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Tumor suppressive *microRNA-1* mediated novel apoptosis pathways through direct inhibition of *splicing factor serine/arginine-rich 9* (*SRSF9/SRp30c*) in bladder cancer

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ABSTRACT

We have previously found that restoration of tumor suppressive *microRNA-1* (*miR-1*), induced cell apoptosis in bladder cancer (BC) cell lines. However, the apoptosis mechanism induced by *miR-1* was not fully elucidated. Alternative splicing of mRNA precursors provides cancer cells with opportunities to translate many oncogenic protein variants, which promote cell proliferation and survival under unpreferable condition for cancer development. Serine/arginine-rich (SR) protein family, which involved in alternative pre-mRNA splicing, plays a critical role for regulating apoptosis by splicing apoptosis-related genes. However, transcriptional regulation of SR proteins, themselves, has not been elucidated. In this study, we focused on *splicing factor serine/arginine-rich 9* (*SRSF9/SRp30c*) on the basis of our previous genome-wide gene expression analysis using *miR-1*-transfected BC cell lines because putative target sites of *miR-1* are existed in 3′-untranslated region (UTR) of *SRSF9* mRNA. The expression levels of mRNA of *SRSF9* were extremely reduced in the *miR-1* transfectants. A luciferase activity significantly decreased in the transfectants suggesting that actual binding occurred between *miR-1* and 3′UTR of *SRSF9* mRNA. Loss-of-function assays demonstrated that significant inhibitions of cell proliferation, migration, and invasion were observed in the si-SRSF9 transfectants. Apoptosis assays demonstrated that cell apoptosis fraction increased and that caspase-3/7 was activated in the si-SRSF9 transfectants. Our data indicated that tumor suppressive *miR-1* induces apoptosis through direct inhibition of SRSF9 in BC. The identification of molecular mechanisms between miRNAs and SR proteins could provide novel apoptosis pathways and their epigenetic regulations and offer new strategies for BC treatment.

1. Introduction

Bladder cancer (BC) is a common malignancy with high mortality in the world [1]. The disease divides into non-muscle-invasive tumors and muscle-invasive cancer. Although 70–80% of BC are diagnosed as non-muscle-invasive tumors at the first treatment, recurrence occur about 70% of them. Among recurrent tumors, 10–15% proceed to muscle invasion and metastasis [2]. There have been significant advances in medical science and treatment, including surgical techniques and adjuvant chemotherapy; however, the biology of BC is incompletely understood, and muscle-invasive BC is especially difficult to cure. Therefore, new treatment modalities based on novel molecular networks in BC are desired.

Alternative splicing is indispensable for allowing individual genes to express multiple mRNAs, which provide proteins with diverse and even antagonistic functions [3]. In cancer field, different splice variants caused by splice change are associated with proliferation, adhesion, differentiation, and invasion [4]. *Serine/arginine-rich splicing factor 9* (*SRSF9*) is a member of the serine/arginine-rich (SR) protein family, which involved in alternative pre-mRNA splicing. Recent studies have revealed that changes in levels of various SR proteins might be associated with different mRNA products, and it can be linked to cancer development, and that depletion of SRSF9 reduced cancer viability in ovarian cancer and prostate cancer (PC) [5–7]. However, functional role of SRSF9 is still unknown as of now.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules of 20–22 nucleotides that post-transcriptionally modulate protein expression. Although their biological functions remain

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largely unknown, recent studies have reported that miRNAs have emerged as central regulators of development, differentiation, and cancer [8]. *MiR-1* has been reported as a down-regulated miRNA in various human malignancies and have tumor suppressive function [9–13]. Our previous study demonstrated that *miR-1* might function as tumor suppressor through repression of oncogenic *transgelin-2* (*TAGLN2*) and *LIM* and *SH3 protein 1* (*LASP1*) [9,13], and induced apoptosis in BC cell lines [13]. However, the functional roles of both the miRNA and its target genes have not been thoroughly investigated.

