Research Article

Ginsenoside Rg3 and Korean Red Ginseng extract epigenetically regulate the tumor-related long noncoding RNAs RFX3-AS1 and STXBP5-AS1

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ABSTRACT

Background: Ginsenoside Rg3, a derivative of steroidal saponins abundant in ginseng, has a range of effects on cancer cells, including anti-cell proliferation and anti-inflammation activity. Here, we investigate two long noncoding RNAs (lncRNAs), STXBP5-AS1 and RFX3-AS1, which are hypomethylated and hypermethylated in the promoter region by Rg3 in MCF-7 cancer cells.
Methods: The lncRNAs epigenetically regulated by Rg3 were mined using methylation array analysis. The effect of the lncRNAs on the apoptosis and proliferation of MCF-7 cells was monitored in the presence of Rg3 or Korean Red Ginseng (KRG) extract after deregulating the lncRNAs. The expression of the lncRNAs and their target genes was examined using qPCR and Western blot analysis. The association between the expression of the target genes and the survival rate of breast cancer patients was analyzed using the Kaplan-Meier Plotter platform.
Results: STXBP5-AS1 and RFX3-AS1 exhibited anti- and pro-proliferation effects, respectively, in the cancer cells, and the effects of Rg3 and KRG extract on apoptosis and cell proliferation were weakened after deregulating the lncRNAs. Of the genes located close to STXBP5-AS1 and RFX3-AS1 on the chromosome, STXBP5, GRM1, RFX3, and SLC1A1 were regulated by the lncRNAs on the RNA and protein level. Breast cancer patients that exhibited a higher expression of the target genes of the lncRNAs had a higher metastasis-free survival rate.
Conclusion: The current study is the first to identify lncRNAs that are regulated by the presence of Rg3 and KRG extract and that subsequently contribute to inhibiting the proliferation of cancer cells.

1. Introduction

The ginsenoside Rg3 is an active monomer found in ginseng roots [1] that has a wide range of antitumor effects, including inhibiting proliferation, metastasis, and angiogenesis, inducing apoptosis, increasing susceptibility to chemotherapeutic treatment, and stimulating the immune system [2]. This anti-cancer activity is achieved by boosting or suppressing several cancer pathways. For example, in colorectal cancer cells, Rg3 exerts powerful antitumor effects through the inhibition of the Wnt/β-Catenin, NF-κB, PBK/TOPK, and MAPK/ERK signaling pathways [3], while in glioma cells, treatment with Rg3 arrests senescence-like growth *via* the Akt and p53/p21 pathways [4].

Rg3 induces apoptosis *via* two major pathways: the mitochondrial-dependent intrinsic apoptotic pathway [5] and the death receptor-dependent extrinsic pathway [6]. In both of these pathways, many proto-oncogenes and/or tumor suppressor genes are deregulated. For example, P53 is a well-known tumor suppressor that is promoted by Rg3 treatment in gallbladder cancer cells [7], while the oncogenes C/EBPβ [3], HIF1α [8], and MMP-9 [9] are downregulated by Rg3 in a variety of cancer cells. Recently, Rg3 was shown to epigenetically regulate tumor-related genes by modulating the methylation of cytosine residue at the promoter [10]. In addition, BCL2 and VEGF proto-oncogenes are hypermethylated and P53 is hypomethylated by Rg3 in HepG2 hepatocarcinoma cells.

Long noncoding RNAs (lncRNAs) are RNA molecules longer than 250 nucleotides which act as RNA and are not translated into proteins. They assist in the control of fundamental biological processes by regulating almost all aspects of gene expression at the epigenetic [11], transcriptional, and post-transcriptional levels [12]. The aberrant expression of lncRNAs has been linked to a number of malignancies, including cancer [13], thus providing new insights into the development and progression of cancer. In recent years, significant advances have been made in understanding the mechanisms by which lncRNAs function. Some well-characterized nuclear lncRNAs, such as XIST, have been shown to modulate gene expression *in cis* by locally regulating chromatin architecture [14]. It has also been proposed that a class of lncRNAs that includes lincRNA-p21 regulates gene expression *in trans* by directing the chromatin localization of protein binding partners [15].

Despite the growing knowledge base regarding the role of lncRNAs in cancer cells, little research has been conducted to investigate the relationship between ginsenosides and lncRNAs. It has been reported that Rg1 downregulates the lncRNA RP11-982M15.8 in Muller cells in high-glucose cultures, inhibiting the mesenchymal activation induced by the high-glucose conditions [16]. In another study, levels of the lncRNA H19 increased significantly in Rh2-treated MC3T3-E1 cells, resulting in the overexpression of osteopontin [17]. This suggests that H19 is an important contributor to Rh2-mediated MC3T3-E1 proliferation *via* the regulation of osteopontin.

Rg3 has been shown to regulate a large number of protein-coding genes, thus participating in important cellular activities, but little is known about its relationship with lncRNAs. In this study, two lncRNAs, RFX3-AS1 and STXBP5-AS1, whose promoter methylation levels were affected by Rg3 in the breast cancer cell line MCF-7, were identified. The expression of these lncRNAs and their effect on cancer cell proliferation and apoptosis were examined at both the molecular and cellular level. Cis genes located close to the two lncRNAs were also identified and monitored to determine how they are affected by the lncRNAs. The contribution of the lncRNAs and their associated cis-regulatory genes to the cancer-free survival of breast cancer patients was then analyzed using The Cancer Genome Atlas (TCGA) database. The current study, to the best of the authors' knowledge, is the first to address the role of lncRNAs epigenetically regulated by Rg3 in cancer cell proliferation.

2. Materials and methods

2.1. Cell cultures, transfection, and chemical treatment

The breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% FBS (Capricorn, Ebsdorfergrund, Germany) and 1% penicillin-streptomycin. The ginsenoside Rg3 and Rh2 (LKT Labs, St. Paul, Minnesota, USA), Korean Red Ginseng (KRG) extract (kindly donated by Korea Ginseng Co., Daejon, Korea), and 5-Aza-2′-deoxycytidine (5-Aza; Sigma-Aldrich, St. Louis, MO, USA) were used to treat the cells in a 60-mm culture dish at a confluence of 50-70%. The contents of various ginsenosides in KRG was determined as described previously [18]. Rg3 and Rh2 stocks were prepared with 20 mM in 100% ethanol and used in final concentrations of 20 and 50 μM. KRG extract was dissolved in distilled water and treated to cells at final concentrations of ∼200 μg/ml. 5-Aza stock was prepared with 10 mM in 100% DMSO (Sigma-Aldrich) and treated to cells in final concentrations of 2.5, 5, and 10 μM. Twenty-four hours after treatment by Rg3, Rh2, KRG extract, or 5-Aza, the cells were harvested, washed with PBS, and trypsinized using 0.05% trypsin-EDTA (Gibco BRL).

