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Research Article

Ginsenoside Rh2 upregulates long noncoding RNA STXBP5-AS1 to sponge microRNA-4425 in suppressing breast cancer cell proliferation

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ABSTRACT

Background: Ginsenoside Rh2, a major saponin derivative in ginseng extract, is recognized for its anticancer activities. Compared to coding genes, studies on long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) that are regulated by Rh2 in cancer cells, especially on competitive endogenous RNA (ceRNA) are sparse.

Methods: LncRNAs whose promoter DNA methylation level was significantly altered by Rh2 were screened from methylation array data. The effect of STXBP5-AS1, miR-4425, and RNF217 on the proliferation and apoptosis of MCF-7 breast cancer cells was monitored in the presence of Rh2 after deregulating the corresponding gene. The ceRNA relationship between STXBP5-AS1 and miR-4425 was examined by measuring the luciferase activity of a recombinant luciferase/STXBP5-AS1 plasmid construct in the presence of mimic miR-4425.

Results: Inhibition of STXBP5-AS1 decreased apoptosis but stimulated growth of the MCF-7 cells, suggesting tumor-suppressive activity of the lncRNA. MiR-4425 was identified to have a binding site on STXBP5-AS1 and proven to be downregulated by STXBP5-AS1 as well as by Rh2. In contrast to STXBP5-AS1, miR-4425 showed pro-proliferation activity by inducing a decrease in apoptosis but increased growth of the MCF-7 cells. MiR-4425 decreased luciferase activity from the luciferase/STXBP5-AS1 construct by 26%. Screening the target genes of miR-4425 and Rh2 revealed that Rh2, STXBP5-AS1, and miR-4425 consistently regulated tumor suppressor RNF217 at both the RNA and protein level.

Conclusion: LncRNA STXBP5-AS1 is upregulated by Rh2 *via* promoter hypomethylation and acts as a ceRNA, sponging the oncogenic miR-4425. Therefore, Rh2 controls the STXBP5-AS1/miR-4425/RNF217 axis to suppress breast cancer cell growth.

1. Introduction

Ginsenoside Rh2 is a major anticancer saponin of *Panax ginseng* [1]. Rh2 has displayed a wide spectrum of pharmaceutical effects, including antiproliferative, antimetastatic, pro-apoptotic, and multidrug-resistant activities against various tumors *in vitro* and *in vivo* [2]. Investigations into the potential signaling pathways and underlying mechanisms of the antitumor activity of Rh2 have revealed NF-κB [3], TNF-α [4], Wnt/β-catenin [5], PI3K/AKT/mTOR [6], and Ras/Raf/ERK/p53 as the representative pathways [7]. Its involvement in these signaling pathways led to altered expression of the associated target genes, leading to changes in cellular activities. Among the target genes are cyclins, CDKs, MMPs, TGF-β, and cadherins, all pivotal in regulating cell cycle, cell proliferation, or tumor development [8]. Rh2 regulates many noncoding RNAs (ncRNAs). Notably, microRNAs (miRNAs) have been shown to enhance Rh2-induced antitumor activities [9]. For example, the promotion of apoptosis and autophagy [6] and the inhibition of cancer cell migration [10] and growth [11] were accompanied by Rh2-mediated regulation of miR-638, miR-491, and miR-4295, respectively. In MCF-7 breast cancer cells, Rh2 mediated reversal of miR-34a, miR-130a, and miR-29a expression that had been deregulated during acquired resistance to docetaxel [12]. Compared to miRNAs, fewer long noncoding RNAs (lncRNAs) are known to be regulated by Rh2. A genome-wide expression screening identified over 700 lncRNAs that were deregulated in Rh2-treated HepG2 cells [13]. Jeong et al. identified lncRNA C3orf67-AS1 as epigenetically regulated by Rh2, leading to suppression of breast cancer cells [14]. In MC3T3-E1 cells, lncRNA H19

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contributed to Rh2-mediated bone cell proliferation by up-regulation of osteopontin [15].

Intriguingly, cross-talk between lncRNAs and miRNAs allows a further modulation of ncRNA-based regulation. If the lncRNA has the same binding sequence as the target gene of a specific miRNA, the lncRNA can sponge the miRNA, leading to suppression of the miRNA activity. This so-called competitive endogenous RNA (ceRNA) plays an essential role in the precise control of target gene expression in cancer [16]. Despite the accumulating number of identified ceRNAs associated with cancer development, few have been linked with the anticancer action of ginsenosides, with a few cases known only for Rg3. One study showed that Rg3 prevented PKM2-targeting miR-324–5p from sponging H19 to oppose the Warburg effect in ovarian cancer cells, contributing to the accumulating evidence proving lncRNAs as sponges to suppress miRNA function [17]. LncRNA ATXN8OS, which sponged the tumor-suppressive miR-424–5p in MCF-7 breast cancer cells, was also downregulated by Rg3 [18].

In the present study, STXBP5-AS1 was identified as a lncRNA whose promoter DNA methylation level was highly altered by Rh2 treatment in MCF-7 cells. Then, the expression of STXBP5-AS1 was examined in multiple mammary gland-originated cell lines. To examine the role of STXBP5-AS1 in cancer cell growth, the effect on cell proliferation and apoptosis was monitored after inducing down-regulation of the gene. MiR-4425 that interacted with STXBP5-AS1 was screened *in silico*, and their sponging relationship was elucidated in the context of the miRNA-mediated gene regulation by Rh2. Based on the established regulatory scenario, the Rh2/STXBP5-AS1/miR-4425/RNF217 axis was proposed.

2. Materials and methods

2.1. Cell culture

Breast cancer cell lines MDA-MB231 and MCF-7 and a normal breast cell line MCF-10A were purchased from the American Type Culture Collection (Manassas, VA, USA). T47D was purchased from the Korean Cell Line Bank (Seoul, Korea). The cancer cell lines and the normal line were cultured in RPMI-1640 (Welgene, Gyungsan, Korea) and MEGM (Lonza, Basel, Switzerland) supplemented with 100 ng/ml cholera toxin, respectively. All cultures were supplemented with 10% fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany) and 2% penicillin/streptomycin (Capricorn Scientific) and maintained at 37 °C in a humidified incubator containing 5% CO_2.