SRSF9 was listed in the down-regulated genes, which have a putative target site of *miR-1*, in our previous oligo-microarray study of the *miR-1* transfectant [13]. We hypothesized that *miR-1* directly regulate *SRSF9* and that this gene has oncogenic activity through anti-apoptotic function in BC. We performed a luciferase reporter assay to determine whether *SRSF9* mRNA was actually targeted by *miR-1* and loss-of-function studies using BC cell lines to investigate functional roles of SRSF9 in BC. Furthermore, we performed apoptosis analysis by using flow cytometry with si-SRSF9 transfectants, and caspase-3/7 activity assays by using fluorescence microscopes with *miR-1* and si-SRSF9 transfectants to elucidate apoptosis mechanism of *miR-1* and SRSF9.

2. Materials and methods

2.1. Clinical specimens and cell culture

The tissue specimens for quantitative RT-PCR were from BC patients ($n = 67$) who had received cystectomy or TUR-BT at Kagoshima University Hospital between 2006 and 2009. Normal bladder epitheliums ($n = 32$) were derived from patients with noncancerous disease. These specimens were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer tumor-node-metastasis classification and histologically graded [14]. Our study was approved by the Bioethics Committee of Kagoshima University; written prior informed consent and approval were given by these patients.

We used two human BC cell lines: BOY, which was established in our laboratory from an Asian male patient aged 66 diagnosed with stage III BC with lung metastasis; and T24, which was invasive and obtained from the American Type Culture Collection. These cell lines were maintained in a minimum essential medium (MEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.2. Tissue collection and RNA extraction

Tissues were immersed in RNAlater (QIAGEN, Valencia, CA, USA) and stored at −20 °C until the RNA extraction. Total RNA including miRNA was extracted using the mirVana™ miRNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's protocol. The integrity of the RNA was checked with RNA 6000 Nano Assay Kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.3. Quantitative real-time RT-PCR

TaqMan probes and primers for SRSF9 (P/N: Hs01596548_g1; Applied Biosystems) were assay-on-demand gene expression products. All reactions were performed in duplicate and a negative-control lacking cDNA was included. We followed the manufacturer's protocol for PCR conditions. To normalize the data for quantification of *SRSF9* mRNA, we used human *GUSB* (P/N: Hs99999908_m1; Applied Biosystems), and the delta–delta Ct method was employed to calculate the fold change. As a control RNA, we used Premium Total RNA from normal human bladder (Clontech, Mountain View, CA, USA) as a control RNA.

2.4. Mature miRNA, siRNA, and SRSF9 expression vector transfection

As described elsewhere [15], the BC cell lines were transfected with Lipofectamine™ RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Invitrogen) with 10 nM of mature miRNA molecules. Pre-miR and negative-control miRNA (Applied Biosystems) were used in the gain-of-function experiments, whereas *SRSF9* siRNA (Cat#; HSS112751 and HSS189499, Invitrogen) and negative control siRNA (D-001810-10; Thermo Fisher Scientific, Waltham, MA, USA) were used in the loss-of-function experiments. Cells were seeded in a 10-cm dish for protein extraction (8×10^5 per dish), in a 6-well plate for apoptosis (10×10^4 per well) and for wound healing assay (20×10^4 per well), in a 24-well plate for mRNA extraction and luciferase reporter assay (5×10^4 per well), and in a 96-well plate for XTT assay (3000 per well). A human *SRSF9* expression vector subcloning the full-length cDNA of *SRSF9* (RC210898, OriGene, Rockville, MD, USA) was transfected using FuGENE HD (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol.