2.2. Transfection

The siRNA used to target STXBP5-AS1 was purchased from Qiagen (Redwood City, CA, USA) and an overexpression vector for RFX3-AS was constructed using the pcDNA3.1/Hygro(+) vector (Invitrogen, Carlsbad, CA, USA) by Bionics (Seoul, Korea). All siRNAs and overexpression vectors were diluted in OptiMEM Medium (Gibco BRL). The MCF-7 cells were seeded in a 60-mm dish with a confluence of 50-70% 24 h prior to transfection. The cells were transfected with siRNA at final concentrations of 20 and 40 nM using Lipofectamine RNAiMAX Reagent (Invitrogen) following the manufacturer's instructions. The cells were transfected with the overexpression vectors at a final concentration of 0.5 μg using Lipofectamine 3000 Reagent (Invitrogen) following the manufacturer's instructions. Information about the siRNAs and overexpression vectors are provided in Supplementary Table S1.

2.3. Cell survival and apoptosis assays

The rate of cell growth or survival was analyzed using two independent methods: cell proliferation assays and colony formation assays. For the cell proliferation assays, 3×10^3 cells were seeded in each well of a 96-well plate and cultured for 5 days. Every 24 h, 10 μl of Cell Counting Kit-8 reagent (Dojindo, Kumamoto, Japan) was added to each well. After incubation for 90 min, the absorbance was measured at 450 nm using a microplate reader. For the colony formation assays, 3×10^3 cells were seeded in a 60-mm culture dish. After two weeks, the cells were washed twice with PBS and fixed with a fixation solution (methyl alcohol and acetic acid at a ratio of 7:1). The cells were then stained with 0.2% crystal violet (Gibco BRL). Colony numbers were calculated using ImageJ software. For the apoptosis assays, cells were seeded on a 6-well plate at a confluence of 50-70% and cultured for 24 h. The cells were then transfected with siRNA or recombinant plasmids and cultured for another 24 h for treatment with Rg3. The cells were washed with PBS and trypsinized before harvest. The cells were suspended with a binding buffer to produce a final concentration of 10^6 cells/ml. Cell staining was performed using FITX Annexin V and propidium iodide (PI) reagents in the Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). Apoptosis was analyzed with a flow cytometer (BD Biosciences) with measurements taken using a 488-nm laser.

2.4. Methylation-specific PCR

The genomic DNA from the cultured cells was extracted using a ZR-Duet DNA/RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) following the supplier's instructions. One microgram of DNA was modified for bisulfite conversion using a Zymo Research EZ DNA Methylation Kit (Zymo Research). Methylation levels were measured using PCR on an ABI 7300 system (Applied Biosystems, Foster City, CA) and calculated using the following equation: $1/[1 + 2^{-(CTu - CTme)}] \times 100\%$, where CTu is the average cycle threshold (CT) obtained from duplicate quantitative PCR analyses using an unmethylated primer pair, and CTme is the average CT obtained using a methylated primer pair. Information about the primers is provided in Supplementary Table S2.

2.5. Quantitative RT-PCR

Total RNA was isolated from the cells cultured in a 60-mm culture dish using a ZR-Duet DNA/RNA MiniPrep kit (Zymo Research). cDNA was synthesized from 2 μg of RNA using ReverTra Ace qPCR RT Master Mix with a gDNA remover (Toyobo, Osaka, Japan). PCR was conducted using a Kapa SYBR Fast qPCR kit (Kapa Biosystems,

Woburn, MA, USA). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized using GAPDH. Information about the primers is provided in Supplementary Table S2.

2.6. Western blot analysis

Total protein was extracted from the cultured cells using a chilled RIPA lysis buffer containing a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Thirty micrograms of the protein sample were loaded into a well of SDS-PAGE gel. After electrophoresis, the protein was transferred to polvinylidene difluoride membranes (Whatman, UK). The blots were then incubated at 4 °C with anti-RFX3 (1:1000, Genetex, Irvine, CA, USA), anti-SLCA1A (1:800, Bioss, Woburn, MA, USA), anti-STXBP5 (1:1000, Genetex), and anti-GRM1 (1:1000, Cusabio Biotech, China) antibodies. After rinsing three times in a 0.01% Tween-20 TBS buffer, the blots were incubated with HRP-conjugated anti-rabbit secondary antibodies (1:1000, Genetex) for 1 h. Protein bands were detected using an ECL solution (Abfrontier, Korea) and quantified with Image Lab software (Bio-Rad, Herculer, CA, USA).

Table 1
lncRNAs of Which Methylation Level is Significantly Affected by Rg3

Symbol	Accession no.	Description	Δ β[a]	Fold change[b]
ATXN8OS	NR_002717	ATXN8 opposite strand	0.189	1.546
RFX3-AS1	NR_121586	RFX3 antisense RNA 1	0.188	1.42
LINC00911	NR_102737	long intergenic non-protein coding RNA 911	0.157	1.648
DOCK4-AS1	NR_103806	DOCK4 antisense RNA 1	0.152	2.013
LINC01018	NR_024423	long intergenic non-protein coding RNA 1018	−0.155	−1.521
LINC01477	NR_110791	long intergenic non-protein coding RNA 1477	−0.164	−1.598
STXBP5-AS1	NR_034115	STXBP5 antisense RNA 1	−0.17	−1.605