2.2. Rh2 treatment and transfection

Cells (5×10^4 cells) at 50% confluence were seeded in a 60-mm culture dish and cultured for 24 h before Rh2 treatment or transfection of RNA. Rh2 was added to cells to obtain final concentrations of 20 and 30 μM using a 20 mM stock (LKT Labs, St. Paul, MN, USA) in 100% ethanol. For transfection, siRNA (Bioneer, Daejon, Korea), mimic miRNA (Bioneer), or inhibitor miRNA (Bioneer) of 10 mM stock in distilled water was diluted to final concentrations of 20 and 40 nM in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA), mixed with 5 μl of Lipofectamine RNAiMAX (Invitrogen), and added to the cell culture. For the cotreatment of Rh2 and RNA, Rh2 was added to the cells, and 24 h later, RNA was transfected. The cells were further cultured for 24 h, then harvested with 0.05% trypsin-EDTA (Gibco BRL, Carlsbad, CA, USA).

2.3. Quantitative polymerase chain reaction (qPCR)

Methylation-specific PCR was performed as previously described [19]. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed following a previous description [20] with minor modifications. Briefly, total RNA was extracted from the 60-mm culture dish using a Quick-DNA/RNA Miniprep kit (Zymo Research, Irvine, CA, USA), with a final elution volume of 20 μl. The cDNA for mRNA was synthesized from total RNA (2 μg) using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) in 10-μl reactions. PCR was then conducted from 1 μl cDNA using SYBR Fast qPCR Kit Master Mix (Kapa Biosystems, Wilmington, MA, USA). The cDNA for miRNA was synthesized from total RNA (2 μg) using miScript II RT kit (Qiagen, Valencia, CA, USA) in a 10-μl reaction. PCR was conducted from 1 μl cDNA using miScript SYBR Green PCR kit (Qiagen) and miScript Primer Assay kit (Qiagen). The expression of mRNA and miRNA samples was normalized to glyceraldehyde-3-phosphate dehydrogenase and U6, respectively. PCR primers are listed in Supplementary Table S1.

2.4. Cell proliferation and apoptosis assay

For the analysis of cell proliferation, 1.5×10^3 cells were seeded per well in a 96-well plate and cultured for 24 h. The cells were then treated with either Rh2 or ncRNA and cultured for up to six additional days. On the designated day of culture, cell proliferation was analyzed using Cell Counting Kit-8 (CCK-8) (Enzo Biochem, New York, NY, USA) to measure cell density at OD_{450} using a plate reader. The apoptosis assay was performed, as described previously, with minor modifications [14]. Briefly, 1×10^6 cells were seeded in a 60-mm plate, treated with Rh2 or transfected with ncRNA, and cultured for 24 h. After harvest, 1×10^5 cells were stained with FITC Annexin V (BD Bioscience, San Jose, CA, USA) and PI (Sigma-Aldrich, St. Louis, MO, USA). Fluorescence was detected using a BD Accuri C6 (BD Technologies), and data were analyzed using BD Accuri C6 software (BD Technologies).

2.5. Luciferase assay

STXBP5-AS1 was subcloned into the pEZX-MT05 dual-luciferase reporter vector (GeneCopoeia, Rockville, MD, USA). A total of 3×10^4 HEK-293T cells were seeded per well in a 24-well plate and cultured for 24 h. Then, 20 nM of miR-4425 mimic or negative control miRNA (miR-NC) was co-transfected with 150 ng of the plasmid DNA using Lipofectamine 3000 (Invitrogen). Two days after transfection, luminescence was detected with a Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia) following the manufacturer's protocol. The transfection efficiency variation was eliminated by signal normalization (ratio of *Gaussia* luciferase [GLuc] expression and secreted alkaline phosphatase [SEAP] activities).

2.6. Western blot analysis

Proteins were extracted from the harvested cells using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing a 1% protease inhibitor cocktail (Thermo Fisher Scientific). The proteins (15 μg) were separated on an SDS-PAGE gel, then blotted on a polyvinylidene difluoride membrane (Whatman, Cambridge, UK). After blocking, the membrane was incubated with primary antibodies of anti-RNF217 (1:500; PA5-42360, Invitrogen) and anti-β-actin (1:1000; bs-0061R, Bioss, Woburn, MA, USA) at 4 °C overnight. The blot was then incubated with HRP-conjugated anti-rabbit IgG antibodies (1:1000; GTX213110-01, GeneTex, Irvine, CA, USA) for 2 h. The signals were visualized using the D-Plus ECL Pico Alpha System (Dongin Biotech, Seoul, Korea) and quantified with Image Lab software (Bio-Rad, Hercules, CA, USA) using β-actin for normalization.

2.7. Data mining

LncRNAs whose promoter DNA methylation level was significantly altered by Rh2 were screened from the methylation array data of the NCBI GEO DataSet (GSE93356). MiRNAs that potentially targeted lncRNA were screened using LncBase Predicted v.2 (http://diana.imis.athena-innovation.gr/DianaTools). Potential target genes of miRNA were predicted using MicroT (http://diana.imis.athena-innovation.gr/DianaTools), miRDB (http://www.mirdb.org), TargetScan7 (http://www.targetscan.org), and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2). The relationship between gene expression and survival of breast cancer patients was investigated through the Kaplan–Meier plotter tool (http://kmplot.com/analysis) using data from The Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo, Affymetrix microarrays only), The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov), and The European Genome-phenome Archive (EGA, https://www.ebi.ac.uk/ega).

