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Induction of growth arrest by miR-542-3p that targets survivin

Sena Yoon [a], Young-Chul Choi [a], Suman Lee [b], Yongsu Jeong [a], Jaeseung Yoon [a], Kwanghee Baek [a,*]

[a] Graduate School of Biotechnology, Kyung Hee University, Yongin, Republic of Korea
[b] Cell and Gene Therapy Research Institute, School of Medicine, CHA University, Seongnam-si, Republic of Korea

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ABSTRACT

Survivin is a protein which functions as a mitotic regulator as well as apoptosis inhibitor. In this study, we show that introduction of synthetic miR-542-3p mimetic reduced both mRNA and protein levels of survivin. In A549 cells, luciferase reporter assay revealed that miR-542-3p targeted predicted binding sites in the 3′-untranslated region (3′-UTR) of survivin. We also demonstrate that ectopic expression of miR-542-3p inhibited cell proliferation by inducing Gap 1 (G1) and Gap 2/Mitosis (G2/M) cell cycle arrest. Collectively, these results suggest that survivin is a direct target of miR-542-3p and growth inhibition by miR-542-3p may have a potential utility as an anti-cancer therapy.

1. Introduction

MicroRNAs (miRNAs) are small endogenous RNAs which regulate gene expression at the post-transcriptional level [1,2]. Accumulating evidence indicates that miRNAs play important roles in many cellular processes including proliferation, apoptosis, and differentiation. Recently, several studies showed that miRNAs affect cell proliferation by controlling cell cycle-related genes. For instances, miR-221 and -222 target cyclin-dependent kinase (CDK) inhibitor p27Kip1 and p57Kip2 [3,4]. Up-regulation of those miRNAs in several cancers facilitated cell proliferation by negatively regulating CDK inhibitors. Another CDK inhibitor p21Cip1 was a direct target of miR-106b and ectopic expression of miR-106b resulted in enhanced cell proliferation by facilitating Gap 1 (G1)/S progression [5,6]. miR-34a is transcriptionally activated by tumor suppressor p53 and functions as a downstream mediator by down-regulating several cell cycle genes including CDK4, CDK6, cyclin D1, cyclin E2, and E2 promoter binding factor 3 [7,8]. miR-16 also regulates multiple cell cycle genes including cyclin D1, cyclin D3, cyclin E1, and CDK6 and introduction of miR-16 into A549 cells induced G1 arrest [9,10].

Survivin belongs to a family of the inhibitor of apoptosis protein. In addition to its function as a negative regulator of apoptosis, survivin plays an important role in controlling cell division as a multi-protein complex called the chromosomal passenger complex [11]. Survivin has been an ideal target for anti-cancer therapy since survivin is upregulated in many cancers whereas its expression is low in normal tissues [12] and targeting survivin leads to inhibition of its dual functions, i.e., inhibition of apoptosis and regulation of cell division [13,14]. Recently, it was reported that miRNAs regulated survivin signaling [15,16]. For example, miR-27a regulates the zinc finger ZBTB10 gene, a putative Sp repressor. Down-regulation of ZBTB10 gene by miR-27a results in up-regulation of Sp1, Sp3, and Sp4 proteins and several Sp-dependent genes including survivin [15].

In this study, we show that survivin is a direct target of miR-542-3p as revealed by Western blot, real-time PCR and luciferase reporter analyses. Ectopic expression of miR-542-3p caused cell cycle arrest at both G1 and Gap 2/Mitosis (G2/M) phases. Thus, miR-542-3p appears to be a cell cycle regulator and may have potential for the treatment of cancer.

2. Materials and methods

2.1. RNA oligonucleotides

MicroRNA mimic and a negative control (NC; CN-001000-01) were purchased from Dharmacon (Lafayette, CO). Survivin siRNA

Abbreviations: miRNA, microRNA; G1, Gap 1; G2/M, Gap 2/Mitosis; 3′-UTR, 3′-untranslated region; CDK, cyclin-dependent kinase; E2F3, E2 promoter binding factor 3; IAP, inhibitor of apoptosis; CPC, chromosomal passenger complex; rRNA, ribosomal RNA

* Corresponding author. Fax: +82 31 203 8127.
E-mail address: khbaek@khu.ac.kr (K. Baek).