[a] The values are obtained by subtracting the methylation level of the control cells from that of Rg3-treated cells.
[b] The values are ratios obtained by dividing the level from Rg3-treated cells by that of the control cells.
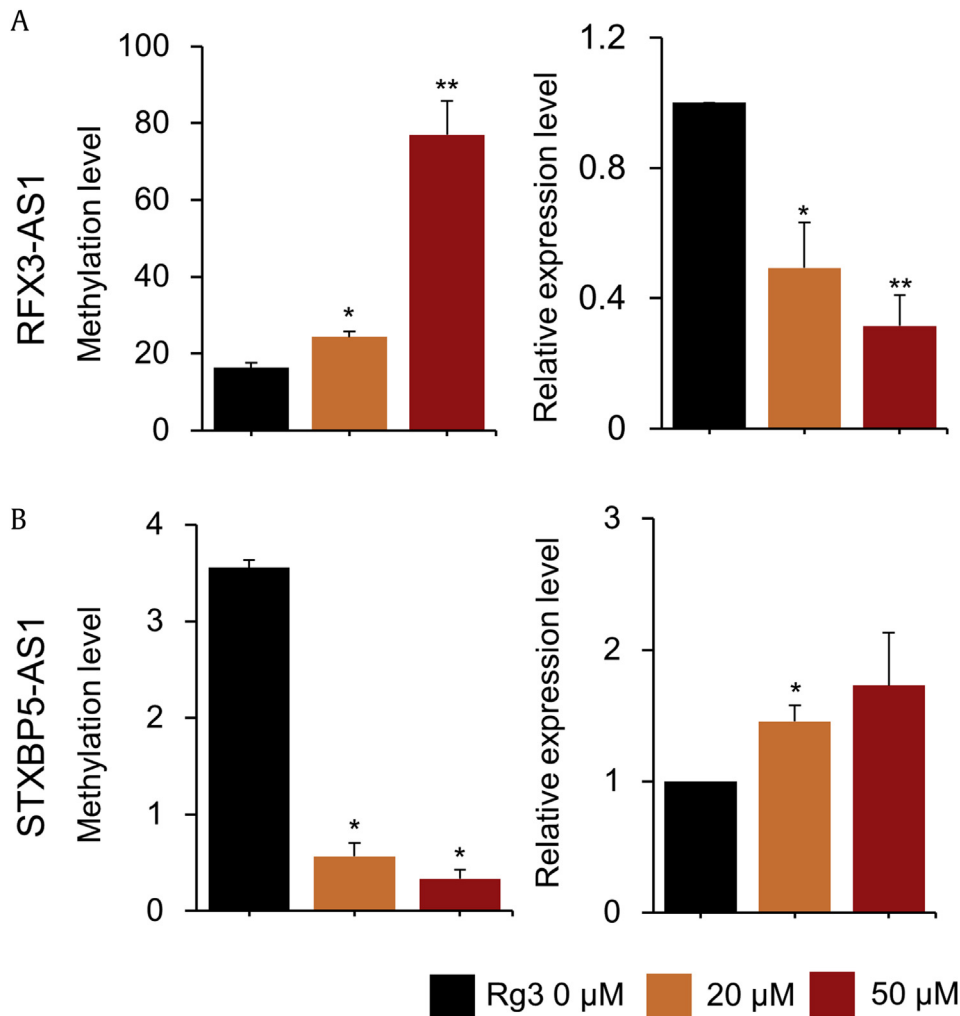
Fig. 1. The effect of Rg3 on the methylation status of RFX3-AS1 and STXBP5-AS1 at the promoter. The methylation and expression of RFX3-AS1 (A) and STXBP5-AS1 (B) after treatment with Rg3 in MCF-7 cells. Changes in the methylation levels of the two lncRNAs were identified using microarray analysis and they were examined further using methylation-specific PCR and qPCR. All of the experiments were performed in triplicate and the values are presented as the mean ± standard error (SE). *$P < 0.05$, **$P < 0.01$.

2.7. Data mining and statistical analysis

All experiments were independently conducted at least three times and plotted using Microsoft Excel 2016 for Windows. Analysis of variance (ANOVA) was used to compare significant differences in all experiments where multiple concentrations of chemicals or siRNAs were used. $P < 0.05$ was considered statistically significant. The association between gene expression levels and the overall survival rate of breast cancer patients was evaluated using the Kaplan-Meier Plotter (http://kmplot.com/analysis) and databases from GEO (https://www.ncbi.nlm.nih.gov/geo, Affymetrix microarrays only), EGA (https://www.ebi.ac.uk/ega), and TCGA (https://cancergenome.nih.gov).

3. Results

3.1. Rg3 alters the methylation levels of the lncRNAs

In our previous study of genome-wide methylation following the Rg3 treatment of MCF-7 breast cancer cells, a group of coding genes exhibited changes in their methylation level at the promoter CpGs [19]. This array contained 11,485 CpGs (2.5%) for lncRNAs and the analysis revealed significant effects on the methylation levels of seven lncRNAs ($|\Delta\beta| > 0.15$; Table 1). After searching the PubMed database, two lncRNAs whose partner coding gene encoded by the DNA sense strand is related to cancer development were selected for further investigation. The first, the 1109-base RFX3-AS1, is the anti-coding strand for the RFX-coding gene. The other, the 3082-base STXBP5-AS1, is the anti-coding strand for the STXBP5-coding gene. Although the functions of their corresponding coding strand genes have been fully characterized, the role of the two lncRNAs in cancer has yet to be determined. Therefore, their expression in cancer cells and their effect on cell proliferation were examined.