2.8. Statistical analysis

Statistical analysis was performed using the SPSS version 23.0 software (SPSS, Chicago, IL, USA). For statistical comparisons, the Wilcoxon test and two-tailed Student's *t*-test were performed. All

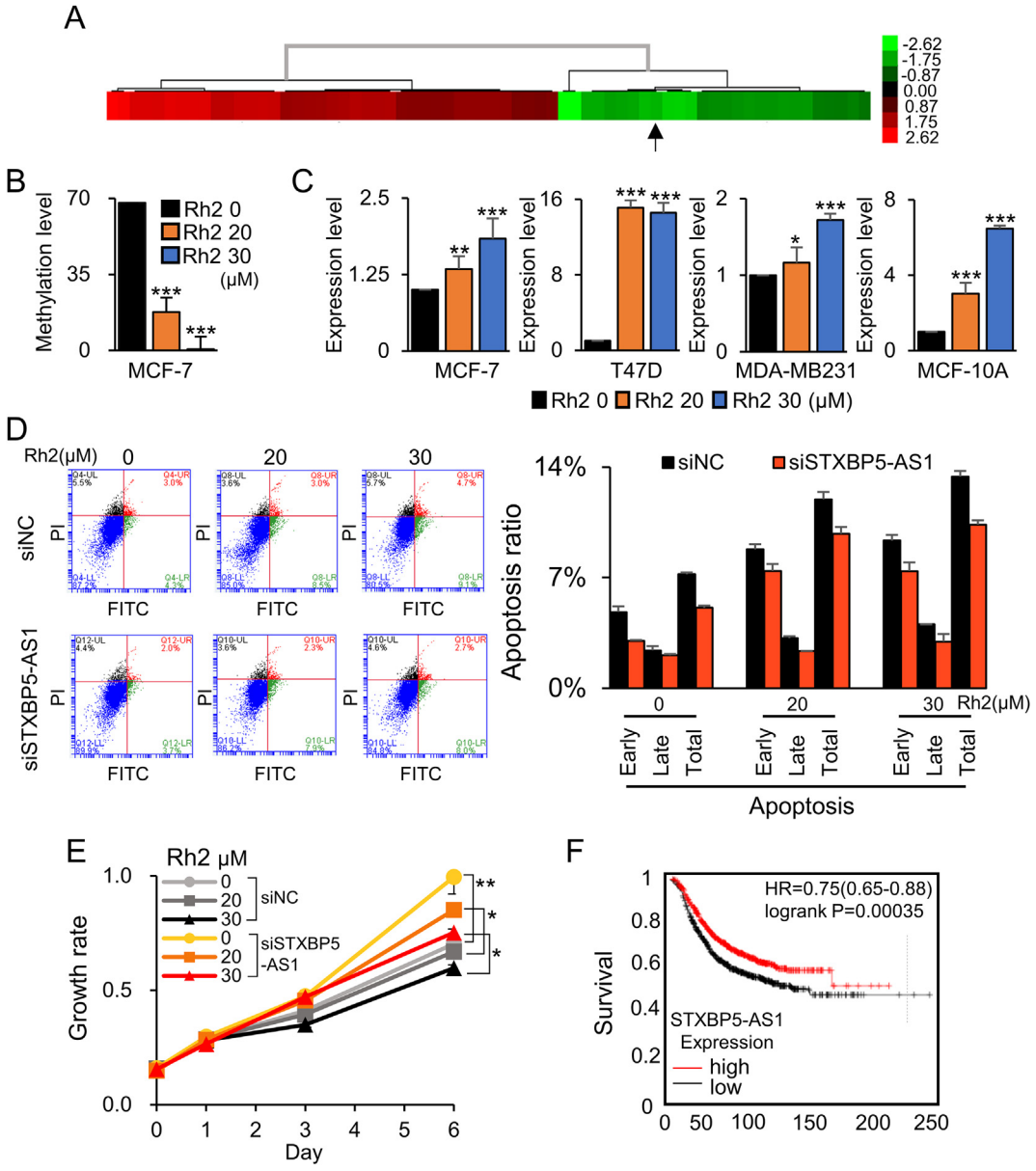

Fig. 1. STXBP5-AS1 is upregulated by Rh2 *via* promoter hypomethylation and shows antiproliferation activity in breast cancer cells. (A) LncRNAs whose promoter CpG methylation were significantly affected by Rh2 in MCF-7 cells ($|FC| \geq 1.5$) were clustered using the methylation array data (GSE93356). STXBP5-AS1 was indicated as an arrow. (B) STXBP5-AS1 is significantly hypomethylated in the methylation array data ($\Delta\beta = -0.21$ and $FC = -1.87$) and confirmed by the methylation-specific PCR. (C) Breast cancer cell lines were treated with Rh2, and the expression of STXBP5-AS1 was examined by qRT-PCR. (D) MCF-7 cells were treated with an STXBP5-AS1-specific siRNA and Rh2, and apoptosis was examined by flow cytometry. (E) The effect of STXBP5-AS1 and Rh2 on cell proliferation was monitored by the CCK-8 assay. (F) Kaplan–Meier survival curves for patients with breast cancer according to the expression level of STXBP5-AS1. Experiments (B–E) were performed in triplicate, and the values are presented as the mean ± SE. FC, fold change; siNC, control siRNA; siSTXBP5-AS1, STXBP5-AS1-specific siRNA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

experiments were independently performed at least three times. Data were presented as mean ± SD and *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Rh2 induces hypomethylation and up-regulation of STXBP5-AS1

In our previous study, genome-wide methylation analyses were performed after treating MCF-7 breast cancer cells with Rh2 [21]. In addition to 850,162 CpGs from coding genes, the array covers over 10,734 CpGs from noncoding genes. Among them, 66 lncRNAs showed a significant change in methylation level higher than 1.5-fold (Fig. 1A). STXBP5-AS1 appeared with the greatest change ($\Delta\beta = -0.21$, 1.87-fold decrease) and was selected for further analysis of its regulatory mechanism in association with Rh2. First, the hypomethylation of STXBP5-AS1 by Rh2 was confirmed by methylation-specific qPCR, which revealed $\Delta\beta$ values ranging from −0.50 to −0.66 compared to the nontreated control (Fig. 1B). qRT-PCR showed up-regulation of STXBP5-AS1 by Rh2 in MCF-7 cells, two other breast cancer cell lines (MDA-MB-231 and T47D), and in a normal cell line, MCF-10A (Fig. 1C). To examine the role of STXBP5-AS1 in cancer cells, the effect of STXBP5-AS1 on apoptosis and cell proliferation was examined in the MCF-7 cells. Down-regulation of STXBP5-AS1 by siRNA (Supplementary Fig. S1) decreased early and late apoptosis, with a 2.1% decrease in the total apoptosis, which was compensated by the co-treated Rh2 (Fig. 1D). Meanwhile, growth of the MCF-7 cells treated with the siRNA for STXBP5-AS1 was retarded by up to 29% in the Rh2-treated cells (Fig. 1E), suggesting pro-apoptotic and antiproliferation effects of STXBP5-AS1. Consistent with these data, breast cancer patients

