Tumor and Stem Cell Biology

Cancer Research

Shrimp miR-S8 Suppresses the Stemness of Human Melanoma Stem-like Cells by Targeting the Transcription Factor YB-1

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Abstract

Cross-species regulation of gene expression by microRNA is a possible untapped opportunity for miRNA-based therapy. In this study, we report a novel approach to ablate melanoma stem-like cells by targeting the transcription factor YB-1, which is significantly and selectively upregulated in these cells in melanoma. Silencing YB-1 expression was sufficient to significantly inhibit the stemness of melanoma stem-like cells. In exploring YB-1 targeting, we discovered that the shrimp microRNA miR-S8 could suppress human YB-1 expression in melanoma stem-like cells. Mechanistic investigations revealed that miR-S8 recognized the 3′UTR of YB-1 mRNA and mediated its degradation. In tumor cell and xenograft experiments, miR-S8 suppressed the tumorigenic capacity of melanoma stem-like cells by targeting human YB-1. Overall, our results illuminated a novel aspect of miRNA-mediated cross-species gene expression and its use in regulating cancer stem-like cells. *Cancer Res; 77(20); 5543–53. ©2017 AACR.*

Introduction

Cancer is a major cause of death because of its high incidence and mortality (1). Owing to the lack of specific therapeutic targets, chemotherapy and biotherapy are the only available treatments for cancer (2). Unfortunately, the repeated clinical use of chemotherapeutic drugs usually results in cancer cell resistance to these treatments, leading to tumor relapses (3). With the discovery of leukemia stem cells (4), there has been a growing understanding that malignant tumors consist of two distinct cell subpopulations, the few cells that continue to generate descendant cells semipermanently (cancer stem cells) and the other cells that eventually stop growing as a consequence of differentiation and aging (cancer non-stem cells). Thus, a tumor can be regarded as a cell mass with a biological hierarchy that is orchestrated by cancer stem cells, which are a group of cancer cells with the ability to self-renew and differentiate similar to normal stem cells (5). In contrast to cancer non-stem cells and general cancer cells, cancer stem cells have their own characteristics, such as high proliferation and strong drug resistance (6, 7). As previously reported, cancer stem cells are associated with treatment failure and tumor relapse (8). These specific features of cancer stem cells may result from a series of regulatory mechanisms of gene expression. Therefore, the regulatory mechanisms of gene expression play important roles in the tumorigenesis of cancer stem cells.

As key regulatory elements of gene expression, transcription factors have critical effects on the proliferation and differentiation of stem cells, including tumor stem cells. Some transcription factors that control developmental decisions can promote the reacquisition of developmental programs required for tumor genesis and function as oncogenes. In multiple malignancies, the pluripotency and neurodevelopmental factor, SRY-related HMG box-2 (SOX2, a transcription factor), is an indispensable driver of stem-like populations (9). Four core transcription factors are reported to identify candidate tumor propagating cells in primary glioblastoma (GBM) tumor stem cells (10). These results indicate that transcription factors play major roles in tumorigenesis of cancer stem cells. However, the regulation of transcription factor expression by miRNAs has not been extensively explored.

miRNAs, small noncoding regulatory RNAs with less than 25 nucleotides, have important roles in the regulation of tumorigenesis (11, 12). By base pairing with the 3′ untranslated regions (3′UTR) of their target mRNAs through the seed sequences of the miRNAs, miRNAs function as a switch of the gene expression regulatory network and are thus involved in virtually all biological processes, including stem cell maintenance, differentiation, and development (13, 14). miRNAs possess multiple targets, implying that an individual miRNA can regulate the expression of different genes from different species. In our previous studies, we show that some shrimp miRNAs were involved in the regulation of phagocytosis of shrimp and human cancer by targeting shrimp genes or human genes (15, 16). At present, this cross-species gene expression regulation of miRNAs remains to be investigated.

To address this issue, the regulation of human Y-box binding protein 1 (YB-1) gene expression by a shrimp miRNA (miR-S8) was characterized in this study. YB-1, a transcription factor, is associated with tumorigenesis. In this study, our results revealed that shrimp miR-S8 could suppress the stemness of melanoma stem cells by targeting YB-1 in a cross-phylum manner.

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Materials and Methods

Cell culture

Melanoma cell line MDA-MB-435 was purchased from the ATCC. MDA-MB-435 cells and cancer non-stem cells were cultured in Leibovitz's L-15 medium (Sigma) supplemented with 10% FBS. Melanoma stem cells were cultured in DMEM/F-12 medium (Invitrogen) supplemented with 20 ng/mL EGF (Beyotime Biotechnology), 10 ng/mL basic fibroblast growth factor (Beyotime), 5 μg/mL of insulin (Beyotime), and 2% of B-27 (Sigma). Melanoma stem cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. MDA-MB-435 cells and non-stem cells were cultured at 37°C with 100% humidified atmosphere.

Sorting cancer stem cells and cancer non-stem cells

The sorting of melanoma stem cells was conducted using an ALDEFLUOR Kit (Cyagen Biosciences Inc.) for detection of aldehyde dehydrogenase 1 (ALDH1), a marker of melanoma stem cells (17). MDA-MB-435 cells were suspended in ALDEFLUOR assay buffer containing ALDH1 fluorescent substrate BODIPY-aminoacetate (BAAA, 1 μmol/L) and incubated for 40 min at 37°C. As a negative control, an aliquot of cells was treated with 50 mmol/L diethylaminobenzaldehyde (DEAB), a specific ALDH1 inhibitor. Following incubation, the cells were centrifuged at 250 × *g* for 5 minutes. After removing the supernatant, the cell pellet was resuspended in 0.5 mL of ALDEFLUOR assay buffer and stored at 4°C for FACS. FACS was conducted with a flow cytometer at an excitation of 575 nm.

Tumorsphere formation assay

Tumorsphere formation assay was conducted under non-adherent and serum-free conditions. The cells were suspended in DMEMT12 medium (Invitrogen). Then, a single cell was plated into an ultralow adherent 96-well plate and cultured in DMEMT12 medium supplemented with 20 ng/mL EGF (Beyotime), 10 ng/mL basic fibroblast growth factor (Beyotime, China), 5 μg/mL of insulin (Beyotime, China) and 2% of B27 (Sigma, USA). The cells were cultured for 2 weeks and examined under a light microscope. The forming spheres were scattered in DMEM/F-12 medium. Subsequently, the tumorsphere formation assay was conducted with a single cell of them. The tumorsphere formation assay was carried out three times.