The expression of the lncRNAs in MCF-7 breast cancer cells after Rg3 treatment at concentrations of 20 μM and 50 μM was examined using RT-PCR. The expression of RFX3-AS1 was reduced in Rg3-treated cells while that of STXBP5-AS1 increased (Fig. 1). The methylation levels of the promoter CpG were also examined using methylation-specific PCR; the results showed that RFX3-AS1 was hypermethylated and downregulated, while STXBP5-AS1 was hypomethylated and upregulated. This suggests that Rg3 regulates the expression of the two lncRNAs by affecting promoter methylation. This is supported by the observation that the treatment of Rg3-treated MCF-7 cells with 5-Aza led to a movement back towards the original lncRNA expression levels, particularly for RFX3-AS1, which was hypermethylated by Rg3 (Fig. 2A). In case of STXBP5-AS1, the methylation level was not significantly decreased by 5-Aza (Fig. 2B). This is possibly due to the same effect of Rg3 and 5-Aza to decrease the methylation level of STXBP5-AS1. There may exist a lower limit of methylation level that could be induced by the two chemicals.
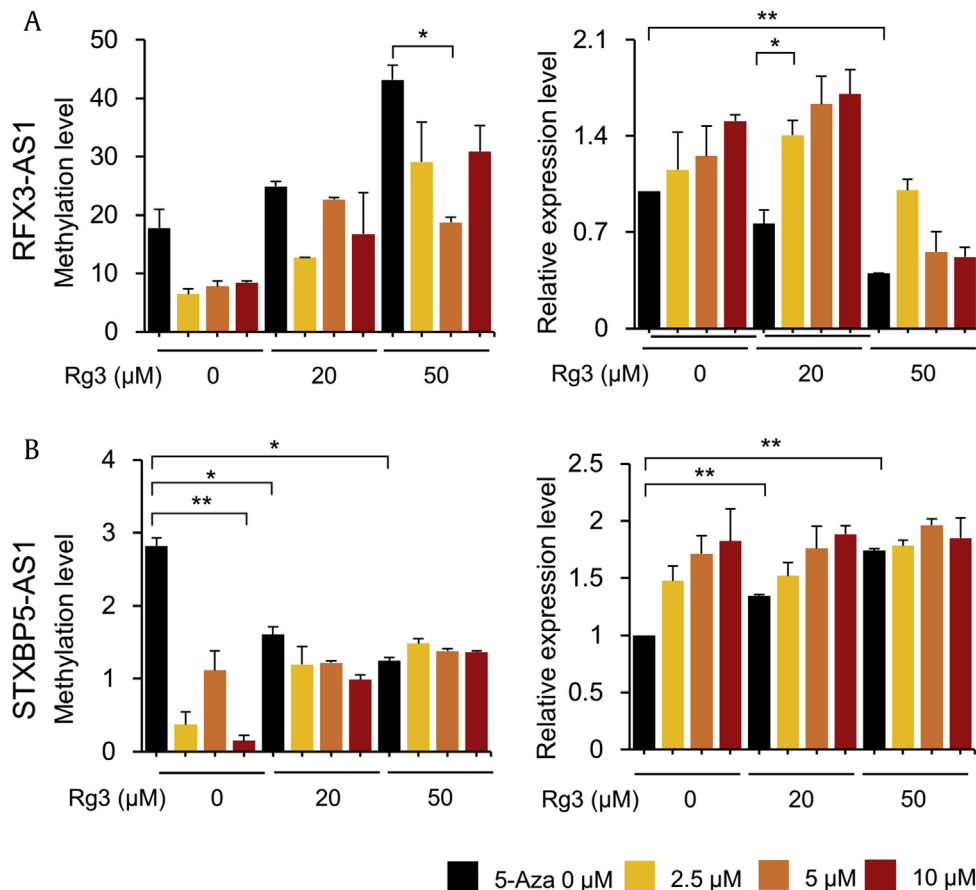
Fig. 2. The effect of the methylation status of RFX3-AS1 and STXBP5-AS1 at the promoter on expression levels. MCF-7 cells were treated with 5-Aza at the indicated concentrations. Dimethyl sulfoxide (DMSO, 0.1%) was used as a control. Rg3 was added together with 5-Aza at concentrations of 20 and 50 μM. The control was 0.1% ethanol, which was used to dissolve the Rg3. The methylation and expression levels of RFX3-AS1 (A) and STXBP5-AS1 (B) were examined using MSP and qPCR. All of the experiments were performed in triplicate and the values are presented as the mean ± SE. *$P < 0.05$, **$P < 0.01$.

### 3.2. STXBP5-AS1 and RFX3-AS1 are involved in the apoptosis and proliferation of MCF-7 cancer cells

To investigate the role of STXBP5-AS1 and RFX3-AS1 in cancer cells, the lncRNAs were deregulated by transiently transfecting the MCF-7 cells with siRNAs or overexpression recombinant plasmids and then monitoring cellular activity, such as apoptosis and proliferation. We decided to overexpress RFX3-AS1 because it was hypermethylated and downregulated by Rg3 (Supplementary Fig. S1). As a result, both early and late apoptosis decreased by 51%; this effect was weakened by Rg3 treatment (Fig. 3A). The cell survival rate increased by up to 16% with the overexpression of RFX3-AS1 based on colony formation assays, but Rg3 treatment lowered this effect (Fig. 3B). The increase in the growth rate was also supported by dye-based proliferation assays, although the increased level was lower than that demonstrated in the colony formation assays (Fig. 3C).

STXBP5-AS1 was downregulated because it was hypomethylated and upregulated by Rg3 (Supplementary Fig. S1). Both early and late apoptosis increased by 27% and 14%, an effect that was diminished by Rg3 treatment (Fig. 4A). The cell survival rate also increased by up to 30% and 20% in the colony formation and the dye-based assays, respectively (Fig. 4B and C).

3.3. Rg3 targets cancer-related genes through RFX3-AS1 and STXBP5-AS1

One of the regulatory mechanisms of lncRNAs is the cis-regulation of neighboring genes on the chromosome. To identify genes that could be potentially regulated by RFX3-AS1 and STXBP5-AS1, those located close to the target lncRNAs were screened and their expression levels examined at the RNA and/or protein level. Four genes were found within 900 kb of RFX3-AS1 (Fig. 5A), three of which (PUM3, RFX3, and SLCA1A) were able to be amplified using RT-PCR. All three genes exhibited increased expression levels in the presence of Rg3 (Fig. 5B). RFX3 and SLCA1A were analyzed further using Western blot analysis, which also found increased expression levels at the protein level following Rg3 treatment (Fig. 5C). To more clearly understand the role of RFX3-AS1 in the expression of these cis genes, their expression levels were examined after the upregulating of RFX3-AS1 *via* transient transfection by recombinant plasmids. There was a subsequent decrease in the expression of the three genes at the RNA level and a decrease in the expression of RFX3 and SLCA1A at the protein level (Fig. 5D).