A

miRNA	Score	MRE	Binding score
hsa-miR-4425	0.985	1731-1757	0.079
hsa-miR-4476	0.994	2864-2881	0.078
hsa-miR-7151-5P	0.989	137-159	0.078
hsa-miR-6881-3P	0.987	565-589	0.076
hsa-miR-6127	0.983	416-436	0.074

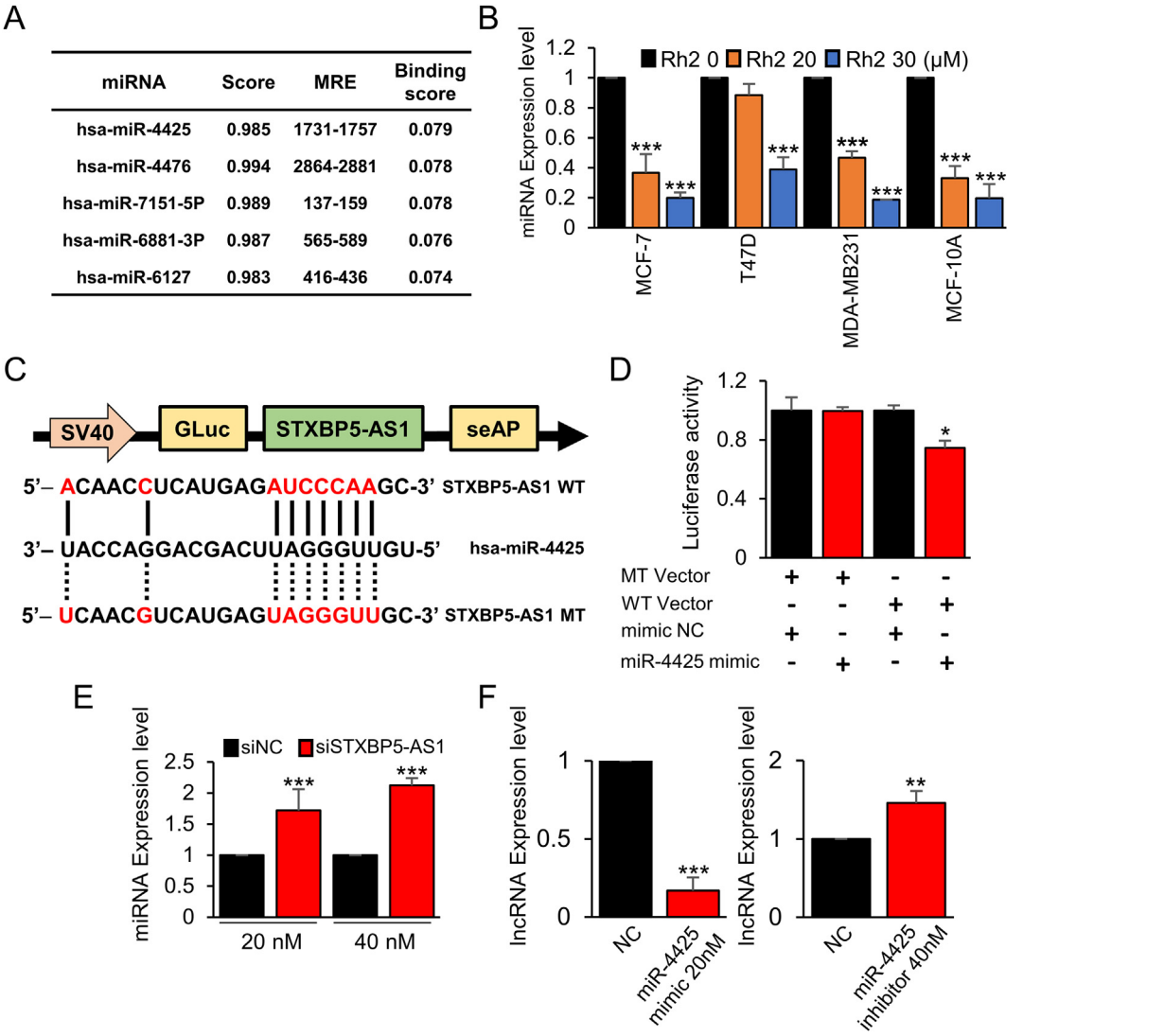

Fig. 2. MiR-4425 is downregulated by Rh2 and sponged by STXBP5-AS1. (A) Five miRNAs that could potentially bind STXBP5-AS1 with a high binding score were screened from miRNA prediction databases. MRE, location of the miRNA recognition element in STXBP5-AS1. (B) MiR-4425, with the highest binding score, was selected, and its expression in Rh2-treated breast cancer cell lines was examined by qRT-PCR. (C) Schematic diagram of the recombinant luciferase/STXBP5-AS1 DNA construct. The binding sequence between STXBP5-AS1 and miR-4425 is denoted. WT, wild type; MT, mutant type. (D) The binding of miR-4425 to STXBP5-AS1 was validated by measuring the luciferase activity of the luciferase/STXBP5-AS1 construct. The regulatory association between STXBP5-AS1 and miR-4425 was examined by monitoring the expression of each RNA after downregulating STXBP5-AS1 using siRNA (E) or overexpressing or inhibiting miR-4425 (F). All experiments were performed in triplicate, and the values are presented as the mean ± SE. siNC, control siRNA; siSTXBP5-AS1, STXBP5-AS1-specific siRNA; mimic NC, negative control mimic for miR-4425; inhibitor NC, negative control inhibitor for miR-4425. $^{**}P < 0.01$, $^{***}P < 0.001$.

with higher expression of STXBP5-AS1 showed a better prognosis of survival than those with lower expression in a cohort of 4934 patients from the TCGA (Fig. 1F).

