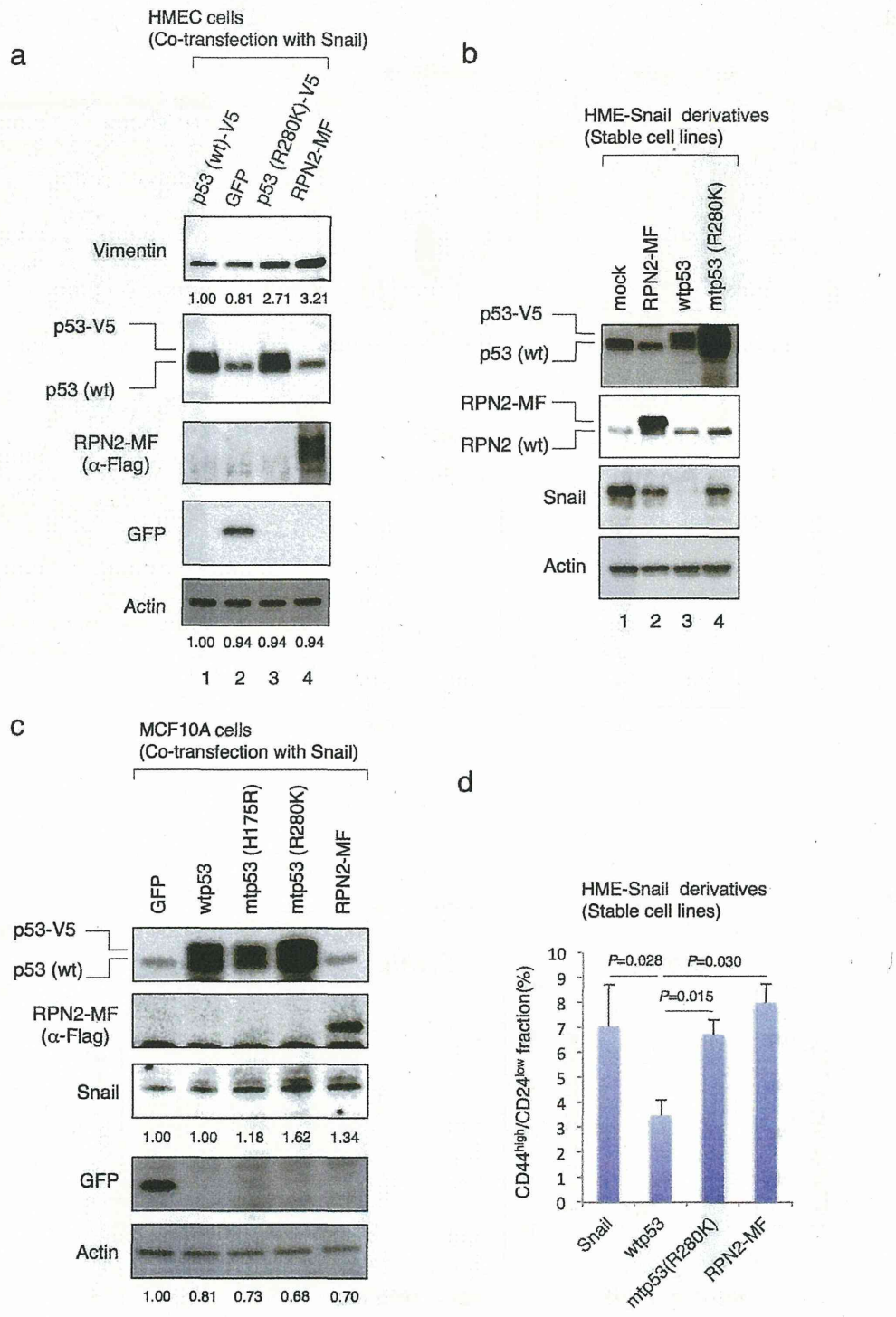

Figure 2 | RPN2 plays the important roles in the generation of CSC fraction in breast cancer cells. (a)–(c) Western blot analysis. Cell lysates were subjected to western blotting with anti-p53, anti-RPN2, anti-Snail, anti-Vimentin, anti-Flag, anti-GFP and anti-actin antibodies. (d) p53 status and RPN2 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