2.5. Plasmid construction and dual-luciferase reporter assay

MiRNA target sequences were inserted between the XhoI–PmeI restriction sites in the 3′UTR of the hRluc gene in the psiCHECK™-2 vector (C8021; Promega, Madison, WI, USA). Primer sequences for full-length 3′UTR of *SRSF9* mRNA (GATCGCTCGAGCCGTGA CTCTCCATACCAAAG and CTCTAGGTTTAAACAATGGGCCGACT-CAGTC) were designed. Following that, T24 cells were transfected with 15 ng of vector, 10 nM of miRNA, and 1 μl of Lipofectamine 2000 (Invitrogen) in 100 μl of Opti-MEM (Invitrogen). The activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (E1910; Promega). Normalized data were calculated as the quotient of *Renilla*/firefly luciferase activities.

2.6. Cell proliferation, migration and invasion assays

According to the manufacturer's instructions, cell proliferation was determined by using an XTT assay (Roche Applied Science) after 72 h transfection. Cell migration activity was evaluated by wound-healing assay. Cells were plated in 6-well dishes, and the cell monolayer was scraped using a P-20 micropipette tip. The initial gap length (0 h) and the residual gap length 24 h after wounding were calculated from photomicrographs. A cell invasion assay was carried out using modified Boyden Chambers consisting of transwell-precoated matrigel membrane filter inserts with 8-mm pores in 24-well tissue culture plates (BD Biosciences, Bedfold, MA, USA). MEM containing 10% fetal bovine serum in the lower chamber served as the chemoattractant as described previously [9]. The number of invading cells were counted after 72 h transfection. All experiments were performed in triplicate.

2.7. Apoptosis assay

BC cell lines transiently transfected with transfection reagent only (mock), si-control and si-SRSF9 in 6-well tissue culture plates as described earlier, were harvested 72 h after transfection by trypsinization and washed in cold PBS. Double staining with FITC-Annexin V and Propidium iodide was carried out using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's recommendations and immediately analyzed within an hour by flow cytometry (FACScan®; BD Biosciences). Cells were discriminated into viable cells, dead cells, early

apoptotic cells, and apoptotic cells by the CellQuest software (BD Biosciences), and then the percentages of early apoptotic and apoptotic cells from each experiment were compared. Experiments were done in triplicate.

2.8. Caspase-3/7 activity assays

Caspase-3/7 activity was measured by CellEvent™ caspase-3/7 Green Detection Reagent (Invitrogen). At first, the BC cell lines grown in a 96-well plate were transfected with mature miRNAs and siRNAs as described above. After 72 h, 5 μM of caspase-3/7 reagents were added to each well and incubated for 30 min. Fluorescence was then measured and recorded for each well. For densitometric analysis, the expression level of sum of fluorescent particles was measured by using ImageJ software (http://rsbweb.nih.gov/ij).

2.9. Statistical analysis

The relationship between two variables and the numerical values obtained by real-time RT-PCR was analyzed using the Mann–Whitney *U* test. The relationship among three variables and the numerical values was analyzed using the Bonferroni-adjusted Mann–Whitney *U* test. Expert StatView analysis software (version 4; SAS Institute Inc., Cary, NC, USA) was used in both cases. In the comparison among three variables, a nonadjusted statistical level of significance of $p < 0.05$ corresponds to a Bonferroni-adjusted level of $p < 0.0167$.

3. Results

3.1. SRSF9 as a target of post-transcriptional repression by miR-1

The expression levels of *SRSF9* mRNA were markedly down-regulated in the *miR-1* transfectants in comparison with the controls or the mocks (Fig. 1A). We performed a luciferase reporter assay to determine whether *SRSF9* mRNA has a target site for *miR-1*. We used a vector encoding full-length 3′UTR of *SRSF9* mRNA and found that the luminescence intensity was significantly reduced in the *miR-1* transfectants compared to the counterparts (Fig. 1B).