3.2. STXBP5-AS1 suppresses cancer cell proliferation via sponging miR-4425

In the view that many lncRNAs are known to regulate miRNAs, as well as their target genes, we hypothesized that STXBP5-AS1 could recognize and regulate specific miRNA(s) to mediate the Rh2-induced inhibition of cancer cell growth. To test this, we first *in silico* searched databases to identify miRNAs that could potentially interact with lncRNAs. As a result, five miRNAs with a high binding score (>0.07) were revealed (Fig. 2A). We decided to focus on miR-4425 because it showed the highest binding score, but also its role in association with Rh2 in cancer cells was not yet known. qRT-PCR revealed up-regulation of miR-4425 in multiple breast cancer cell lines, including MCF-7, MDA-MB-231, and T47D, when treated with Rh2 (Fig. 2B), which indicated that the upregulated STXBP5-AS1 by Rh2 might act as a ceRNA against miR-4425 by sponging its activity. To prove this hypothesis, we first measured the luciferase activity after constructing recombinant plasmids so that the 8597-bp STXBP5-AS1 DNA fragment, including the binding sequence, was placed downstream the luciferase gene (Fig. 2C). The mimic of miR-4425 decreased the luciferase activity by 26%, but the construct containing the mutant binding sequence was not affected at all (Fig. 2D). Next, the expression of miR-4425 was examined after inducing deregulation of STXBP5-AS1, and *vice versa*, in the MCF-7 cells. Down-regulation of STXBP5-AS1 by siRNA accompanied increased expression of miR-4425, and down-regulation of miR-4425 by an inhibitor RNA resulted in increased expression of STXBP5-AS1 (Fig. 2E). The effect of miR-4425 on STXBP5-AS1 was further supported by an experiment using mimic RNA of miR-4425, which showed down-regulation of STXBP5-AS1 (Fig. 2F). These results collectively suggest a ceRNA relationship between the two ncRNAs.

3.3. STXBP5-AS1 regulates miR-4425 target genes

To elucidate the role of miR-4425 in cancer cell proliferation, its effect on cell growth and apoptosis was examined after being deregulated in the MCF-7 cells. First, miR-4425 was upregulated

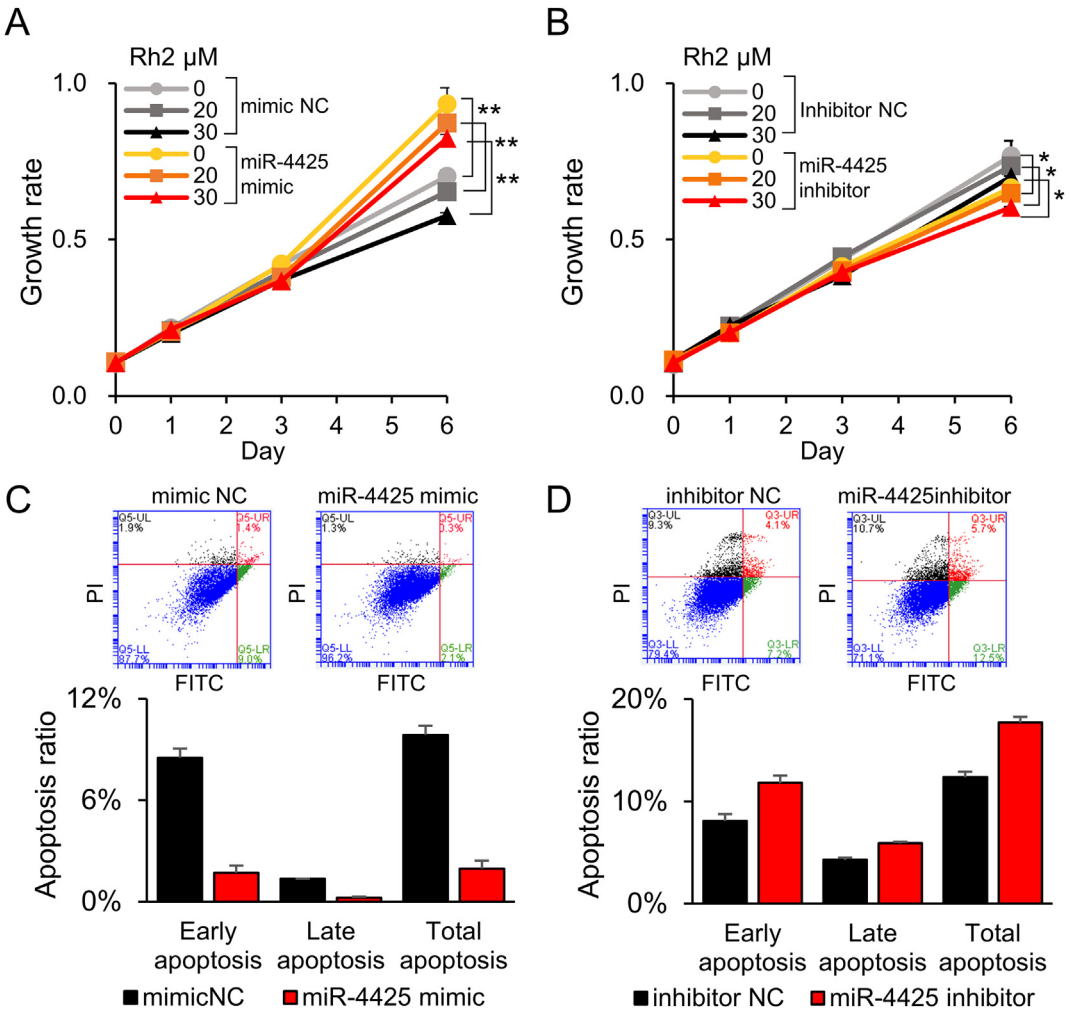

Fig. 3. MiR-4425 stimulates the growth of MCF-7 cancer cells. MiR-4425 was deregulated in MCF-7 by transiently transfecting the cells with mimic (A) or inhibitor RNA (B) in combination with Rh2, and then cell proliferation was assessed by the CCK-8 assay. Effect of miR-4425 on apoptosis was observed by flow cytometry after treating the cells with a mimic (C) or an inhibitor RNA (D). All experiments were performed in triplicate, and the results are presented as the mean ± SE. Representative images are shown for the flow cytometry analysis. Mimic NC, negative control miR-4425 mimic; inhibitor NC, negative control inhibitor RNA for miR-4425. *$P < 0.05$.