Tumorigenicity in nude mice

Cancer stem cells and cancer non-stem cells were collected at 5×10^3 cells/mL in physiological saline. Matrigel (Becton, Dickinson and Company) was added to the cell suspension at a ratio of 1:2. Then, 100 μL of cell suspension was subcutaneously injected into NOD/SCID mice to induce tumor growth. Forty days later, the mice were sacrificed to examine the tumors. All procedures performed on mice in this study were performed in accordance with the protocols approved by The China Institutional Animal Care and Use Committee (IACUC). All the methods were carried out in accordance with the approved guidelines.

Quantification of mRNA with real-time PCR

Total RNAs were extracted using an RNA Isolation Kit (Ambion). The reverse transcription reaction was conducted using PrimeScript RT Reagent Kit (TaKaRa). Quantitative real-time PCR was conducted with 2 × TaqMan Premix Ex Taq (TaKaRa). The PCR amplification reaction mixture (10 μL) contained cDNA, primers [YB-1 (Y box binding protein 1), 5′-GCAGCAGACCG-TAACCATTA-3′, and 5′-CCTCGTTCTTTTCCCCACT-3′; Oct-3/4 (Octumer-binding transcription factor-3/4), 5′-GAGCAAAACCC GGAGGAGT-3′ and 5′-TTCTCTTTCGGGCCTGC AC-3′; Nanog (Nanog homeobox), 5′-GCTTGCCTTGCTTTGAAGCA-3′ and 5′-TT CTTGACTGGG ACCTTGTC-3′; ALDH1 (aldehyde dehydrogenase 1), 5′-TTACCTGTCCTACTCACCGA-3′ and 5′-CTCCTTAT-CTCCTTCTTCTACCT-3′; ABCG2 (ATP binding cassette subfamily G member 2), 5′-GGCCTCAGGAAGACTTATGT-3′ and 5′-AAG-GAGGTGGTGTAGCTGAT-3′; GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), 5′-GGTATCGTGGAAGGACTCA TGAC-3′ and 5′-A TG CCAGTGAGCTTCCCGTTCAG-3′] and TaqMan Universal PCR Master Mix (TaKaRa). GAPDH was included for normalization. The $2^{-(\Delta\Delta C_t)}$ method was used to calculate the relative fold change of mRNA expression. The thermal cycle conditions included maintaining the reactions at 95°C for 30 seconds, and then alternating for 40 cycles between 95°C for 5 seconds and 60°C for 30 seconds.

Western blot analysis

The proteins were separated using 12% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with triethanolamine-buffered saline solution (TBS) containing 5% skim milk. Subsequently, the membrane was incubated overnight with the antibody against YB-1 or β-tubulin, followed by incubation with the alkaline phosphatase-conjugated secondary antibody (Roche) for 2 hours at room temperature. After a rinse, the membrane was detected with BCIP/NBT substrate (Sangon Biotech).

Target gene prediction of miRNA

The targets of miR-S8 were predicted using TargetScan, miRanda, Pictar, and miRInspector algorithms. The seed sequence (the second to the seventh nucleotides) of miR-S8 was complementary to the 3′UTR of its target mRNA. Predictions were ranked based on the predicted efficacy of targeting as calculated using the context$^+$ scores from the sites.

Dual-luciferase reporter assay

The miR-S8 binding sites of the YB-1 3′UTR (5′-AATTGTTTCA-TATCTGGTCAAGTTGAGA-3′) and the YB-1 3′UTR mutant (5′-AATTGTTTCATATCTAACTGGGTTGAGA-3′) were cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) and confirmed by DNA sequencing. Then, 50 nmol/L of miR-S8 or the control miRNA was cotransfected with 0.1 mg of the YB-1 3′UTR plasmid or the YB-1 3′UTR mutant plasmid into MDA-MB-435 cells using Lipofectamine 2000. At 36 hours after cotransfection, the luciferase activity of cells was measured using the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed to evaluate the binding of shrimp miRNA to human Ago2 protein. The recombinant glutathione S-transferases (GST)-Ago2 was purified. Shrimp miR-S8 (40 mmol/L) was incubated with human Ago2 protein at different concentrations. Shrimp miR-S8 was synthesized by Shanghai GenePharma Co. Ltd.. After incubation in the reaction buffer (0.1 M KCl, 1 mmol/L DTT, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, pH 7.6) for 30 minutes at 37°C, the

mixture was separated by 1% agarose gel electrophoresis at 100 V for 25 min and then stained with ethidium bromide to detect RNA or Coomassie blue to detect protein.

miR-S8–mediated degradation of its target mRNA

The 3′UTR of YB-1 mRNA was amplified with sequence-specific primers (5′-ATGCCGGCTTACCATCTCTA-3′ and 5′-AAGTTGAA-ACTGCATTTA AT-3′), which contained T7 promoter sequence at its 5′-end of the forward primer, using a T7 transcription kit (TaKaRa) according to the manufacturer's protocol. To obtain the Ago2 complex, cancer stem cells were homogenized in lysis buffer [20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.25% NP-40 (Nonidet P-40), 1 mmol/L phenylmethanesulfonylfluoride (PMSF), pH 7.5] for 15 minutes on ice and then centrifuged at 10,000 × *g* for 10 minutes at 4°C. The cell lysate supernatant was incubated with the human Ago2-specific antibody, which was prepared in our laboratory, for 1 hour at 4°C, followed by mixing with protein-G–coupled agarose beads for 4 hours at 4°C. After washes with cold PBS, the beads were resuspended in reaction buffer [100 mmol/L KOAc, 40 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mmol/L $MgCl_2$, 2 mmol/L dithiothreitol (DTT), 0.35% Triton X-100, 1 mmol/L PMSF, pH 7.6]. To evaluate the miR-S8–mediated degradation of YB-1 mRNA, the co-immunoprecipitated product of human Ago2, 3′UTR of YB-1 mRNA (100 ng) and miR-S8 at different concentrations were mixed and supplemented with 2 μL of 10 mmol/L ATP and 2 μL of 2 mmol/L GTP at a total volume of 20 μL. After incubation at 30°C for 2 hours, the RNAs were separated by 1% agarose gel electrophoresis and then transferred to a nylon membrane (GE Healthcare). The RNAs were detected using a 5′ digoxigenin (DIG)-labeled YB-1 probe (5′-CCCAAG GTGACAAAACTAAGT-3′).

Sequencing of the degraded mRNA 3′UTR fragment

The YB-1 mRNA 3′UTR was incubated with miR-S8 (20 nmol/L) and Ago2 complex at 30°C for 2 hours. Subsequently, the degraded fragment was separated by 1% agarose electrophoresis. After recovery from the agarose gel, the RNAs were reversely transcribed into cDNAs using PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa). The single-stranded cDNAs were transformed into double-stranded cDNAs with second strand cDNA Synthesis Kit (Beyotime Biotechnology). The cDNAs were cloned into the pEASY-Blunt simple cloning vector (Transgen Biotech) and subjected to sequencing.