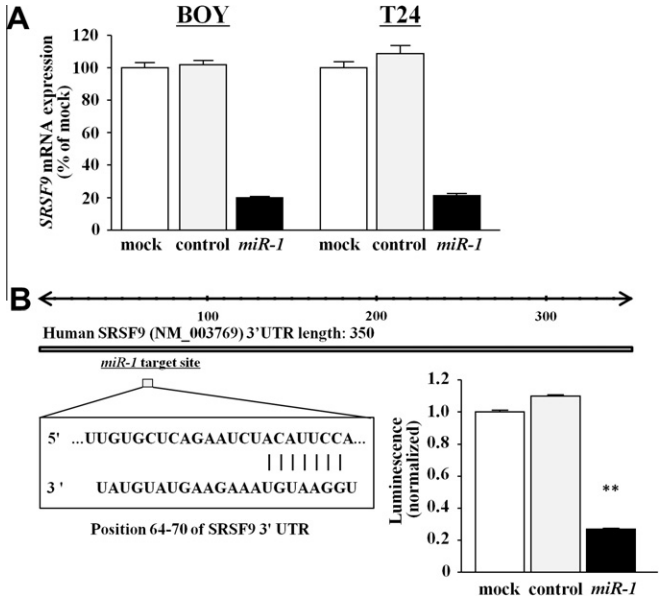

Fig. 1. (A) *SRSF9* mRNA expression after 24 h transfection with 10 nM of *miR-1*. (B) *miR-1* binding sites in 3′-UTR of *SRSF9* mRNA. A luciferase reporter assay using the vector encoding full-length 3′-UTR of *SRSF9* mRNA. The *Renilla* luciferase values were normalized by firefly luciferase values. $^{**}p < 0.0001$.

3.2. SRSF9 mRNA expression in BC cell lines and clinical BC specimens

The quantitative real-time RT-PCR analysis showed that the expression level of *SRSF9* mRNA in BOY and T24 cell lines was higher than that in the normal human bladder RNA (relative to the normal; 11.83 ± 0.55 and 6.09 ± 0.16, respectively; Fig. 2A). The mRNA expression levels of *SRSF9* were significantly higher in the clinical BC specimens than in the normal bladder epitheliums (1.55 ± 0.12, and 1.05 ± 0.55, respectively, $p = 0.0188$; Fig. 2B). No significant relationship was found between the clinicopathological parameters and the expression levels of *SRSF9* mRNA (data not shown).

3.3. Effect of SRSF9 knockdown on cell proliferation, invasion, and migration activity in BC cell lines

The expression levels of *SRSF9* mRNA were markedly decreased in the si-SRSF9_1 and the si-SRSF9_2 transfectants in comparison with the untransfectants (mocks) and the si-control transfectants (% expression relative to the mock; BOY, 35.2 ± 4.2, 11.1 ± 0.9, 100.0 ± 8.8, and 117.2 ± 6.6, respectively, $p < 0.0001$; T24, 22.4 ± 2.7, 7.5 ± 0.3, 100.0 ± 3.3, and 97.7 ± 3.1, respectively, $p < 0.0001$; Fig. 3A). To examine the functional role of SRSF9, we performed loss-of-function studies using two different si-SRSF9-transfected BOY and T24 cell lines. The XTT assay demonstrated significant cell proliferation inhibitions in the two si-SRSF9 transfectants in comparison with the mocks and the si-control transfectants (% of cell viability relative to the mock; BOY, 57.0 ± 1.1, 64.8 ± 2.9, 100.0 ± 0.8, and 100.0 ± 2.2, respectively, $p < 0.0001$; T24, 52.5 ± 1.1, 55.6 ± 2.1, 100.0 ± 1.8, and 102.7 ± 2.6, respectively, $p < 0.0001$; Fig. 3B). The wound healing assay demonstrated significant cell migration inhibitions in the two si-SRSF9 transfectants compared with the counterparts (% of wound closure relative to the mock; BOY, 17.3 ± 2.0, 39.2 ± 2.2, 100.0 ± 3.4, and 96.6 ± 6.3, respectively, $p < 0.0001$; T24, 45.6 ± 2.3, 59.7 ± 3.5, 100.0 ± 3.8, and 93.5 ± 3.3, respectively, $p < 0.0001$; Fig. 3C). The matrigel invasion assay demonstrated that the number of invading cells was significantly decreased in the two si-SRSF9 transfectants compared with the counterparts (% of cell invasion relative to the mock; BOY, 30.8 ± 2.7, 43.8 ± 8.0, 100.0 ± 8.9, and 145.1 ± 9.1, respectively, $p < 0.0001$; T24, 37.9 ± 4.6, 47.0 ± 2.4, 100.0 ± 6.5 and 100.7 ± 10.8, respectively, $p < 0.0001$; Fig. 3D).