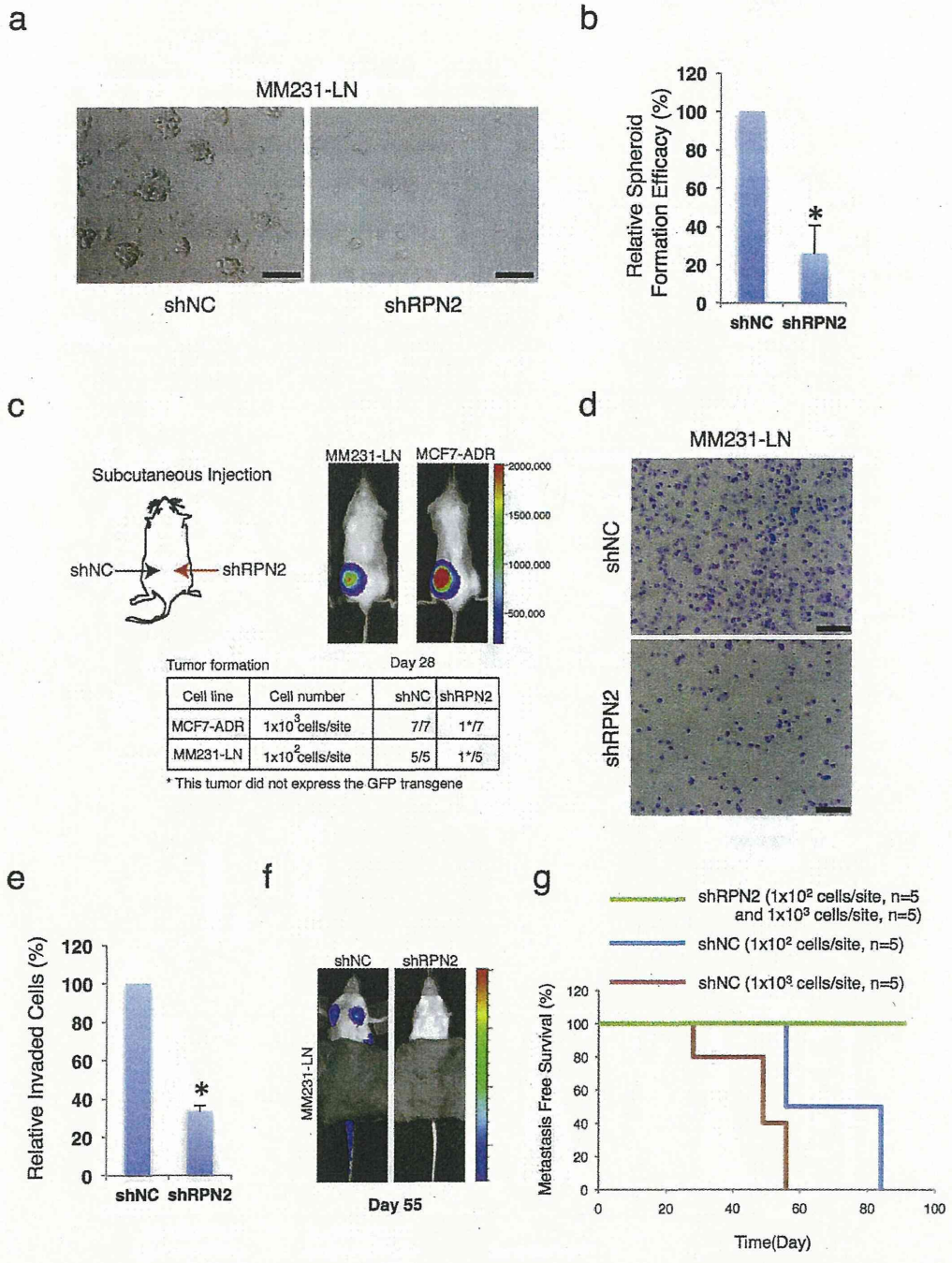

Figure 3 | RPN2 regulates the tumorigenicity and metastatic activity of CSCs. (a) Phase-contrast images of spheroids seeded by MM231-LN shNC (right) and MM231-LN shRPN2 (left) cells. Scale bar, 50 μm. (b) Quantification of spheroid formation in MM231-LN shNC and MM231-LN shRPN2 cells. The data in b) 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-