Five genes were found within 500 kb of STXBP5-AS1 (Fig. 6A). STXBP5 and GRM1 were successfully amplified using RT-PCR; it was found that Rg3 increased the expression of STXBP5 but decreased
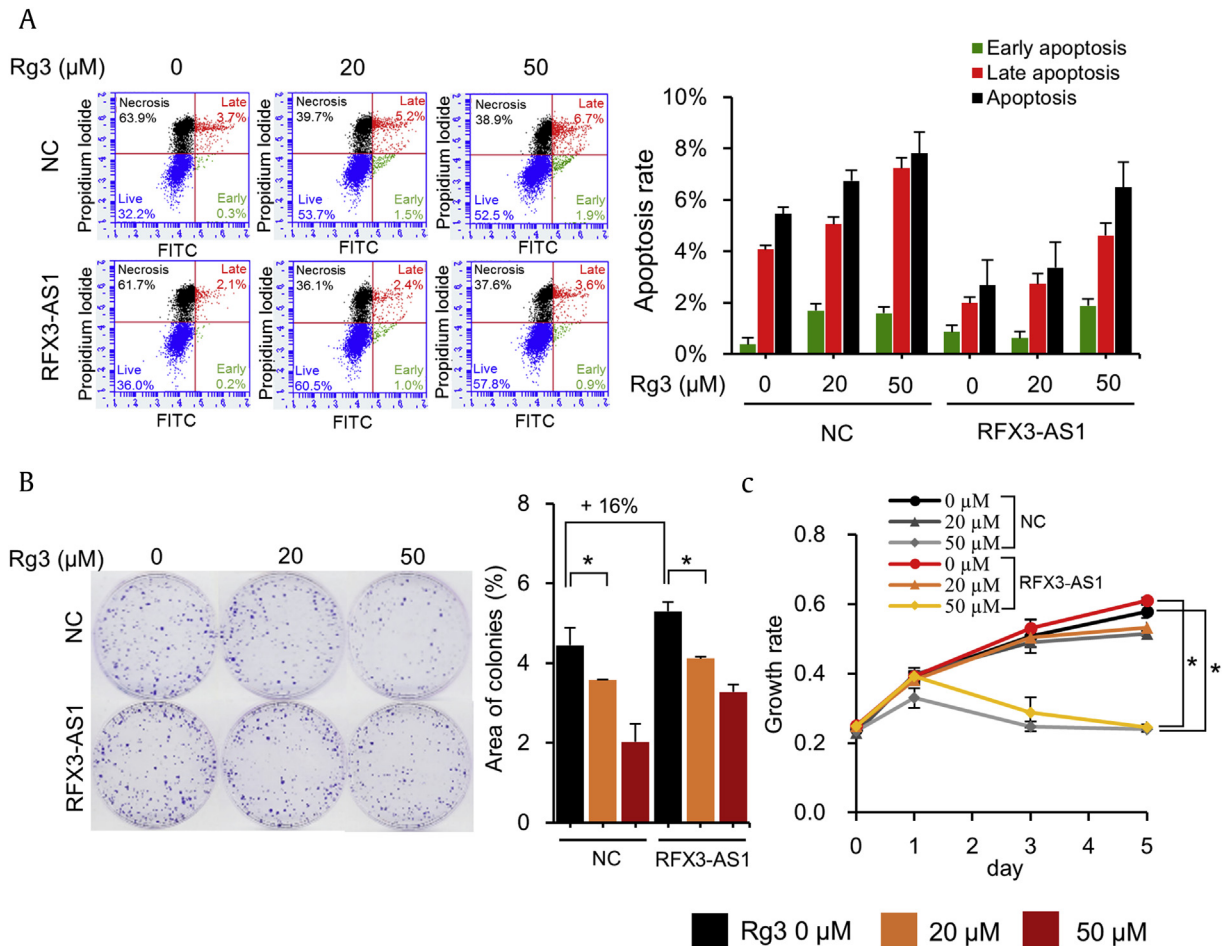
Fig. 3. The effect of RFX3-AS1 on apoptosis and cell survival. RFX3-AS1 was overexpressed in MCF-7 by transiently transfecting MCF-7 cells with an ORF-containing recombinant plasmid. (A) The transfected cells were examined for apoptosis using flow cytometry. The effect of RFX3-AS1 on cell growth was examined using colony formation analysis (B) and CCK-8 assays (C). NC, a control plasmid vector. Representative images are shown for flow cytometry and colony formation. All of the experiments were performed independently at least three times, and the values are presented as the mean ± SE. *$P < 0.05$.
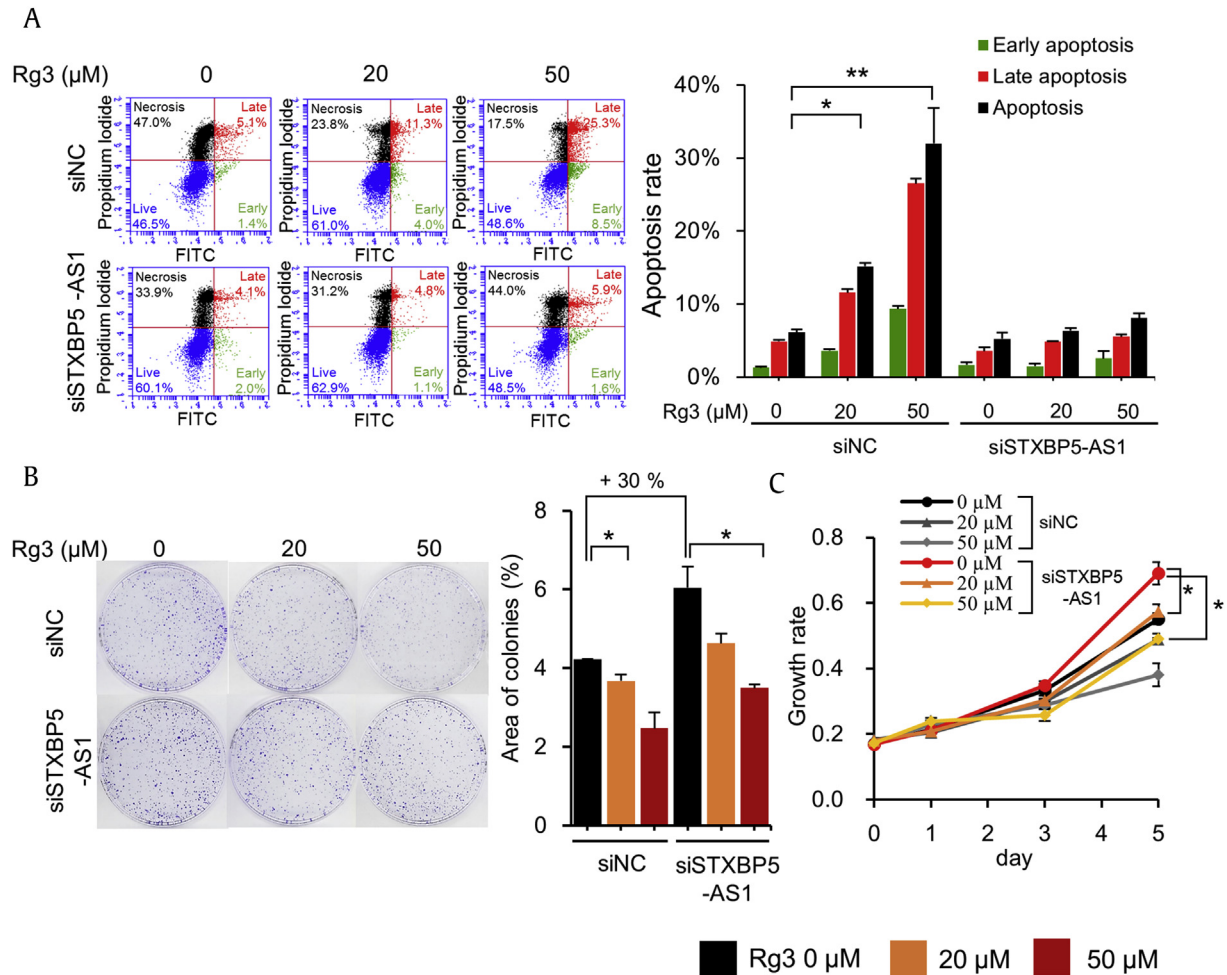
Fig. 4. The effect of STXBP5-AS1 on apoptosis and cell survival. (A) STXBP5-AS1 was downregulated in MCF-7 using siRNA, and apoptosis was examined using flow cytometry. Representative images are shown. The effect of the siRNA-driven downregulation of STXBP5-AS1 on cell proliferation was examined using colony formation analysis (B) and CCK-8 assays (C). Representative images are shown for flow cytometry and colony formation. All of the experiments were performed independently at least three times, and the values are presented as the mean ± SE. siNC, control siRNA. *$P < 0.05$, **$P < 0.01$.