with a mimic RNA, which stimulated the cells to grow faster than the control RNA-treated cells (Fig. 3A). Accordingly, treatment of the cells with an inhibitor RNA suppressed the cell growth (Fig. 3B). Cotreatment of Rh2 with the mimic or inhibitor on the cells acted to inhibit cell growth. Next, apoptosis was monitored in the miR-4425-deregulated cells. The mimic RNA decreased apoptosis, but the inhibitor RNA increased apoptosis (Fig. 3C and D). These data indicate the pro-proliferation activity of miR-4425, which contributes to cell growth in the opposite way to STXBP5-AS1.

As the action mode of miRNAs is to bind and regulate target genes, the target genes of miR-4425 were searched on an *in silico* target gene prediction platform, which represented 246 potential targets from four programs; MicroT, miRDB, miRWalk, and TargetScan (Fig. 4A). The methylation array data obtained by treating the MCF-7 cells with Rh2 (GSE93356) were then applied to further narrow down the number of target genes that would be potentially upregulated *via* promoter hypomethylation by the ginsenoside and targeted by miR-4425, exposing three genes (Fig. 4B and C). To decide whether the selected genes are targets in the Rh2/STXBP5-AS1/miR-4425 regulatory axis, their expression was checked in the MCF-7 cells under various treatment conditions with Rh2, STXBP5-AS1, and miR-4425. As a result, only RNF217 satisfied the criteria of the expression profile, that is, upregulated by Rh2, downregulated by siRNA of STXBP5-AS1, downregulated by mimic RNA of miR-4425, and upregulated by inhibitor RNA of miR-4425 (Fig. 4D–G). Western blot analysis confirmed a similar expression profile between RNF217 and RNA under the same treatment conditions (Fig. 5A–D and Supplementary Fig. S2). Down-regulation of RNF217 increased the MCF-7 cancer cell growth but decreased apoptosis, suggesting tumor-suppressive activity (Fig. 5E and F and Supplementary Fig. S1). Accordingly, the higher the expression of RNF217, the higher the survival rate of breast cancer patients in a cohort of 4934 patients from GEO, EGA, and TCGA (Fig. 5G). In summary, ginsenoside Rh2 upregulates STXBP5-AS1 sponging miR-4425, leading to de-suppression of RNF217, which, eventually, suppresses the proliferation of MCF-7 cells (Fig. 6).

4. Discussion

This study identified a novel Rh2-regulated lncRNA and established a lncRNA/miRNA/target gene regulatory pathway associated with Rh2-induced inhibition of breast cancer cell growth. The study was prompted by the notable observation that Rh2 hypomethylated the promoter region of STXBP5-AS1, resulting in its upregulation in breast cancer cells. STXBP5-AS1 has shown anticancer activity in various cancers by inhibiting oncogenic *cis*-gene expression, such as STXBP5 and GRM1 in pancreatic cancer and breast cancer cells [22,23], or by modulating the activities of miRNAs, such as miR-96–5p in cervical cancer [24], or by targeting specific regulatory pathways, such as the PI3K/AKT pathway [25]. Together with our observation, this knowledge suggests the beneficial application of Rh2 in the chemoprevention of various cancers *via* inducing the tumor-suppressive STXBP5-AS1.

Although we revealed the molecular pathway initiated by Rh2 and its target genes suppressing cancer cell growth, how Rh2 regulates STXBP5-AS1 is still elusive. Similarly, the underlying mechanisms of other ginsenosides are yet unknown, although their potential targets are well described. As the chemical structure of