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was synthesized as described previously [17]. All siRNA and miRNA mimics were resuspended in DEPC-treated water to a final concentration of 20 μM.

2.2. Cell lines and transfection

Human cell lines A549 (human epithelial, lung carcinoma derived; KCLB 10 185), HeLa (human epithelial, cervix adenocarcinoma derived, KCLB 10 002), and MCF7 (human epithelial, breast adenocarcinoma derived, KCLB 30 022) were obtained from Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37 °C in a humidified 5% CO_2 incubator. Transfection was carried out using Lipofectamine RNAiMAX (Invitrogen) as described previously [18]. After incubation with a transfection solution for 5 h, cells were cultured at 37 °C in standard culture media for different times before harvested for Western blot and other experiments.

2.3. Western blot analysis

Western blot analysis was performed as described previously [18]. Primary antibody specific for survivin (#2803) was obtained from Cell Signaling Technology (Danvers, MA). Anti-β-actin (C-11) antibody, which was used to monitor equal loading of protein samples, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4. Quantitative real-time PCR analysis

Real-time PCR reactions were performed in 96-well plates using ABI 7900 HT system (Applied Biosystems, Foster City, CA). Each PCR reaction mixture (20 μl) contained 10 μl of 2X SYBR Green Master Mix (Applied Biosystems) and 0.8 μl of forward and reverse primers (10 pmol/μl). The cycling conditions were as follow: initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The sequences of the primers specific for survivin were: forward primer, 5′-GCACCACTTCCAGGGTTTAT-3′; reverse primer, 5′-CTCTGGTGCCACTTTCAAGA-3′. The amount of survivin transcript was divided by that of GAPDH as a means to obtain normalized expression levels. To quantitate the amount of 18S ribosomal RNA (rRNA), a plasmid DNA containing the sequence between 873 and 1398 nt of 18S rRNA gene was used to construct a standard curve. The DNA concentration was determined by optical density measurement and tenfold serial dilutions were used to generate the standard curve of 18S rRNA. The primers for 18S rRNA had the following sequences: forward primer, 5′-CGCCGCTAGAGGTGAAATTC-3′; reverse primer, 5′-TTGGCAAATGCTTTCGCTC-3′. The amount of 18S rRNA was obtained by calculating the absolute 18S rRNA concentration using the standard curve.

2.5. Cell proliferation assay

For cell proliferation assay, 1.8×10^4 A549 cells were seeded on 24-well plates and next day, cells were transfected with miRNA mimics (20 nM). At 72 h after transfection, the cell proliferation was measured using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to manufacturer's protocol. Briefly, 1/10 volume of CCK-8 solution was added to each well and incubated for 1 h at 37 °C. The plates were read in a microplate reader (model 550; Bio-Rad Laboratories, Hercules, CA) at 450 nm.

2.6. Colony formation assay

One day before transfection, 8×10^4 A549 cells were seeded in 12-well plates. Next day, cells were transfected with 20 nM miRNA mimics. Twenty-four hours after transfection, 200 cells were seeded in 6-well plates and cultured for 10 days in RPMI-1640 media. Colonies were then fixed with methanol and stained with 0.5% crystal violet in 25% methanol for 10 min at room temperature. Total number of colonies per 6-well were counted in triplicate for each sample.