RNA interference assay in melanoma stem cells

To silence the expression of YB-1 in cancer stem cells, the RNA interference (RNAi) assay was conducted using YB-1-specific siRNA (YB-1-siRNA, 5′-GGAACGGAUAUGGUUUCAUTT-3′). The melanoma stem cells (1×10^5) were transfected with 50 nmol/L of YB-1-siRNA using Lipofectamine 2000 (Invitrogen). As a control, YB-1-siRNA-scrambled (5′-UUCUCCGAACGUGU-CACGUTT-3′) was included in the transfection. All the siRNAs were synthesized by Shanghai GenePharma Co., Ltd. At different times after transfection, the cells were harvested for later use.

The transfection of shrimp miR-S8 into melanoma stem cells

The melanoma stem cells were cultured in a six-well plate at a density of 1×10^5/well. Aliquots of 100 nmol/L of miR-S8 (5′-GUUGACCGAAGCGGAGGAG-3′) or miR-S8-scrambled (5′-UUCUCCGAACGUGUCACGUTT-3′) were transfected into cancer stem cells using Lipofectamine 2000 (Invitrogen). The cells were harvested after transfection for 36 hours. The miR-NAs were synthesized by Shanghai GenePharma Co., Ltd.

Cell viability and proliferation analysis

Cell viability and proliferation analysis was conducted with MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assays (Promega). Cells were seeded onto a 96-well plate with 100 μL of culture medium and 20 μL of MTS reagent. Then, the plate was incubated for 1.5 hours at 37°C in a humidified incubator containing 5% CO_2. The absorbance was recorded at 450 nm. Cell proliferation rate analysis was performed via calculating cell viability of time-course assays. All experiments were repeated three times.

Cell-cycle analysis

Cell-cycle analysis was conducted with flow cytometry. Cell samples were fixed in ice-cold ethanol for 4 hours. Then the cells were incubated with DNase-free RNase A (20 μg/mL) for 30 minutes. After centrifugation at 500 × *g* for 5 minutes, the cells were stained with propidium iodide (PI; 50 μg/mL). The fluorescence intensity of 1×10^4 cells was measured with a flow cytometer at an excitation wavelength of 488 nm.

Analysis of caspase-3/7 activity

The caspase-Glo 3/7 assay (Promega) was used to evaluate the activities of caspase-3/7 according to the manufacturer's protocol. Cells at a density of 1×10^4/well were plated in a 96-well plate and transfected with siRNA or miRNA. Thirty-six hours later, 100 μL of caspase-Glo 3/7 reagent (Promega) was added to each well. After incubation in the dark at room temperature for 30 minutes, the luminescence of cells was measured.

Apoptosis detection with Annexin V

The FITC Annexin V Apoptosis Detection Kit I (Becton, Dickinson and Company) was used to examine the cell apoptosis. Cells were harvested and rinsed with cold PBS and then resuspended in 1 × Annexin binding buffer at 1×10^6 cells/mL. Subsequently, 5 μL of Alexa Fluor 488 Annexin V and 0.1 μg of PI were added to the cells. After incubation at room temperature for 15 minutes in the dark, 400 μL of 1 × Annexin binding buffer was added to the sample. The sample was analyzed with a flow cytometer at an excitation of 575 nm.

Tumorigenicity in nude mice

Experiments to evaluate the effects of miR-S8 on solid tumors were performed in nude mice. Melanoma stem cells and cancer non-stem cells were suspended in physiological saline at 5×10^3 cells/mL. Then matrigel (Becton, Dickinson and Company) was added to the cell suspension at a ratio of 1:2. The cell suspension (100 μL) was subcutaneously injected into NOD/SICD mouse to induce tumor growth. Six weeks later, when the tumor volume was around 15 mm^3, mice were injected via the lateral tail vein with 80 mg/kg of miR-S8 or miR-S8-scrambled every 3 days. The tumor volume was measured every week. At 45 day after the first miRNA injection, the mice were sacrificed. The tumor sizes and tumor weights were examined. All procedures performed on mice in this study were performed in accordance

with the protocols approved by the IACUC. All the methods were carried out in accordance with the approved guidelines.

Northern blot analysis

The small RNAs were extracted from tumors using a mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's manual. Then, the RNAs were electrophoresed on a 15% polyacrylamide gel containing 7 M urea for 2 hours at 5 mA, followed by transfer to a nylon membrane (Amersham Biosciences) at 300 mA for 2 hours. After UV cross-linking, the membrane was prehybridized in prehybridization solution (Roche) for 30 minutes. Subsequently, the membrane was hybridized with DIG-labeled miR-S8 probe (5′-CTCCTCCGCTTCGGTCAAC-3′) overnight. The membrane was rinsed and then blocked in a blocking solution (Roche) for 1 hour at room temperature. The membrane was incubated with the antibody against DIG-labeled alkaline phosphate (Roche) for 2 hours at room temperature and detected with the substrate BCIP/NBT solution (Roche).

Immunhistochemical analysis

To detect YB-1 protein in solid tumors of mice by immunhistochemical staining, 5-μm-thick section was placed on a precoated slide with 3-triethoxysilylpropylamine (Merck). The slide was soaked in xylol for 1 hour and washed in series of decreasing alcohol concentrations. After deparaffinizing the tissue, antigen retrieval of the section was performed in a microwave for 5 minutes in TEC-buffer (0.05 M Tris-HCl, 0.05 M ethylenediaminetetraacetic acid, 0.02 M Na-Citrate, pH 7.8), followed by peroxidase blocking. After incubation with the primary antibody against YB-1 for 12 hours, the slide was incubated with a biotinylated rabbit anti-mouse antibody (Vector) for 30 minutes. Subsequently, the slide was stained with diaminobenzidine (Sigma) for 10 minutes to label YB-1 protein and then counterstained with hematoxylin to label nuclei.

Statistical analysis

All data were presented as mean ± SD. Numerical data were processed using one-way ANOVA, and Student *t* test was used to assess the significant difference. All assays were biologically repeated for three times.

Results

The upregulation of YB-1 in melanoma stem cells

To characterize the expression of YB-1 in melanoma stem cells, the melanoma stem cells and the corresponding cancer non-stem cells were sorted from MDA-MB-435 cells by detecting the activity of ALDH1, a marker of cancer stem cells (17). The ALDH1-positive cells were potentially cancer stem cells (Fig. 1A, P4 region), whereas the ALDH1-negative cells were cancer non-stem cells (Fig. 1A, P3 region). The potential cancer stem cells were subjected to tumorsphere formation assay to examine whether the cells could self-renew under serum-free conditions. The results of three tumorsphere formation assays showed that the ALDH1-positive cells formed large floating spheres (Fig. 1B), suggesting these cells might be cancer stem cells. To evaluate the tumor formation ability of ALDH1-positive cells *in vivo*, 500 cells isolated from the spheres of tumorsphere formation assays were subcutaneously injected into immunodeficient mice. Our results indicated that tumors formed in five mice injected with the ALDH1-positive cells (Fig. 1C). However, tumors were not observed in five mice injected with the ALDH1-negative cells (Fig. 1C). These data revealed that the ALDH1-positive cells were melanoma stem cells.