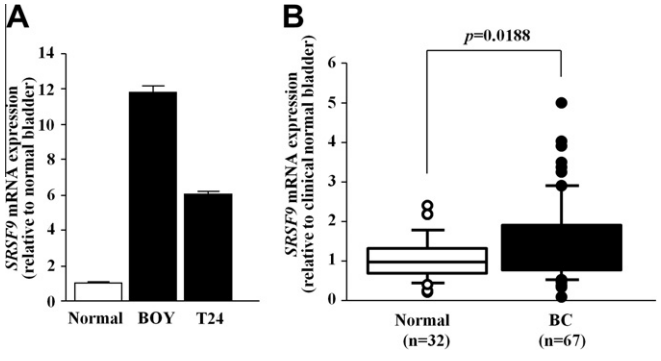

Fig. 2. (A) *SRSF9* mRNA expression in BOY and T24 cell lines and normal human bladder RNA. Quantitative real-time RT-PCR analysis showed that the innate expression level of *SRSF9* mRNA in BC cell lines was higher than that in normal human bladder RNA. (B) mRNA expression levels of *SRSF9* in BC clinical samples. Relative mRNA expression level of *SRSF9* is expressed in box plots.

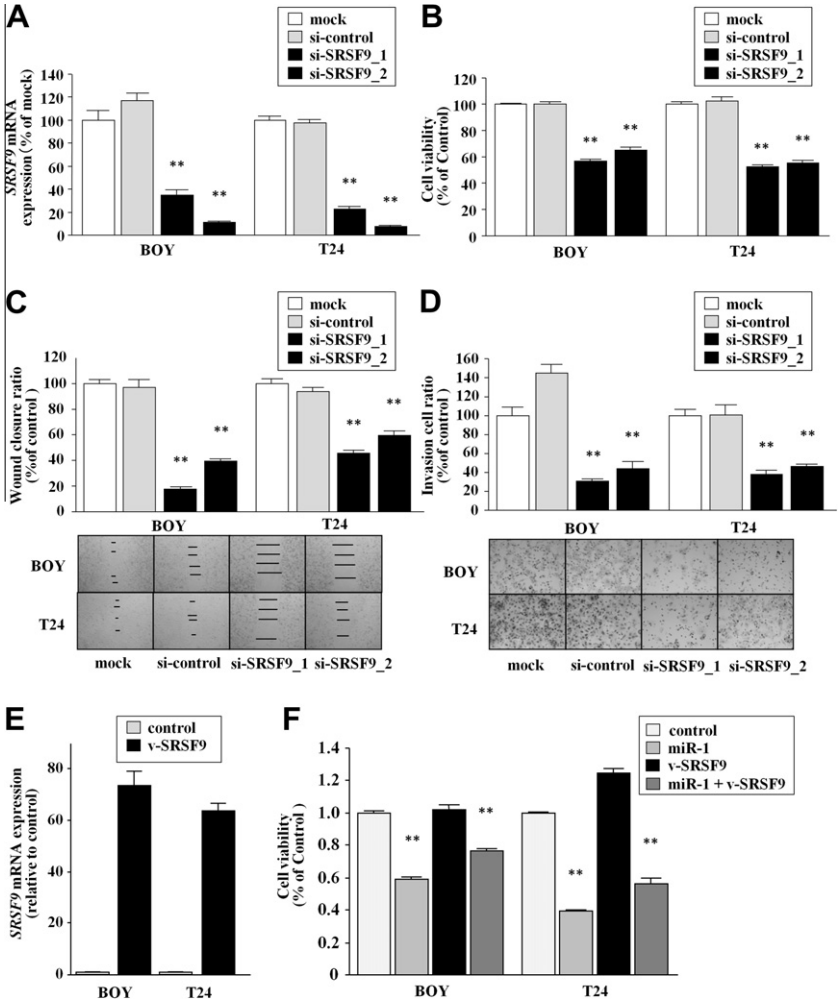

Fig. 3. (A) *SRSF9* mRNA expression after 24 h of transfection with 10 nM of si-SRSF9. *SRSF9* mRNA expression was repressed in si-SRSF9 transfectants. (B–D) SRSF9 – knockdown effects on BC cell viability by siRNA. (B) Cell proliferation determined by the XTT assay after 72 h transfection of si-SRSF9; (C) cell migration activity determined by the wound healing assay after 24 h transfection; and (D) cell invasion activity determined by the matrigel invasion assay in BOY and T24 cell lines after 72 h transfection by si-SRSF9. $^{**}p < 0.0001$; (E) *SRSF9* mRNA expression in SRSF9-transfected BOY and T24 compared with the controls; (F) cell proliferation determined by the XTT assay after 72 h transfection of *miR-1* and *SRSF9* vector. $^{**}p < 0.0001$.

3.4. Effect of SRSF9 overexpression on cell proliferation in miR-1 transfected BC cell lines

Our previous study demonstrated that a marked cell proliferation inhibition occurred in *miR-1* transfected BC cell lines. To investigate whether oncogenic SRSF9 depends on *miR-1* regulation, we performed gain-of-function study of SRSF9 by using *miR-1*-transfected BC cell lines. *SRSF9* mRNA expression was markedly increased after *SRSF9* expression vector transfection in BOY and T24 cell