2.7. Construction of 3′-untranslated region (3′-UTR) reporter plasmids and luciferase assay

The full-length cDNA clone containing the 3′-UTR of survivin gene (NM_001168) was obtained from Open Biosystems (Huntsville, AL). The sequence between 551 and 1670 nt was amplified using primers Surv-551L and Surv-1670R and cloned into XhoI/NotI sites of psiCHECK-2 vector (Promega) to give rise to pSurv-3′-UTR plasmid. To delete three predicted target site of miR-542-3p from pSurv-3′-UTR plasmid, PCR approach was used as described previously [18]. Briefly, to delete 5′-most target site, DNA fragments containing 379 bp upstream and 719 bp downstream of the target site were amplified using primers Surv-551L/929R and Surv-952L/1670R, respectively. The amplified DNA fragments were digested with XbaI, ligated at 4 °C overnight, digested with XhoI and NotI, and then cloned into psiCHECK-2 vector. Similar cloning strategy was used to delete two other target sites using primers 992R/1015L and 1461R/1484L, respectively. Primer sequences were as follow: Surv-551L; 5′-GATCTCGAGGGCCTCTGGCCGGAGCTGCCTGG-3′, Surv-1670R; 5′-GATGCGGCCGCTCAGGAACAGCCGAGATGACCT-3′, 929R; 5′-CATTCTAGAGGTCCAGACACATTCACTGTGG-3′, 952L; 5′- CATTCTAGAGTCCTGAGTGTGGACTTGGCAG-3′, 992R; 5′-CATAAGCTTGCTCAGATTCAACAGGCACCTG-3′, 1015L; 5′-CATAAGCTTCCTGTGCCTCCTCAGAGGACAG-3′, 1461R; 5′-CATGAATTCCTGACAGACACACGGCCTGCAG-3′, 1484L; 5′-CATGAATTCTTCTCCACACGGGGGAGAGACG-3′. For luciferase assay, 8×10^4 A549 cells in 12-well plate was cotransfected with 250 ng of luciferase vector and miRNA mimics (10 nM) using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, firefly and Renilla luciferase activities were measured using Dual-luciferase assay (Promega) following the manufacturer's protocol.

2.8. Microarray experiments

Microarray analysis was performed using the Illumina Human Ref-8 bead array (Illumina, Inc., San Diego, CA) as described previously [18]. The array was scanned with an Illumina BeadArray Reader and Data analysis was performed using the Illumina BeadStudio program.

2.9. Cell cycle analysis

Cells were harvested at 72 h after transfection. After fixing at 70% ethanol, cells were treated with RNase A (1 mg/ml) for 30 min and then, stained with propidium iodide (50 μg/ml). DNA content was analyzed using FACS Vantage SE flow cytometer (Becton Dickinson, Franklin, NJ). For mitotic arrest, A549 cells were treated with 100 ng/ml of nocodazole (Sigma, St Louis, MO) 48 h post-transfection and subjected to FACS analysis after 16 h nocodazole treatment.

3. Results and discussion

3.1. Target identification using microarray analyses

Recently, we found that several miRNAs including miR-542-3p targeted 3′-UTR of cyclooxygenase-2 in luciferase reporter assay. Since little is known about functional aspects of miR-542-3p, we have chosen this miRNA for further analyses. Since a miRNA con-

tains hundreds of target genes, we first performed microarray analyses to identify additional target(s) of miR-542-3p [19]. In addition to translation repression, miRNAs cause target mRNA degradation and these changes in mRNA level can be detected by microarray experiments [20]. Compared to NC-transfected cells, 431 transcripts and 340 transcripts were down- and up-regulated by two-fold or greater in cells transfected with miR-542-3p, respectively. Among 431 down-regulated transcripts, 184 transcripts were predicted to be a target of miR-542-3p by TargetScan including evolutionary non-conserved target genes whereas only 21 transcripts were predicted among 340 up-regulated genes, indicating that down-regulated transcripts are enriched for a predicted target of miR-542-3p. Of note, the most down-regulated candidate gene was survivin, whose expression was decreased by 17.99-fold. As predicted by TargetScan, there were three binding sites for miR-542-3p in the 3′-UTR of survivin, whose location is schematically represented in Fig. 1A. In Supplementary Table 1, top-ranked 20 candidate genes predicted by TargetScan were displayed according to significantly decreased expression in miR-542-3p-transfected cells compared to NC-transfected cells.