To reveal the role of YB-1 in the progression of cancers, the expression level of YB-1 in melanoma stem cells and cancer non-stem cells was investigated. Quantitative real-time PCR data showed that YB-1 was significantly upregulated in the melanoma stem cells compared with the cancer non-stem cells (Fig. 1D). Western blots yielded the similar results (Fig. 1E). Our findings presented that YB-1 might play an important role in melanoma stem cells.

The requirement of YB-1 for the stemness of melanoma stem cells

To explore the effects of YB-1 on the stemness of melanoma stem cells, YB-1 expression was knocked down, followed by evaluation of stemness. Western blots showed that the YB-1 protein level was very low when the YB-1-specific siRNA (YB-1-siRNA) was transfected into the melanoma stem cells at 36 hours after transfection (Fig. 2A). The low YB-1 protein level in the stem cells treated with YB-1-siRNA resulted from the low *YB-1* mRNA level compared with the control (Fig. 2B), providing strong evidence that the YB-1 expression was silenced by YB-1-siRNA in melanoma stem cells.

To evaluate the effects of YB-1 silencing on the stemness of melanoma stem cells, the expression levels of stemness genes in YB-1-silenced cancer stem cells were examined. Our results showed that the YB-1 silencing led to significant downregulation of stemness genes (*Oct-3/4*, *Nanog*, *ALDH1*, and *ABCG2*) compared with the control (Fig. 2C), indicating that YB-1 played a positive role in the stemness of melanoma stem cells. The data from tumorsphere formation assays revealed that the sphere-forming capacity of melanoma stem cells was significantly suppressed when YB-1 expression was knocked down (Fig. 2D). Our findings indicated that YB-1 was required for the stemness of melanoma stem cells.

To further explore the effects of YB-1 silencing on the growth of melanoma stem cells, MTS assays were conducted. Our results indicated that YB-1 silencing led to a significant decrease of cancer stem cell viability (Fig. 2E). In contrast, there was no significant effect on the growth of cancer non-stem cells after YB-1 knockdown (Fig. 2E). To reveal the mechanism of YB-1 in melanoma stem cell proliferation, the cell cycle of YB-1-silenced cancer stem cells was evaluated. The results from flow cytometry showed that the percentage of cells in the G_1 phase following the YB-1-siRNA treatment was much higher than that of the control (Fig. 2F), indicating that the YB-1 silencing could cause cancer stem cell-cycle arrest at G_1 phase. Therefore, YB-1 had positive effects on the proliferation of melanoma stem cells.

To assess whether the cancer stem cell-cycle arrest triggered apoptosis, the apoptotic activity of cancer stem cells transfected with YB-1-siRNA was characterized. Caspase-3/7 activity detection showed that the YB-1 knockdown could induce melanoma stem cell apoptosis compared with the controls (Fig. 2G), indicating that YB-1 silencing induced cancer stem cell-cycle arrest and further promoted apoptosis of cancer stem cells. The data from Annexin V assays revealed that following the transfection of YB-1-siRNA into cells, the percentage of apoptotic cells (Annexin V–positive) was significantly increased in cancer stem cells but not in cancer non-stem cells (Fig. 2H). Our findings indicated that

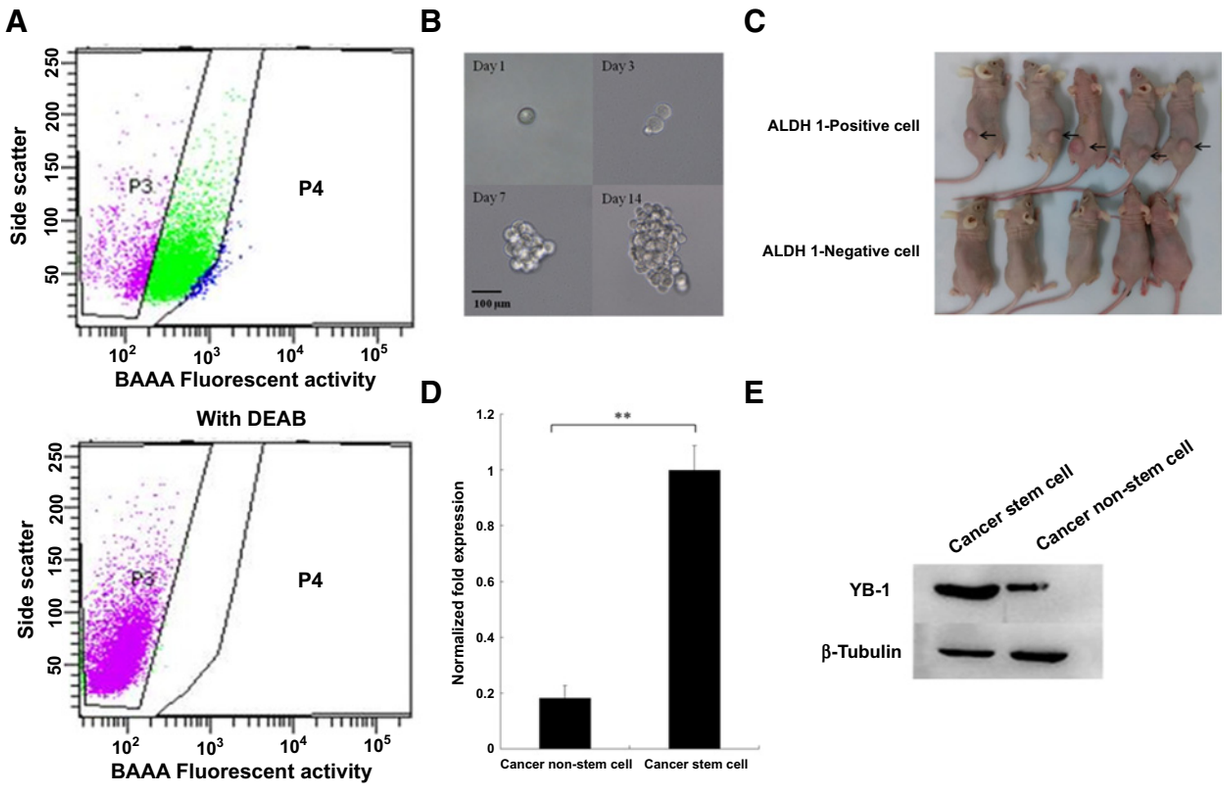

Figure 1.