lines (relative to the control; BOY, 73.82 ± 5.21; T24, 63.98 ± 2.51; Fig. 3E). The XTT assay demonstrated that obvious increase of cell proliferation was not found in *SRSF9*-transfected cells compared with the controls and that cell proliferation of *miR-1*-transfected cells was significantly reduced even in the cells with co-transfection of *SRSF9* expression vector (each, $p \leqslant 0.0001$; Fig. 3F). However, we found modest increase of cell viabilities in the cells with co-transfection of *miR-1* and *SRSF9* vector compared to the cells with solo transfection of *miR-1* ($p \leqslant 0.0001$; Fig. 3F).

3.5. miR-1 restoration and SRSF9 knockdown induced cell apoptosis through caspase-3/7 activation in BC cell lines

Cell apoptosis in si-SRSF9 transfectants was detected using flow cytometry. The apoptotic cell fractions (early apoptotic and late apoptotic; bottom right and upper right quadrant, respectively) were greater in the two si-SRSF9 transfectants than in the mocks and the si-control transfectants at 72 h after transfection (relative to mock; BOY, 7.20 ± 1.58, 3.55 ± 1.26, 1.00 ± 0.00, and 1.48 ± 0.35, respectively, $p < 0.01$; T24, 4.27 ± 0.53, 2.04 ± 0.26, 1.00 ± 0.00, and 0.91 ± 0.06, respectively, $p < 0.01$; Fig. 4A). In terms of *miR-1*, we have previously reported that apoptotic cell fraction was greater in the *miR-1*-transfected BOY and T24 BC cell lines [13]. Caspase-3/7 activity assay showed that fluorescent intensity was markedly increased in the *miR-1* transfectants in comparison with the miR-control transfectants ($p < 0.05$; Fig. 4B), and it was also markedly increased in the two si-SRSF9 transfectants compared with the si-control transfectants ($p < 0.05$; Fig. 4C).