3.2. Survivin is a direct target of miR-542-3p

To validate survivin as a target of miR-542-3p, down-regulation of survivin at the mRNA and protein levels was examined by real-time PCR and Western blot analyses. As can be seen in Fig. 1B, survivin mRNA in miR-542-3p-transfected cells was significantly decreased compared to NC-transfected cells (~16%). In Western blot analysis (Fig. 1C), very little survivin was detected in both A549 and HeLa cells transfected with miR-542-3p, thus indicating that miR-542-3p down-regulates endogenous survivin at both the mRNA and protein levels.

To demonstrate direct interaction between miR-542-3p and 3′-UTR of survivin, miR-542-3p was cotransfected with luciferase reporter plasmid bearing 3′-UTR of survivin. As can be seen in Fig. 1D, cotransfection with miR-542-3p resulted in significant decrease in luciferase activity compared to that in NC-transfected cells. To verify that miR-542-3p interacts with predicted binding sites in 3′-UTR of survivin, all three predicted binding sites for miR-542-3p as predicted by TargetScan were deleted from survivin 3′-UTR using PCR approach and the resulting luciferase reporter plasmid was cotransfected with miR-542-3p. As shown in Fig. 1D, luciferase activity was not decreased when three predicted binding sites are deleted, indicating specificity of this interaction. To identify which sites are functional, 2 sites out of 3 predicted binding sites were deleted to construct reporter plasmids containing only one intact site. As can be seen in Fig. 1D, when site 1 (M2,3) or 3 (M1,2) is intact, luciferase activity was not decreased. However, when site 2 (M1,3) is intact, luciferase activity was significantly decreased, suggesting that site 2 is functional. Same results were obtained with cotransfection of mutant plasmids containing two intact sites and miRNA mimics: when site 2 (M2) is deleted, only a slight decrease in luciferase activity was observed whereas luciferase activity was significantly decrease when site 1 (M1) or site 3 (M3) is deleted, indicating that site 2 functions as a major site for post-transcriptional repression of survivin expression by miR-542-3p. Taken together, these data indicate that miR-542-3p directly regulate survivin mainly through interaction with predicted binding site 2 in the 3′-UTR of survivin.

3.3. Growth inhibition by miR-542-3p

Since miR-542-3p targets survivin which plays an important role in cell division, we have examined the effects of miR-542-3p