The upregulation of YB-1 in melanoma stem cells. **A,** The sorting of melanoma stem cells. The fluorescence-activated cell sorting was performed based on the detection of ALDH1 activity using the ALDH1 fluorescent substrate BODIPY-aminoacate (BAAA). As a control, the activity of ALDH1 was inhibited by DEAB. The ALDH1-positive cells were potential melanoma stem cells (P4 region) and ALDH1-negative cells were noncancer stem cells (P3 region). **B,** Tumorsphere formation assay. The ALDH1-positive cells were subjected to tumorsphere formation assay. The sphere formation was examined with a light microscope at day 1, 3, 7, and 14. Scale bar, 100 μm. **C,** Tumorigenicity of cancer stem cells in nude mice. Five mice were subcutaneously injected with the cells isolated from the spheres of tumorsphere formation assays (the ALDH1-positive cells). As controls, the ALDH1-negative cells were subcutaneously injected into five mice. Forty days later, the tumors were examined. Arrows, tumors. **D,** The differential expression of YB-1 in melanoma stem cells and cancer non-stem cells. Quantitative real-time PCR was conducted to detect the YB-1 mRNA level. **, $P < 0.01$. **E,** Western blot analysis of YB-1 in melanoma stem cells and cancer non-stem cells. β-Tubulin was used as a control.

YB-1 played a positive role in the regulation of apoptosis of melanoma stem cells.

Taking the above data together, YB-1 was required for cancer cell stemness and played important roles in the progression of melanoma stem cells.

The regulation of YB-1 expression by shrimp miR-S8 in melanoma stem cells

Based on the target predictions, a shrimp microRNA miR-S8 might target the *YB-1* gene (Fig. 3A). The seed sequence of miR-S8 was complementary to the 3′UTR of the *YB-1* gene.

To explore the interaction between miR-S8 and *YB-1*, dual-luciferase reporter assays were conducted in MDA-MB-435 cells. Our results showed that the luciferase activity of the cells transfected with miR-S8 and YB-1 3′UTR was significantly decreased compared with the controls (Fig. 3B), showing that miR-S8 directly interacted with the YB-1 gene.

To investigate the targeting of *YB-1* gene by miR-S8, miR-S8 was overexpressed in melanoma stem cells, followed by the evaluation of YB-1 expression level. The results of quantitative real-time PCR and Western blots showed that the expression level of YB-1 was significantly decreased when miR-S8 was overexpressed in cancer stem cells (Fig. 3C and D). Our findings revealed that *YB-1* was a target gene of miR-S8.

To further evaluate whether the exogenous shrimp miR-S8 could function in human cancer cells, miR-S8–mediated degradation of YB-1 mRNA in the Ago2 complex was examined. The results of EMSA showed that miR-S8 was bound to human Ago2 protein, indicating that shrimp miR-S8 could be loaded into the Ago2 complex (Fig. 3E). Northern blots revealed that miR-S8 mediated the degradation of the YB-1 mRNA in a concentration-dependent manner (Fig. 3F). To confirm the miR-S8-mediated degradation of target mRNA, the degraded 3′UTR fragment of YB-1 mRNA was sequenced. The data demonstrated that miR-S8 could mediate the degradation of target mRNA 3′UTR and the miR-S8–mediated degradation stopped in the vicinity of the 3′UTR sequence complementary to the seed sequence of miR-S8 (Fig. 3G). Our results indicated that miR-S8 mediated the degradation of its target mRNA in the human Ago2 complex.

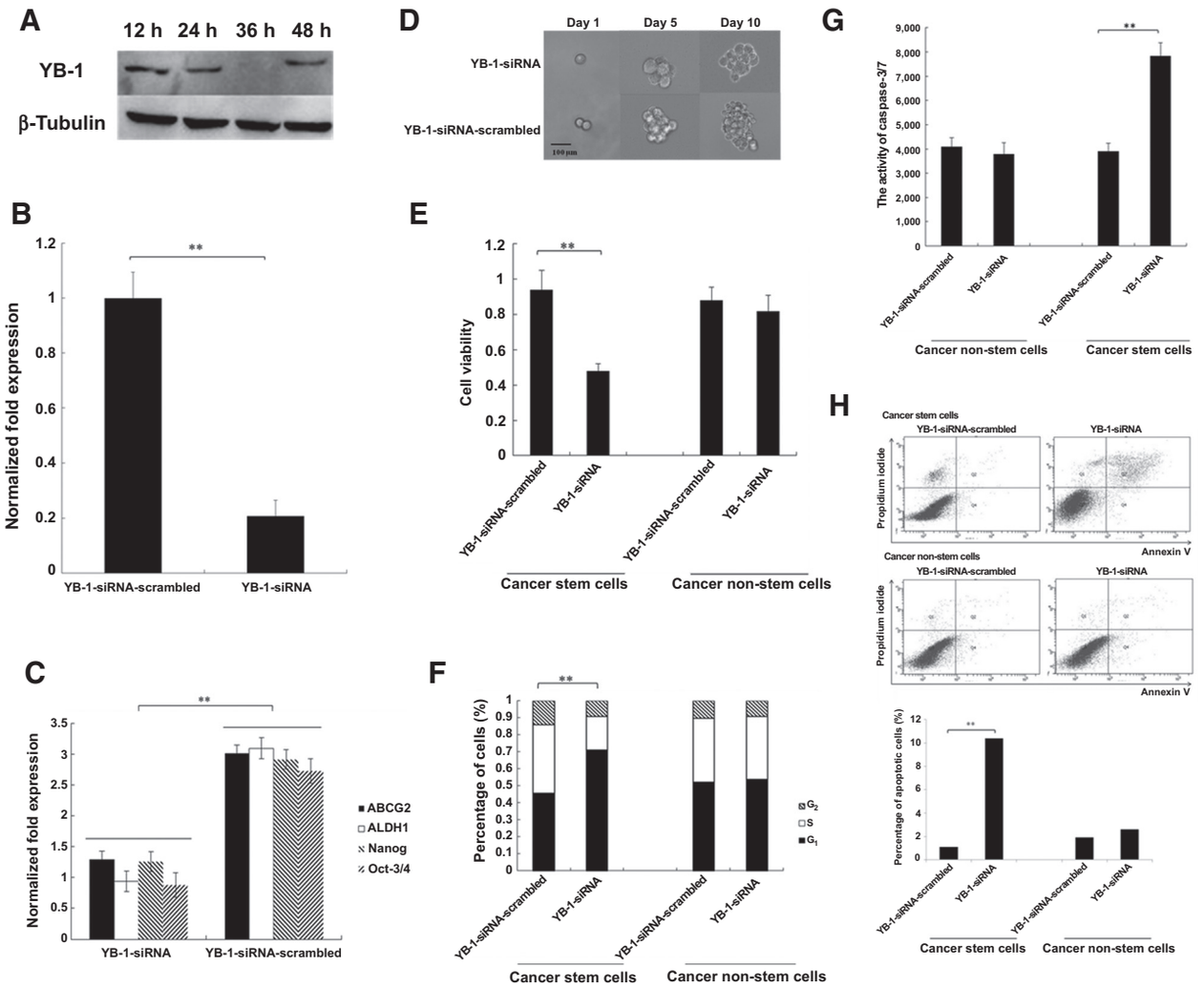

Figure 2.