that of GRM1, an observation confirmed at the protein level by Western blot analysis (Fig. 6B and C). The downregulation of STXBP5-AS1 using siRNA reversed the effect of Rg3 on the expression of the two genes, indicating that STXBP5 and GRM1 are downstream target genes of STXBP5-AS1 (Fig. 6D).

The oncogenic or tumor suppressive properties of specific genes are closely associated with the survival of cancer patients. The association between the genes regulated by the lncRNAs affected by Rg3 treatment and the development of cancer in cancer patients was investigated by analyzing the distant metastasis-free survival (DMFS) rate of breast cancer patients using the GOBO database. Higher DMFS rates were observed in cancer patients with a greater expression of STXBP5-AS1, STXBP5, and RFX3, all three of which are elevated by Rg3 treatment ($P < 0.05$; Supplementary Fig. S2). These findings support the conclusion that Rg3 has a beneficial effect on the survival of cancer patients by regulating lncRNAs and thus affecting the expression of tumor-related genes.

3.4. Korean Red Ginseng extract regulates RFX3-AS1 and STXBP5-AS1

Because the saponin fraction of KRG extract has a high Rg3 content (Supplementary Table S3), its efficacy in the regulation of RFX3-AS1 and STXBP5-AS1 was examined using the same experimental methodology as that used in the testing of Rg3. The saponin fraction of KRG extract induced the hypermethylation of the promoter region and a decrease in the expression of RFX3-AS1, while hypomethylating and increasing the expression of STXBP5-AS1 (Supplementary Fig. S3). Notably, a high concentration of KRG extract (200 μg/ml) was needed to affect the methylation levels. In contrast, expression levels were altered even at low concentrations of KRG extract (10 μg/ml). In the colony formation assay, the saponin fraction of KRG extract