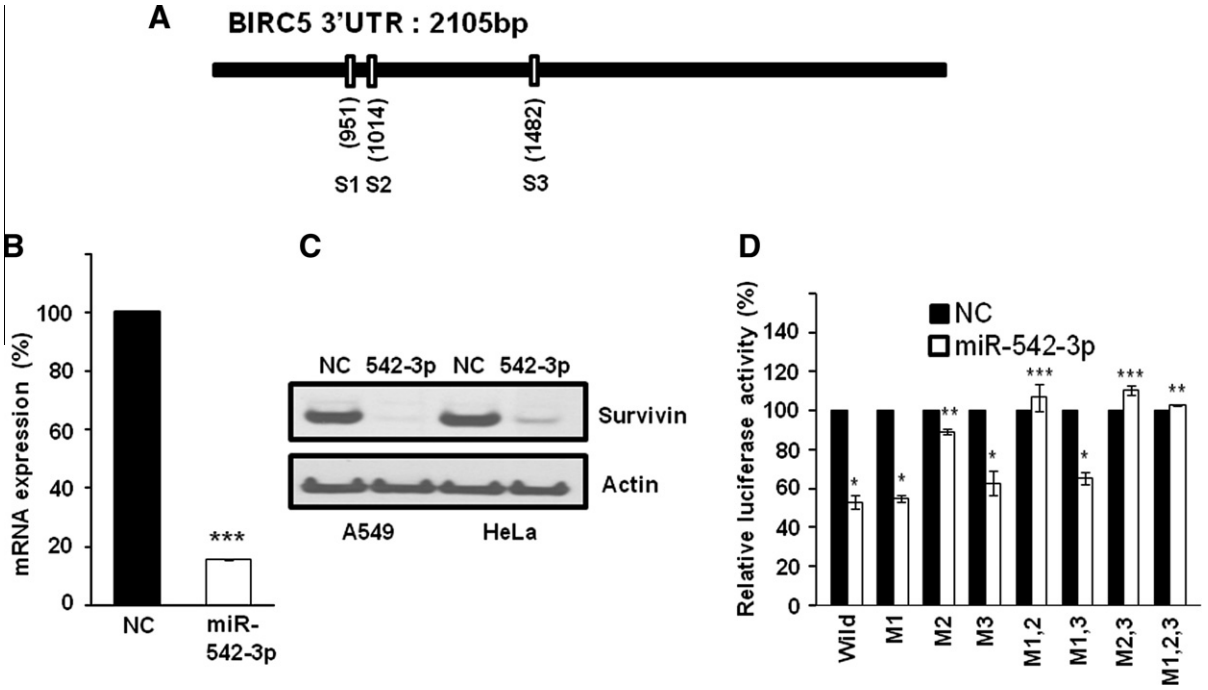

Fig. 1. Down-regulation of survivin by miR-542-3p at mRNA and protein levels via direct targeting of 3′-UTR. (A) A schematic diagram of 3 binding sites (S1, S2, and S3) for miR-542-3p predicted by TargetScan in the 3′-UTR of survivin. Numbers in parentheses represent the nucleotide position in survivin gene. (B, C) At 48 h after transfection with miR-542-3p and NC mimetics (20 nM), cells were subjected to real-time PCR (B) and Western blot (C) analyses. In (B), survivin mRNA level of miR-542-3p-transfected cells was expressed as the percentage of that in NC-transfected cells, which was set at 100%. The experiments in (B) were performed in triplicate and repeated twice. $^{***}P < 0.05$. (D) Direct targeting of survivin 3′-UTR by miR-542-3p. A549 cells were cotransfected with miRNA (10 nM) and luciferase reporter plasmids carrying 3′-UTR of survivin. At 48 h after transfection, luciferase activity was measured using dual-luciferase assays (Promega). Normalized Renilla luciferase activity in cells transfected with NC was set at 100%. In (D), the experiments were performed in triplicate and repeated thrice. $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$. M1, mutated at S1; M2, mutated at S2; M3, mutated at S3; M1,2, mutated at S1 and S2; M1,3, mutated at S1 and S3; M2,3, mutated at S2 and S3; M1,2,3, mutated at S1, S2, and S3.