The requirement of YB-1 for the stemness of melanoma stem cells. **A,** The knockdown of YB-1 expression in cancer stem cells. The cancer stem cells were transfected with YB-1-siRNA. At different times after transfection, the YB-1 protein was detected with Western blot analysis. β-Tubulin was used as a control. **B,** The *YB-1* mRNA level in cancer stem cells transfected with YB-1-siRNA. The YB-1-siRNA-scrambled was used as a control. At 36 hours after transfection, the mRNA level of the cells was evaluated using quantitative real-time PCR. **, $P < 0.01$. **C,** The effects of YB-1 silencing on the expression of stemness genes in melanoma stem cells. At 36 hours after transfection of YB-1-siRNA in cancer stem cells, quantitative real-time PCR was used to evaluate the expression levels of stemness genes. **, $P < 0.01$. **D,** The influence of YB-1 knockdown on the tumorsphere formation capacity of cancer stem cells. The expression of YB-1 was silenced by YB-1-siRNA in melanoma stem cells. At different times after siRNA treatment, the tumorsphere formation capacity of cancer stem cells was examined. YB-1-siRNA-scrambled was used as a control. Scale bar, 100 μm. **E,** The impact of YB-1 silencing on the proliferation of melanoma stem cells. **, $P < 0.01$. **F,** The role of YB-1 in the cell cycle of melanoma stem cells. The cancer stem cells and non-stem cells were transfected with YB-1-siRNA. At 36 hours after transfection, the cell cycle was evaluated. **, $P < 0.01$. **G,** The role of YB-1 in the regulation of cancer stem cell apoptosis. The cancer stem cells and non-stem cells were treated with YB-1-siRNA. Thirty-six hours later, apoptosis was examined with the caspase-3/7 activity assay. **, $P < 0.01$. **H,** The detection of apoptosis using Annexin V assay. Apoptosis of cancer stem cells and cancer non-stem cells was evaluated by flow cytometry at 36 hours after the transfection of YB-1-siRNA. **, $P < 0.01$.

The influence of miR-S8 on the stemness of melanoma stem cells

To explore the role of miR-S8 in regulating the stemness of melanoma stem cells, the shrimp miR-S8 was transfected into melanoma stem cells. The quantitative real-time PCR results showed that the expression of cancer stem genes (*Oct-3/4, Nanog, ALDH1*, and *ABCG2*) was significantly decreased in the miR-S8–transfected stem cells compared with the control (Fig. 4A), indicating that miR-S8 had a negative effect on the stemness of melanoma stem cells. The data from tumorsphere formation assays indicated that the sphere forming ability of miR-S8–transfected stem cells was inhibited compared with the control (Fig. 4B). Together, these data strongly revealed that miR-S8 played a negative role in regulating the stemness of melanoma stem cells.

To reveal the miR-S8-mediated inhibitory mechanism on tumorsphere formation of melanoma stem cells, the viability of miR-S8-transfected cancer stem cells was evaluated. The MTS data

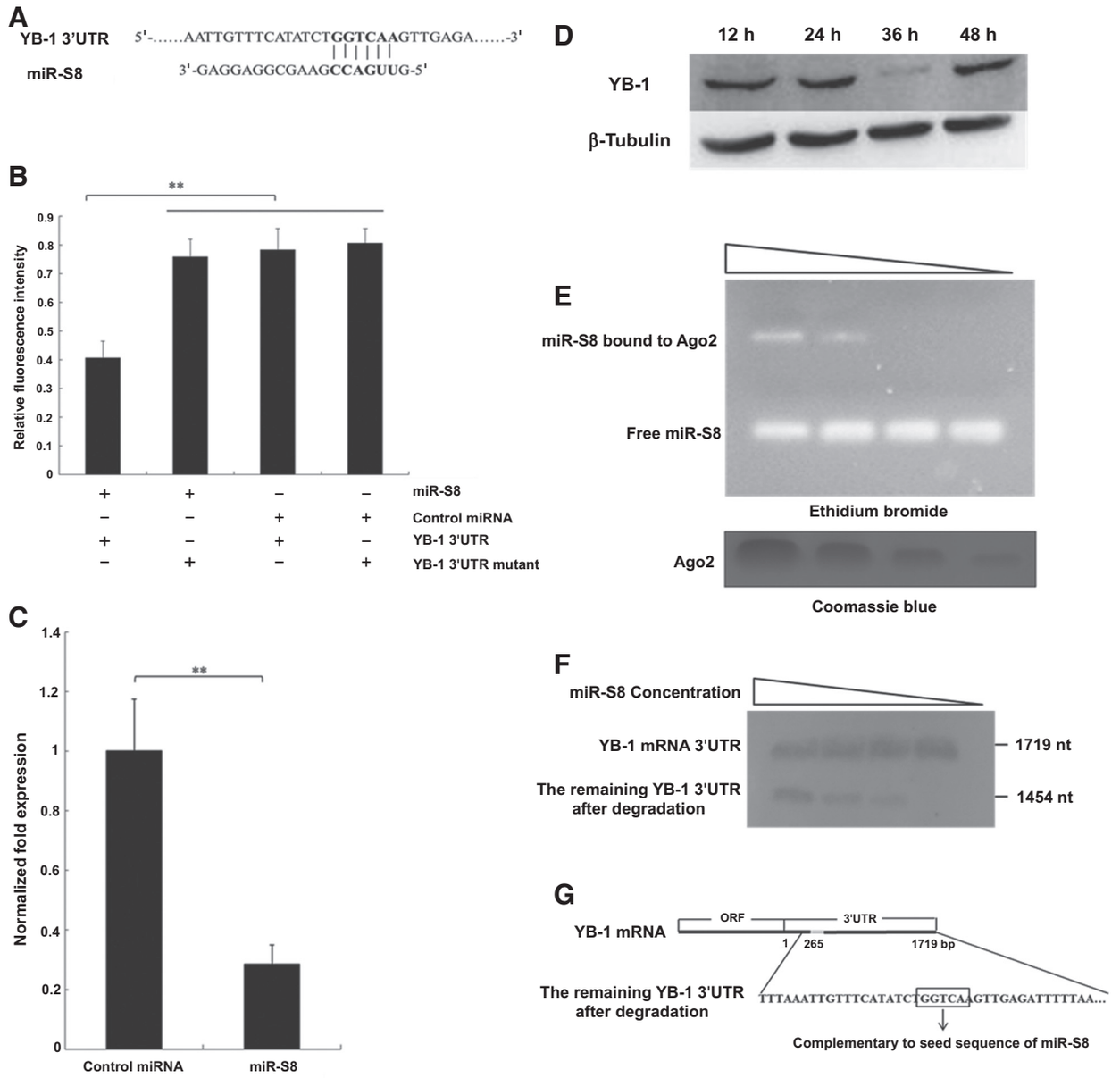

Figure 3.