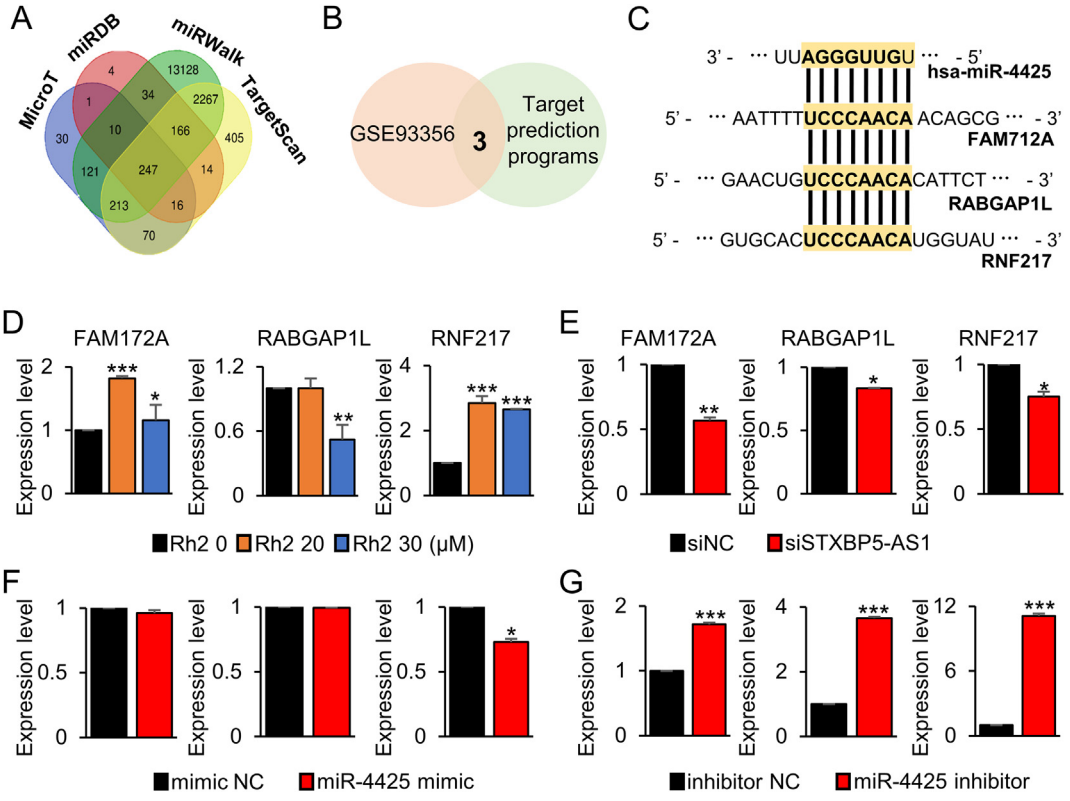

Fig. 4. Regulation of miR-4425 target genes by Rh2, STXBP5-AS1, and miR-4425. Potential miR-4425 target genes were screened using four public database platforms (MicroT, miRDB, miRWalk, TargetScan) (A), then further curtailed by comparison with the methylation array data of the Rh2-treated MCF-7 cells (GSE93356) (B). (C) Binding sequence on the target genes for miR-4425. The seed sequence is denoted in bold. Target gene expression was examined by qRT-PCR for Rh2-regulated genes. The MCF-7 cells were treated with Rh2 (D), STXBP5-AS1-specific siRNA (E), miR-4425 mimic (F), and miR-4425 inhibitor (G). siNC, control siRNA; mimic NC, negative control mimic for miR-4425; inhibitor NC, negative control inhibitor for miR-4425. All experiments were performed in triplicate, and the results are presented as the mean ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

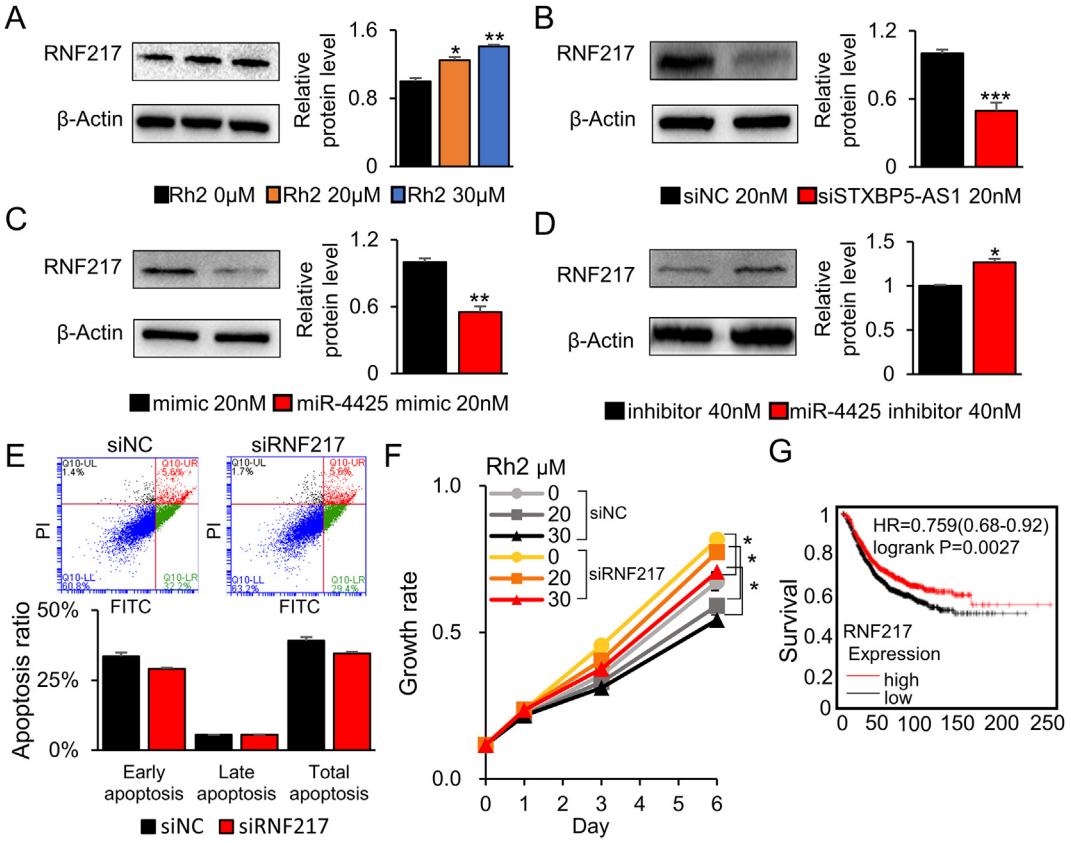

Fig. 5. Effect of RNF217 on cancer cell growth and apoptosis. Western blot analysis of RNF217 was performed after treating MCF-7 cells with Rh2 (A), STXBP5-AS1-specific siRNA (B), miR-4425 mimic (C), or miR-4425 inhibitor (D). The band intensity was measured using the Image Lab software and indicated as bar graphs. MCF-7 cells were treated with a siRNA to RNF217, and apoptosis (E) and cell growth rate (F) were examined. (G) Kaplan–Meier survival curves for patients with breast cancer according to the expression level of RNF217. siNC, control siRNA; mimic NC, negative control mimic for miR-4425; inhibitor NC, negative control inhibitor for miR-4425. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Rh2 is similar to the synthetic glucocorticoid dexamethasone, it is speculated that Rh2 might bind with glucocorticoid receptor (GR) or its analogous nuclear receptor and stimulate translocation of GR [8]. Rh2 has also been shown to affect cellular functions *via* changing the physical properties of cholesterol-regulated membrane lipid bilayers [26] or binding the lipid portion of the cell membrane [27]. Examining whether STXBP5-AS1 is responsive to glucocorticoid would provide insight into the hormone-like regulation of STXBP5-AS1.