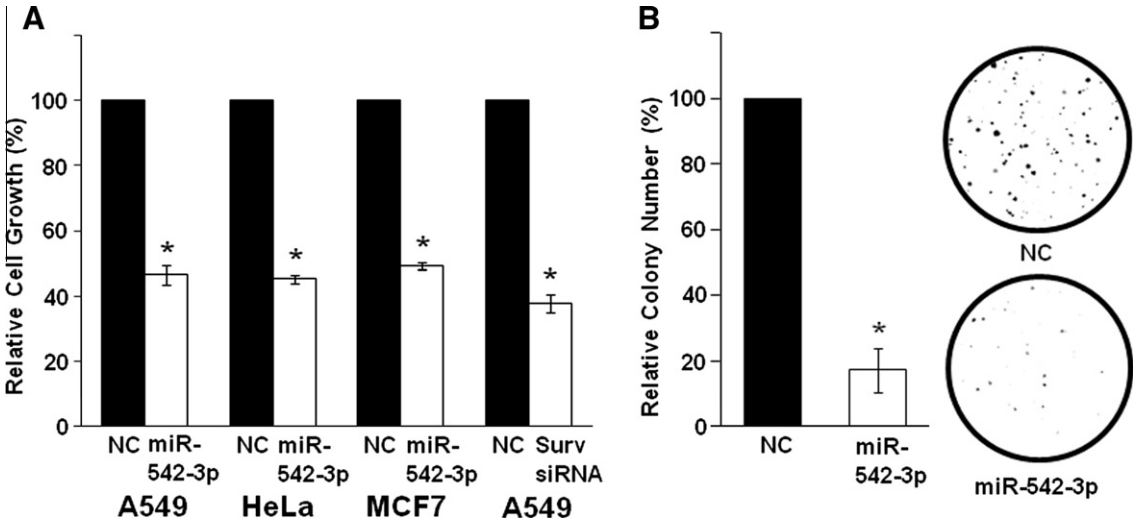

Fig. 2. Inhibitory effects of miR-542-3p on cell proliferation and colony formation. (A) Cell proliferation assay by CCK-8. A549, HeLa, and MCF7 cells were transfected with miR-542-3p (20 nM). NC and survivin siRNA were used as the negative and positive controls, respectively. At 72 h after transfection, cell growth was measured using CCK-8 following manufacturer's instruction. In (A), the experiments were performed in triplicate and repeated twice. $^{*}P < 0.001$. (B) Colony formation assay to determine growth inhibition by miR-542-3p. At 24 h after transfection, A549 cells were harvested and 200 transfected cells were plated on each well of 6-well plates. After culturing for 10 days, the number of stained colonies was counted to measure cell proliferation. The relative cell growth is shown after setting the number of colonies formed from cells transfected with NC mimetic to 100%. In (B), the experiments were performed in triplicate and repeated twice. $^{*}P < 0.001$. On the right side of Fig. 2B, the photograph showed 6-wells containing colonies grown from cells transfected with NC or miR-542-3p.

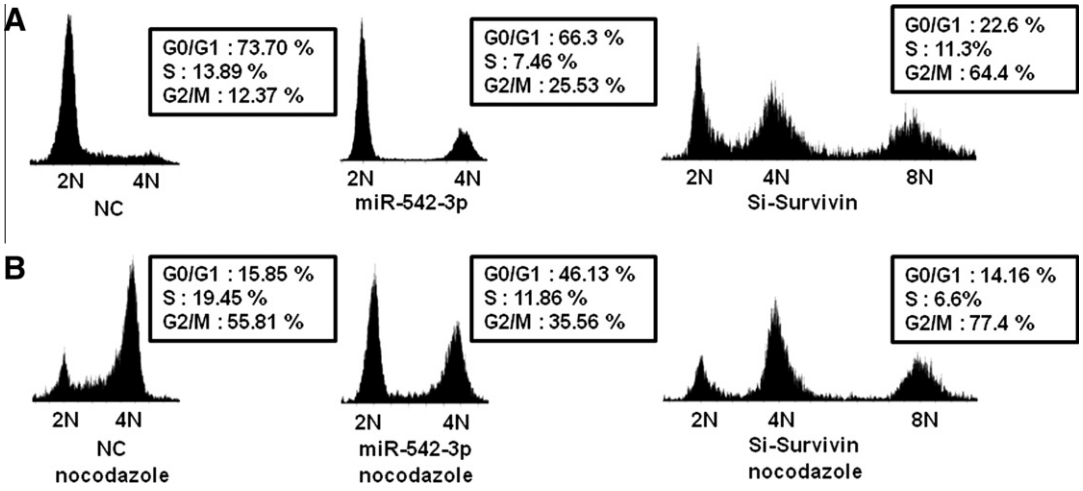

Fig. 3. G1 and G2/M arrest by miR-542-3p. A549 cells were transfected with miR-542-3p and NC mimetics (20 nM). With (B) or without (A) nocodazole treatment, transfected cells were stained with propidium iodide and analyzed with flow cytometer. Si-survivin; survivin siRNA.

on the proliferation of several cell lines by CCK-8 and colony formation assays. As shown in Fig. 2A, transfection of miR-542-3p into several cell lines significantly inhibited cell proliferation in all tested cell lines by CCK-8 assay. When transfected with survivin siRNA, similar level of inhibition was observed in A549 cells when compared to miR-542-3p. In colony formation assay, ectopic expression of miR-542-3p in A549 cells resulted in significant inhibition of colony formation compared to NC-transfected cells (Fig. 2B). Taken together, these results indicate that miR-542-3p has an adverse effect on cell proliferation.