The regulation of YB-1 expression by shrimp miR-S8 in melanoma stem cells. **A,** The target prediction. As predicted, *YB-1* was a target gene of miR-S8. The bold letters represent the seed sequence of miR-S8. **B,** The direct interaction between miR-S8 and the YB-1 gene. MDA-MB-435 cells were cotransfected with miR-S8 and a luciferase reporter fused with YB-1 3′UTR. At 36 hours after transfection, the firefly and *Renilla* luciferase activities were analyzed. As controls, control miRNA and YB-1 3′UTR mutant were included in the cotransfections. **C,** The interaction between miR-S8 and the YB-1 gene in melanoma stem cells. miR-S8 or the control miRNA was transfected into cancer stem cells. At 36 hours after transfection, the YB-1 mRNA was detected using quantitative real-time PCR. **D,** Western blot analysis of YB-1 in miR-S8-overexpressing melanoma stem cells. At different times after miR-S8 transfection, the YB-1 protein in cancer stem cells was analyzed with Western blot analysis. Numbers represent the time points after the miR-S8 transfection. **E,** The interaction between miR-S8 and human Ago2 protein. Shrimp miR-S8 was incubated with recombinant human Ago2 protein. The mixture was separated by 1% agarose gel and stained with ethidium bromide to visualize the miRNA (top), followed by staining with Coomassie blue (bottom). The wedges indicate the concentration gradient of recombinant protein used. **F,** The influence of miR-S8 on the degradation of its target mRNA. The 3′UTR of YB-1 mRNA and Ago2 complex was incubated with miR-S8 at different concentrations for 2 hours, followed by Northern blot analysis to detect the mRNA using a YB-1 probe. The wedges indicate the concentration gradient of miR-S8 used. **G,** The sequencing of degraded target mRNA 3′UTR mediated by miR-S8. The significant differences between treatments are indicated with asterisks (**, $P < 0.01$).

showed that the viability of miR-S8-transfected cancer stem cells was significantly suppressed compared with the control (Fig. 4C). However, miR-S8 had no effect on the viability of cancer non-stem cells (Fig. 4C). These findings demonstrated that the downregulation of YB-1 by miR-S8 could inhibit the cell proliferation of melanoma stem cells but not cancer non-stem cells. The results of

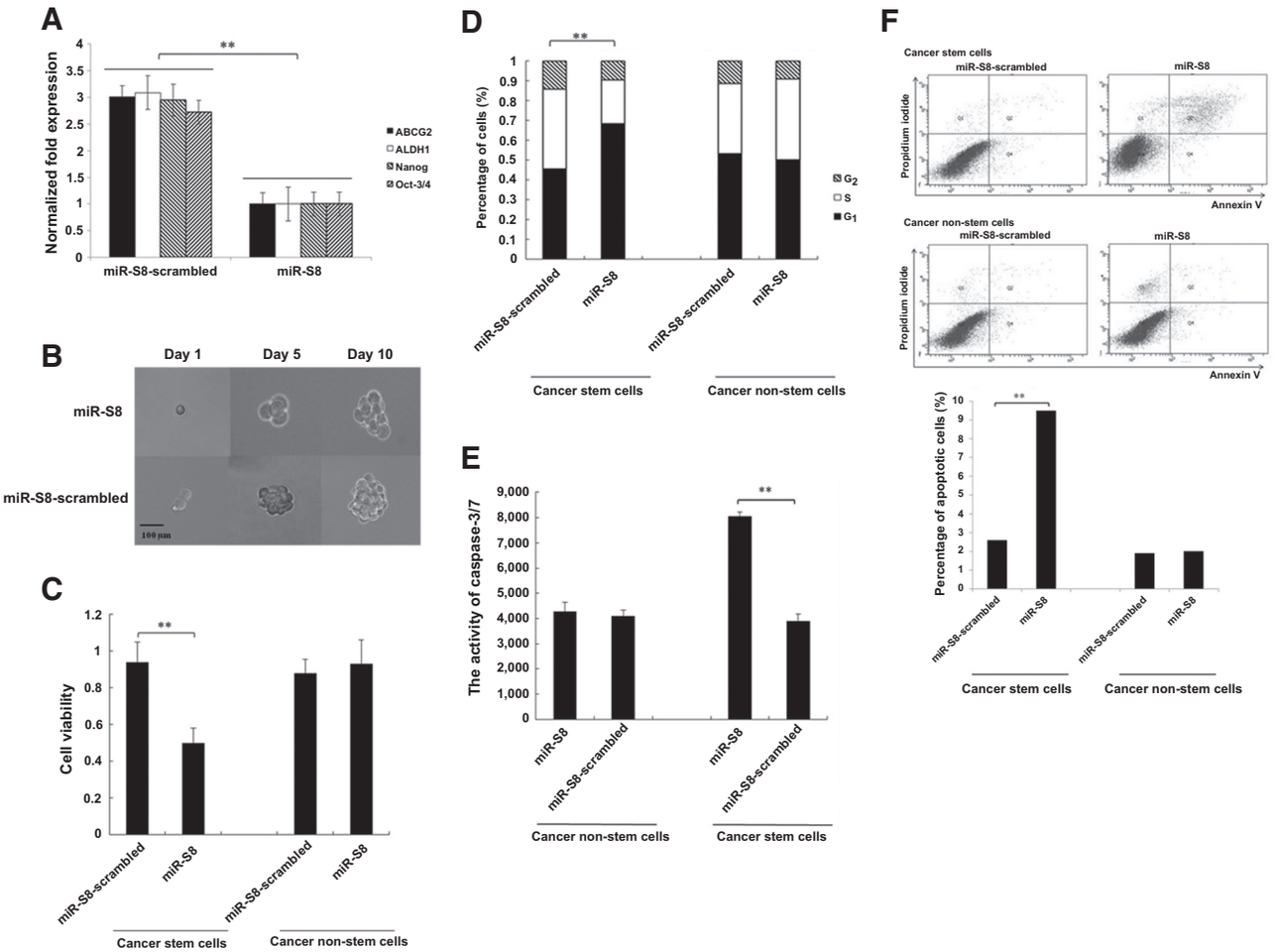

Figure 4.