also suppressed pro-proliferation activity in MCF-7 cells in which RFX3-AS1 was overexpressed or STXBP5-AS1 was downregulated (Supplementary Fig. S4). The regulatory effects on RFX3-AS1 and STXBP5-AS1 were additionally examined for Rh2 as a negative control of which content in KRG extract was very low. The results indicated no significant association between the methylation and gene expression (Supplementary Fig. S5). In detail, RFX3-AS1 showed no significant change of methylation but was downregulated by Rh2. The altered expression level was not remarkable compared to Rg3. STXBP5-AS1 was hypermethylated by Rh2, which had been hypomethylated by Rg3. The expression of STXBP5-AS1 did not show consistency at Rh2 concentrations of 20 and 50 μM. These results imply that the altered methylation and expression of the two lncRNAs by KRG extract are mainly contributed by Rg3. These results imply that the methylation and expression of the two lncRNAs could be altered by Rg3 and KRG extract.
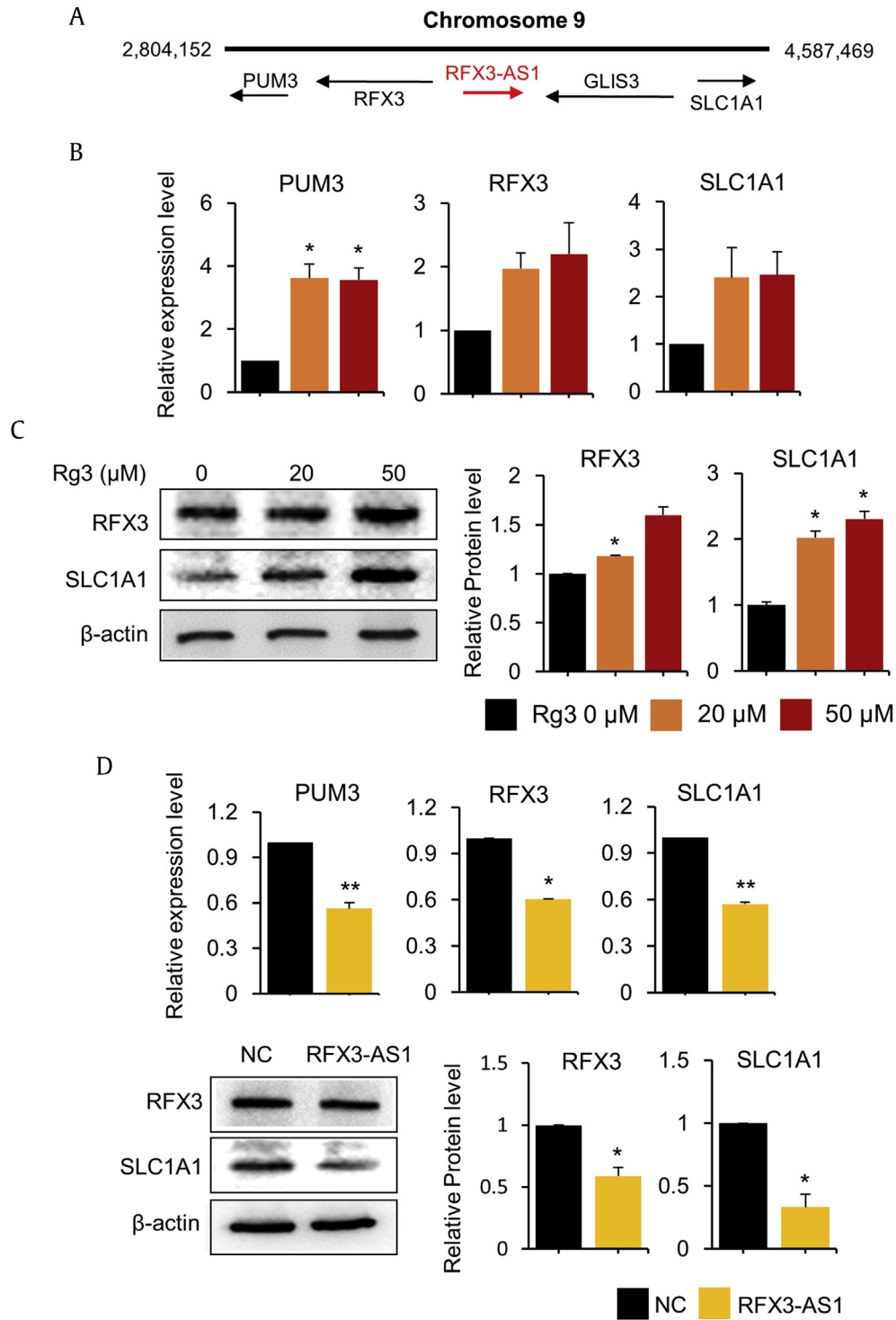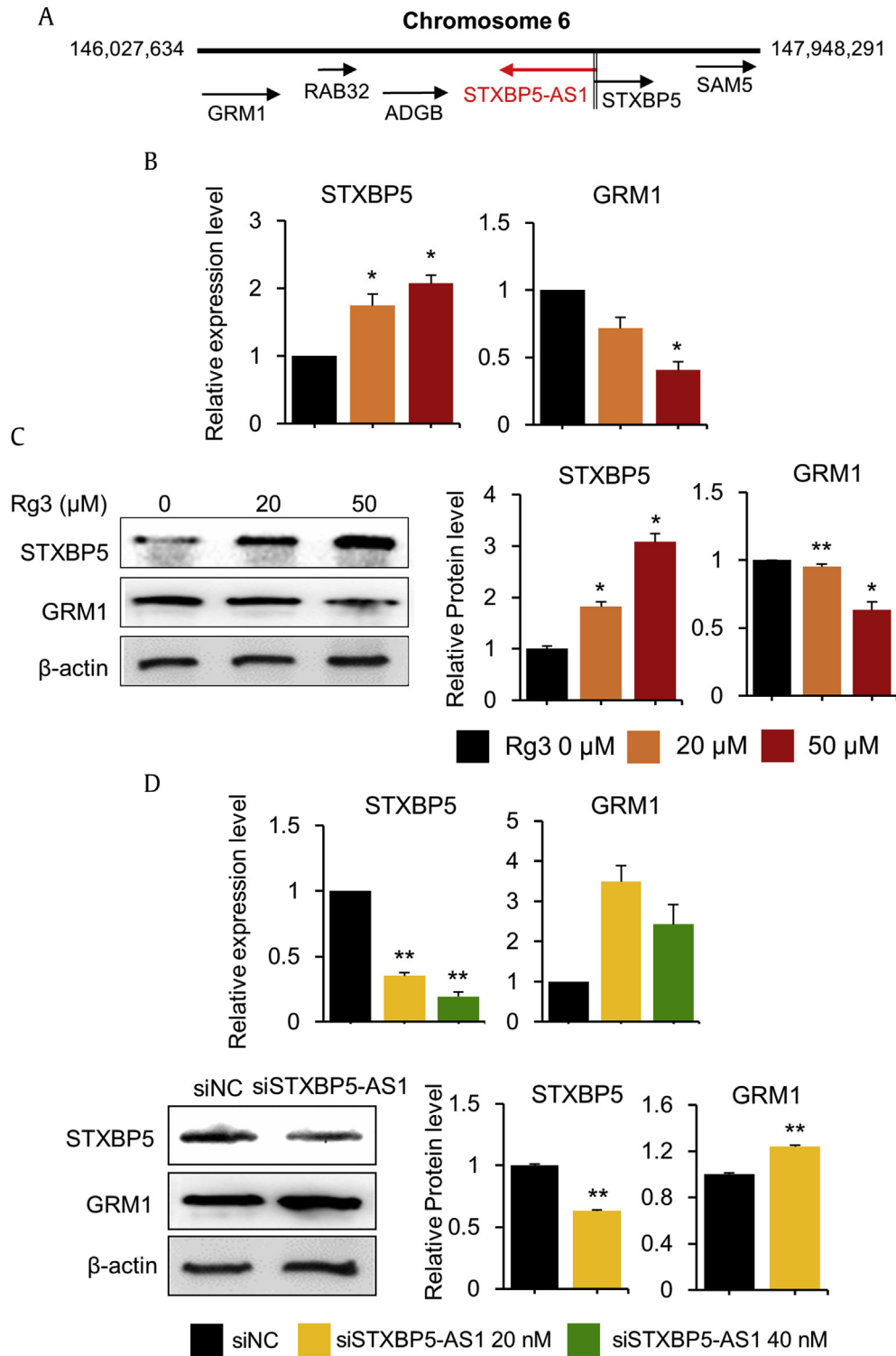
Fig. 5. The effect of Rg3 and RFX3-AS1 on the regulation of cis genes. (A) A schematic map of the relative position of RFX3-AS1 and its nearby genes. The numbers at the ends of the horizontal line are the first and last nucleotide of a sub-fragment on chromosome 9. The arrows indicate the expression direction. (B) The MCF-7 cells were treated with Rg3 and the expression levels of the cis genes were examined using qPCR. (C) Protein expression levels of RFX3 and SLC1A1 were examined using Western blot analysis. (D) Expression levels of the cis genes after the overexpression of RFX3-AS1 was measured using qPCR and Western blot analysis. All of the experiments were performed independently at least three times, and the values are presented as the mean ± SE. *P < 0.05, **P < 0.01.