3.4. miR-542-3p induces G1 and G2/M cell cycle arrest

To determine which stage of cell cycle is arrested during cell cycle distribution, we examined DNA content by flow cytometry after staining with propidium iodide. As can be seen in Fig. 3A, the percentage of miR-542-3p-transfected cells at the S phase was decreased while that of miR-542-3p-transfected cells at the G2/M phase was increased compared to NC-transfected cells. When treated with survivin siRNA, however, much more cells were accumulated in G2/M phase than miR-542-3p-transfected cells in which survivin is also down-regulated. One possibility for the discrepancy was that miR-542-3p also induces G1 arrest in addition to G2/M arrest. To test the possibility, cells were treated with nocodazole, a microtubule destabilizer, which arrests at G2/M phase. In NC-transfected cells, nocodazole treatment resulted in substantial arrest in G2/M phase (Fig. 3B). However, significant number of miR-542-3p-transfected cells remained in G1 phase even in the treatment of nocodazole. These results indicate that in addition to G2/M arrest, miR-542-3p also induces G1 arrest, which may be mediated through down-regulation of unidentified target(s) by miR-542-3p.

Thus, like miR-34 and miR-16, miR-542-3p seems to coordinately regulate cell proliferation by down-regulating multiple cell

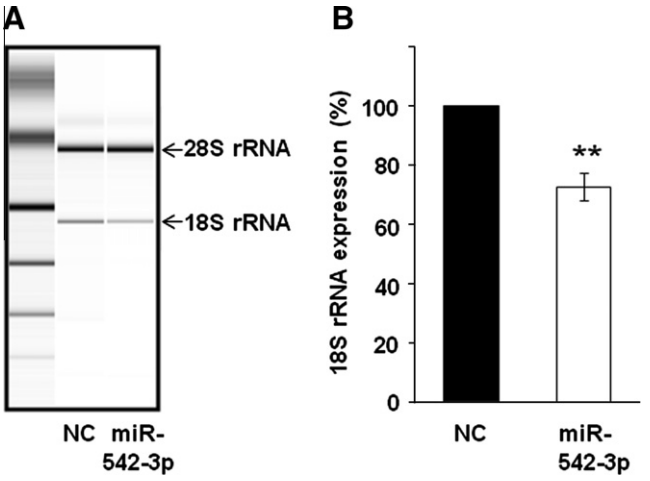

Fig. 4. miR-542-3p down-regulates 18S rRNA. A549 cells were transfected with miR-542-3p and NC mimetics (20 nM) and at 48 h after transfection, total RNA was prepared and subjected to denaturing gel electrophoresis on Agilent 2100 Bioanalyzer (Agilent Technologies, CA) (A) and quantitative real-time PCR (B). In (B), the amount of 18S rRNA in NC-transfected cells was set at 100%. The experiments in (B) were performed in triplicate and repeated twice. $^{**}P < 0.01$.

cycle-related genes. When examining microarray data, potential candidate targets include CDK6 at G1/S phase and TUBB2B at G2/M phase. Additionally, when examining RNA quality in microarray experiments, we found that 18S rRNA was significantly decreased in cells transfected with miR-542-3p compared to NC-transfected cells (Fig. 4A). To measure the amount of 18S rRNA in miR-542-3p-transfected cells compared with that in NC-transfected cells, we have performed quantitative real-time PCR. The result showed that the level of 18S rRNA in miR-542-3p-transfected cells was decreased to 70% of that in NC-transfected cells (Fig. 4B). Since rRNAs play crucial roles in the process of protein synthesis, it is possible that reduction in 18S rRNA may affect cell proliferation. Considering miRNA target(s), miR-542-3p may decrease 18S rRNA directly or indirectly by targeting other gene(s) whose down-regulation in turn affects 18S rRNA.

miR-542-3p regulates cell cycle by inducing both G1 and G2/M arrests. At G2/M phase, growth inhibition was, at least in part, mediated by down-regulation of survivin which is an attractive target for anti-cancer therapy. Thus, on the therapeutic side, miR-542-3p has the potential for the application in the treatment of cancers. In the future, identification of more targets of miR-542-3p, especially target(s) responsible for G1 arrest, may be necessary to gain more insight into the functional significance of miR-542-3p as a regulator of cell cycle progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.08.025.

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