4. Discussion

We previously demonstrated that *miR-1* and *miR-133a*, which are located on the same chromosomal loci (18q11.2 and 20q13.33), had tumor suppressive function through regulating oncogenic *TAGLN2* and restoration of the miRNA induced apoptosis in BC [13]. These miRNAs are cardiac and skeletal muscle–specific miRNAs transcriptionally controlled by some major regulators of muscle differentiation [16]. Occasionally, these miRNAs have opposite function; *miR-1* promotes differentiation of cardiac pro-

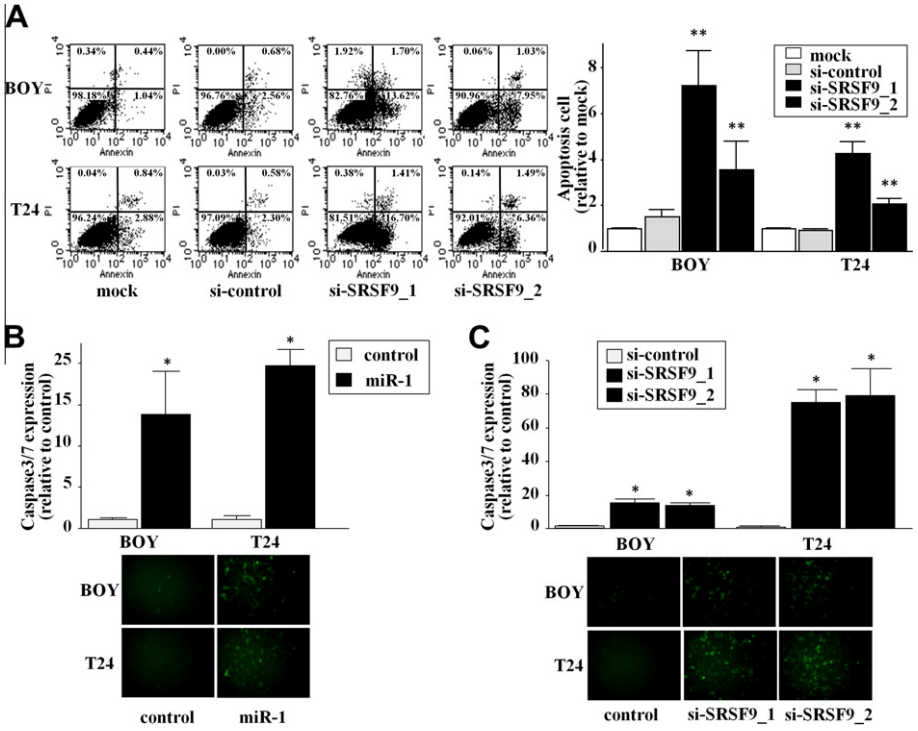

Fig. 4. Effect of SRSF9 knockdown on apoptosis, and detection of caspase-3/7 activation in *miR-1* and si-SRSF9 transfected BC cell lines. (A) Apoptosis assay determined by flow cytometry. Early apoptotic cells can be seen in bottom right quadrant and late apoptotic cells are in upper right. The normalized ratio of the apoptosis assay is shown in the histogram. $^{**}p < 0.01$. (B and C) caspase-3/7 activity was measured by fluorescence intensity in BOY and T24 cell lines transfected with *miR-1* and si-SRSF9. $^{*}p < 0.05$.