One way lncRNAs impact cellular events is by controlling the expression of *cis*-genes, which are on the chromosomal DNA flanking the lncRNA gene and spanning a few hundred kilobases. One study identified several genes *cis* to STXBP5-AS1 and RFX-AS1 regulated by the lncRNAs on the RNA and protein level in Rg3-treated MCF-7 cancer cells [23]. Another study found that STXBP5-AS1 suppressed the stem cell-like properties of pancreatic cancer cells by inhibiting neighboring androglobin gene expression [22]. Notably, all the regulated *cis*-genes are known as oncogenes. However, none of them appeared in the list of miR-4425 target genes, suggesting separate pathways by which STXBP5-AS1 regulates its *cis*-genes and the miRNA target genes. LncRNAs can control the expression of coding genes by modulating miRNAs [28]. In detail, lncRNAs can sponge miRNAs by binding to target sites within miRNAs, potentially affecting the regulation of its original targets, thereby acting as a ceRNA [29]. In this respect, STXBP5-AS1 could be a ceRNA of miR-4425, which was supported by the fact that they interacted through the target gene binding sequence and that each RNA was downregulated by the other.

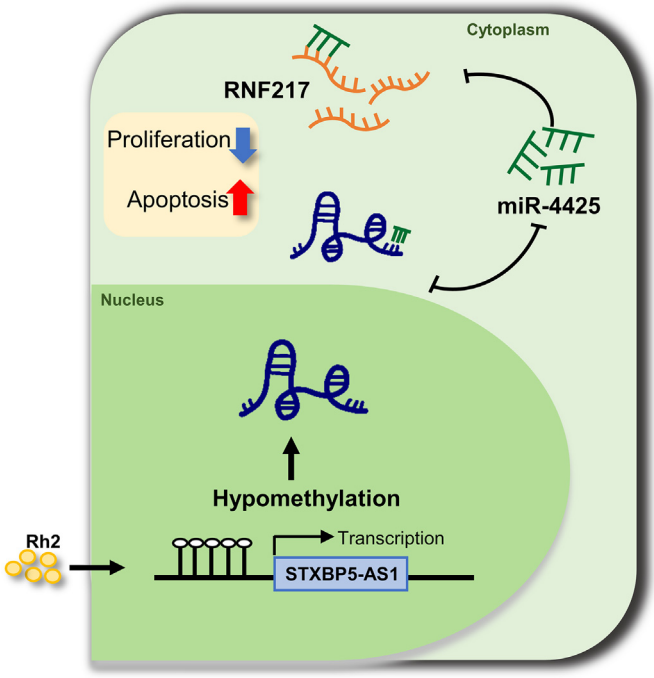

Fig. 6. Schematic diagram of the Rh2/STXBP5-AS1/miR-4425/RNF217 regulatory axis. Upregulation of STXBP5-AS1 by Rh2 *via* promoter hypomethylation suppresses the oncogenic miR-4425, resulting in activation of the tumor-suppressive gene, RNF217. The regulatory axis eventually contributes to the antiproliferation of cancer cells.

It should be mentioned that although miR-4425 is dysregulated in various cancers and targeted oncogenic genes, its target genes were not found in the list of this study. This discrepancy may be attributed to the diverse regulatory factors acting on miR-4425, such as Rh2/STXBP5-AS1, as in this study, or other ginsenosides and lncRNAs. For example, lncRNA HCG11 suppressed glioma growth by cooperating with the miR-4425/MTA3 axis [30]. In addition, Rg3 upregulated FDFT1 *via* reducing miR-4425 to inhibit ovarian cancer progression [31]. Notably, up-regulation of STXBP5-AS1 was also observed in Rg3-treated MCF-7 cells [23], implicating Rh2 and Rg3 as common regulators of the STXBP5-AS1/miR-4425 pathway. It has already been shown that Rh2 and Rg3 affect various pharmacological events, such as immunosuppression, anti-proliferation, and pro-apoptosis, by acting alone, as well as synergistically [32]. The structural similarity between the two ginsenosides sharing the protopanaxadiol-type compound group could explain their common regulation of STXBP5-AS1, but this is yet to be determined.

RNF217 encodes a highly conserved RING finger protein and interacts with the anti-apoptotic protein HAX1 [33]. Expression of RNF217 was highly detected in testis and skeletal muscle, but no data are available regarding its expression in breast cancer. Therefore, elucidating the molecular mechanism by which RNF217 contributes to the antiproliferation of cancer cells should be followed up to complete the picture of Rh2-induced inhibition of cancer cell growth *via* the Rh2/STXBP5-AS1/miR-4425/RNF217 axis. Our revelation of the antiproliferation effect of RNF217 and its up-regulation by Rh2 indicates that the anticancer activity of Rh2 is, at least in part, due to the tumor-suppressive activity of RNF217. However, how RNF217 inhibits cancer cell growth still remains elusive, although downregulation of the gene resulted in decreased apoptosis but increased proliferation of the MCF-7 cancer cells. A comprehensive analysis of the affected gene after deregulating RNF217, STXBP5-AS1, or miR-4425 would give an insight to reveal its activity in apoptosis.

In conclusion, lncRNA STXBP5-AS1 was upregulated by ginsenoside Rh2 *via* inducing hypomethylation at the promoter CpG in breast cancer cells. Rh2 augmented the suppression of cancer cell growth by STXBP5-AS1. MiR-4425 was downregulated by STXBP5-AS1 and also downregulating STXBP5-AS1, suggesting a sponging relationship between them. A target gene of miR-4425, RNF217, was also regulated by Rh2 and STXBP5-AS1. These results suggest that ginsenoside Rh2 suppresses MCF-7 cancer cell proliferation through targeting the STXBP5-AS1/miR-4425/RNF217 axis.

Declaration of competing interest

The authors declare that they have no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.08.006.

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