4. Discussion

This study aimed to identify lncRNAs that were affected by Rg3 and KRG extract treatment and to understand the molecular mechanisms employed by these lncRNAs during cancer cell proliferation. Our initial methylation array analysis identified seven lncRNAs whose methylation levels were significantly affected by Rg3 treatment. The array covered 11,485 CpGs for lncRNAs; considering the growing number of newly identified lncRNAs, it is expected that even more lncRNAs would be affected by Rg3. It is

Fig. 6. The effect of Rg3 and STXBP5-AS1 on the regulation of cis genes. (A) A schematic map of the relative position of STXBP5-AS1 and its nearby genes. The numbers at the ends of the horizontal line are the first and last nucleotide of a sub-fragment on chromosome 6. The arrows indicate the expression direction. (B) The MCF-7 cells were treated with Rg3 and the expression levels of the cis genes examined using qPCR. (C) Protein expression levels of STXBP5 and GRM1 were examined using Western blot analysis. (D) Expression levels of the cis genes after the downregulation of STXBP5-AS1 were determined using qPCR and Western blot analysis. All of the experiments were performed independently at least three times, and the values are presented as the mean ± SE. *$P < 0.05$, **$P < 0.01$.

also possible that lncRNAs could be regulated by Rg3 *via* other routes such as transcriptional factors, microRNAs (miRs), and/or histone modification. As an example of a transcriptional factor, SP1 binds directly to the lncRNA PANDAR promoter region, which is

dysregulated in several cancer types, and activates its transcription [20]. lncRNA molecules may also act as a molecular sponge for miRs by sharing the same miR response elements with the target genes [21]. lncRNA PTEN1 is such a case, acting as an endogenous RNA

(ceRNA) that competes with miR-19b in breast cancer cells [22]. In terms of histone modification, H3K27 acetylation activates the expression of colon cancer-associated transcript-1 (CCAT1) [23].

Of the seven lncRNAs identified as being affected by Rg3, four were hypermethylated and three were hypomethylated. Previous research has also reported protein-coding genes that represented either hyper- or hypomethylation depending on specific CpGs in Rg3-treated MCF-7 cells [19]. These results indicate that, rather than having a uniform effect, Rg3 finely modulates DNA methyl-transferase so that it acts in a gene-specific manner. Little is known about the function of the seven lncRNAs whose methylation was affected by Rg3. ATXN8OS, which is the ataxin 8 opposite strand, was hypermethylated by Rg3 ($\Delta\beta = 0.189$). Interestingly, both (CUG) expansion in ATXN8OS and (CAG) expansion in ATXN8 were observed in patients with spinocerebellar ataxia type 8, indicating the involvement of noncoding (CUG) expansion transcripts in neurodegenerative pathogenesis [24].

Both of the lncRNAs investigated in this study, RFX3-AS1 and STXBP5-AS1, affected a set of nearby genes, suggesting that they operate in cis-acting mode. Cis-acting lncRNAs can differ in terms of their origin; they may arise from promoters, enhancers, or the antisense transcripts of protein-coding genes [25]. As indicated by their name, RFX3-AS1 and STXBP5-AS1 are the antisense transcripts of RFX3 and STXBP5, respectively. Antisense lncRNAs can positively or negatively affect the expression of their corresponding protein-coding genes. The two lncRNAs inhibited the expression of their partner genes, but the molecular mechanisms behind this remain to be determined.

Although the regulation of novel lncRNAs and their target genes by Rg3 was identified in the present study, except for a few genes, their specific role in cancer development has yet to be fully determined. It has been reported that STXBP5-AS1 may intervene in breast cancer pathogenesis by competitively binding to miR-190b, thus acting as a ceRNA [26]. Some targets of miR-190b, such as ERG, STK38L, and FNDC3A, are positively associated with STXBP5-AS1 as the result of this competition. The results of our cell proliferation and apoptosis assays suggest that RFX3-AS1 is a non-coding RNA with pro-proliferation activity, while STXBP5-AS1 is characterized by anti-proliferation activity.

RFX3, a target gene for RFX3-AS1 that is upregulated by Rg3, is a transcriptional target for SPEN that stimulates breast cancer cell migration [27]. The expression levels of these two proteins are strongly associated in cancer patients, indicating that RFX3 has metastasis-enhancing activity. When overexpressed, GRM1, a target gene for STXBP5-AS1 that is downregulated by Rg3, promotes increased proliferation, anchorage-independent growth, and invasiveness [28]. This pro-proliferation activity of GRM1 and its downregulation by Rg3 represent a good coincidence with the anti-proliferation activity of Rg3. However, not all of the target genes were deregulated by Rg3 in a way that suppressed cell proliferation. For example, RFX3, which exhibits oncogenic activity in breast cancer cells, was upregulated by Rg3. Nonetheless, as a whole, the data suggests that the anti-proliferation effect of Rg3 in cancer cells is in part achieved *via* the action of cancer-related lncRNAs.

A limitation of the current study is that the possible influence of trans-acting mode in the two target lncRNAs, which is a major regulatory mechanism in many other lncRNAs, was not considered. An example of this is the lncRNA H19, which was the first lncRNA to be identified and which is involved in EMT, migration, metastasis, and carcinogenesis [29]. The tumor suppressor protein and cell-cycle regulator p53 negatively regulate H19 in tumor cells. In a few cancers, H19 is known to physically interact with p53 and inactivate it. The identification of partner proteins that interact with RFX3-AS1 and STXBP5-AS1 and their mode of action should thus be a focus of future research.

In conclusion, two lncRNAs, RFX3-AS1 and STXBP5-AS1, were identified as being regulated by Rg3 and KRG extract *via* the alteration of methylation levels at the promoter. These lncRNAs play a role in the apoptosis and proliferation of MCF-7 cancer cells, regulating the expression of both their partner gene encoded by the opposite DNA strand and a number of cis genes, which then mediated the regulatory activity of the lncRNAs. The expression levels of the cis genes were closely associated with the survival rate of breast cancer patients. Overall, the current study provides insight into the anti-proliferation activity of Rg3 in cancer cells, which is achieved *via* the action of lncRNAs.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.02.004.

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