genitors and exit from the cell cycle, on the other hand *miR-133a* inhibits differentiation of skeletal myoblasts and maintains them in a proliferative state [17]. We speculated these miRNAs function as either individually or simultaneously in some cases. Therefore, we focused on the genes targeted by a single miRNA in this study. M*iR-1* was reported to be down-regulated and associated with apoptosis in human malignancies [13,18,19]. Furthermore, ectopic *miR-1* induced apoptosis through enhanced activation of caspase-3 and -7, and depletion of anti-apoptotic *Mcl-1* in lung cancer [18]. Caspase-3 and -7 activities are most important mediators for cell apoptosis. Though *miR-1* was reported to be associated with apoptosis, there have been scarcely reports about *miR-1* and caspase-3/7. In this study, we firstly reported that caspase-3/7 activation could be visible in BC cells, and that these activities were robustly increased by *miR-1* transfection and SRSF9 knockdown. However, increased caspase-3/7 activity did not synchronized with increased apoptotic cell number counted by flow cytometry in the si-SRSF9-transfectants (BOY and T24). Because of the wide-spread regulation by a single miRNA, it is plausible that *miR-1* may regulate pro-apoptotic as well as anti-apoptotic genes, which expression differs according to cell lines. Further examinations are necessary to elucidate this.

In terms of SR proteins, activation of signaling pathways, caused by protein kinases that phosphorylate SR protein, might have relationship with carcinogenesis [4]. Previous studies reported that several SR proteins, such as SRp30a, SRp30b, and SRp20 were elevated expression in malignant tumor; SRp30a, SRp30b, and SRp20 in ovarian cancer; SRp30a in gastric cancer [20]. In particular, SRp30b has been described as a proto-oncogene in various tumors [20]. Recent studies demonstrated that SRp75, one of the SR proteins, was phosphorylated and activated by SR protein kinase 2 (SRPK2), and SRPK2 inhibitors might be clinically useful as antiviral agents [21]. In another aspect, regulations of SR proteins by miRNAs could become promising therapeutic modalities. Therefore, it seems to be important to elucidate apoptosis mechanism induced by *miR-1* in detail. SRSF9 (/SRp30c) is one of SR protein family, which plays important roles in splicing, as both general splicing factors and regulators of alternative splicing [22]. Alternative splicing is a crucial mechanism for providing protein diversity. However, different splice variants can lead different and antagonistic biological functions and are commonly found to be enriched in cancer tissue compared to the normal tissue [4,23]. Different splice variants was mainly caused by genetic mutations that create or disrupt splice sites or splicing enhancers and silencers, and activation of signaling pathways that can affect the activity of splicing regulatory factors [4]. SRSF9 knockdown by siRNAs reduced migration ability in ovarian cancer and antagonized bommesin's effect involved in PC progression on glucocorticoid resistance [5,7]. In addition, increase level of SRSF9 may facilitate the production of the anti-apoptotic Bcl-xL isoform [24]. Consistent with previous reports, we found that cell viability was markedly attenuated in SRSF9-knockdown cell lines. Moreover, there was a significant difference in mRNA expression levels of *SRSF9* between clinical BC specimens and the normal bladder epitheliums. There were no significant relationships of SRSF9 expression with the clinicopathological parameters. These results suggest that SR protein family involved in early tumorigenesis of BC. In this study, the anti-tumor effect of siRNA against SRSF9 was not paralleled with repression of cell viability and induction of apoptosis. It might be caused by off-target effects that is defined as unintended up regulation or down regulation of genes within transfected cells.

In this study, cell proliferation hardly changed in the exogenous expression of *SRSF9* induced cells compared with control, even though *SRSF9* mRNA expression was markedly increased. We speculated that innate expression levels of *SRSF9* in BC cells were high enough to activate cell proliferation, and this might be a reason why cell proliferation did not increase in the exogenous expression of *SRSF9* induced cells. On the other hand, their tumorigenicity was not enough but partially rescued in the cells with co-transfection of *miR-1* and *SRSF9* vector compared to the cells with solo transfec-

tion of *miR-1*, suggesting that *SRSF9* might work downstream of *miR-1* but might be tremendously suppressed under the environment of *miR-1* overexpression.

In summary, we revealed that *miR-1* may function as tumor suppressors through repression of oncogenic *SRSF9* in BC, and restoration of *miR-1* and SRSF9-knockdown increased caspase-3/7 activity, resulting in apoptosis. Novel molecular networks provided by miRNAs may provide new insights into the underlying molecular mechanisms of BC.

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