The influence of miR-S8 on the stemness of melanoma stem cells. **A,** The effects of miR-S8 on the expression of cancer stem genes. Melanoma stem cells were transfected with miR-S8. At 36 hours after transfection, the stem gene expression levels in stem cells were examined with quantitative real-time PCR. **B,** The influence of miR-S8 on the tumorsphere formation capacity of cancer stem cells. Days represent the time after the miR-S8 transfection. Scale bar, 100 μm. **C,** The effects of miR-S8 on the cell viability. The cell viability was evaluated at 36 hours after transfection of melanoma stem cells and cancer non-stem cells with miR-S8. **D,** The influence of miR-S8 on the cell cycle of melanoma stem cells and cancer non-stem cells. At 36 hours after transfection of miR-S8, the cell cycle was examined. **E,** The role of miR-S8 in apoptosis of melanoma stem cells. The cancer stem cells and cancer non-stem cells at 36 hours after the transfection of miR-S8 were subjected to the caspase-3/7 activity analysis. **F,** The detection of apoptosis of miR-S8–transfected cells using Annexin V assays. Apoptosis of melanoma stem cells and cancer non-stem cells was examined by flow cytometry at 36 hours after the transfection of miR-S8. In all panels, the statistical significance between treatments is indicated with stars (**, $P < 0.01$).

cell-cycle assays showed that the percentage of cancer stem cells in the G_1 phase following treatment with miR-S8 was significantly higher than that of the control (Fig. 4D). The cell cycle of cancer non-stem cells was not affected by miR-S8 (Fig. 4D). These data indicated that the YB-1 knockdown by miR-S8 resulted in the cancer stem cell-cycle arrest in the G_1 phase.

To explore whether the YB-1 silencing induced by miR-S8 caused apoptosis, the apoptotic activity of miR-S8-transfected melanoma stem cells was examined. Our results showed that the miR-S8-mediated YB-1 silencing led to a significant increase of caspase-3/7 activity in melanoma stem cells but not in cancer non-stem cells compared with the controls (Fig. 4E). Annexin V assays revealed that the apoptotic percentage of cancer stem cells transfected with miR-S8 was significantly increased compared with the control (Fig. 4F). But miR-S8 had no effect on apoptosis of cancer non-stem cells (Fig. 4F). The data indicated that miR-S8 could promote apoptosis of melanoma stem cells.

The above data provided compelling evidence that shrimp miR-S8 had a negative effect on the stemness of melanoma stem cells by targeting the *YB-1* gene.

The effects of miR-S8 on tumorigenesis of melanoma stem cells *in vivo*

To evaluate the impact of miR-S8 on tumorigenesis of melanoma stem cells *in vivo*, melanoma stem cells or non-stem cells were injected into nude mice, followed by injection of miR-S8 or miR-S8-scrambled and tumor examination (Fig. 5A). Our results indicated that the tumor sizes were significantly reduced in mice

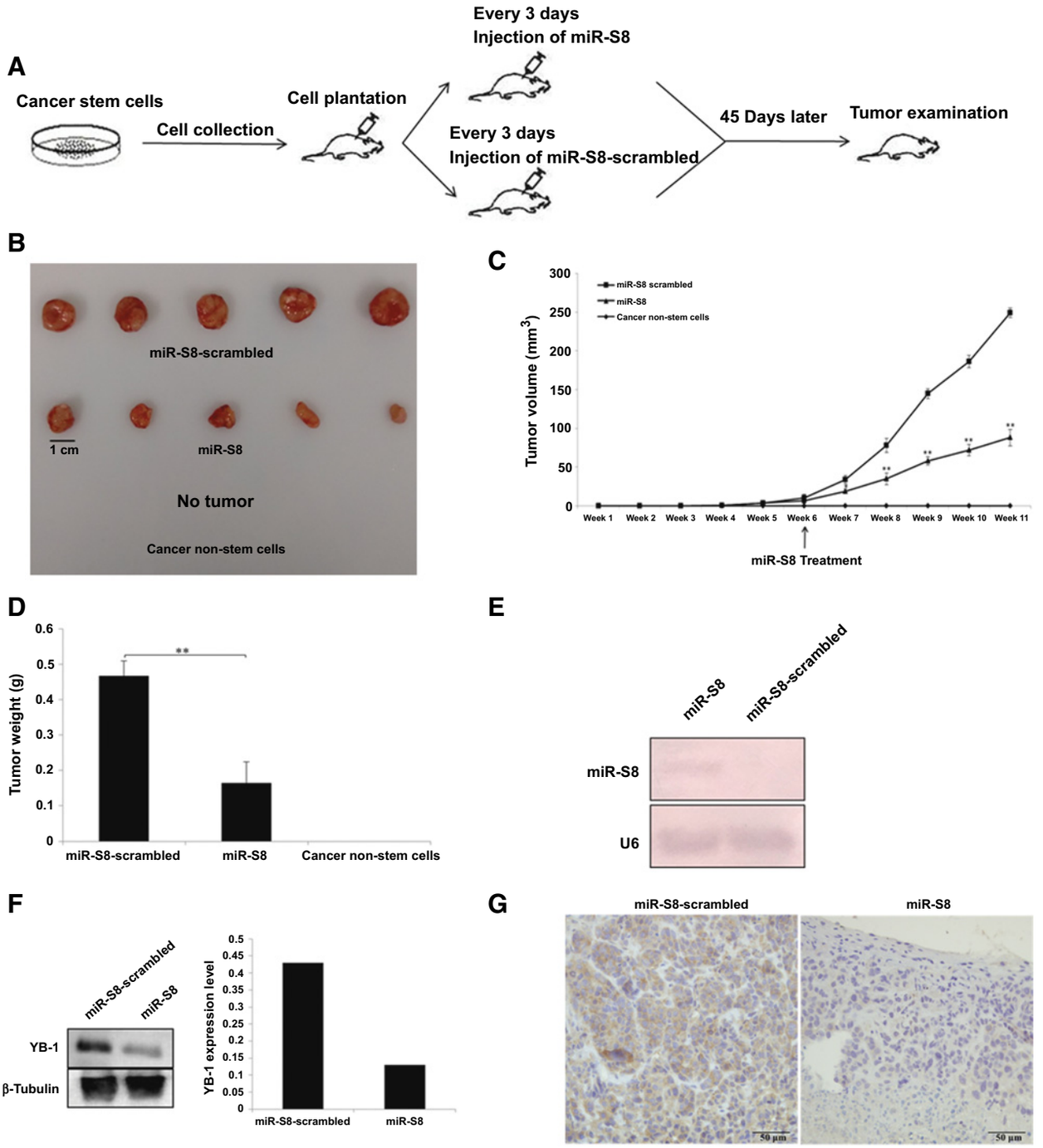

Figure 5.

The effects of miR-S8 on tumorigenesis of melanoma stem cells *in vivo*. **A,** A flow diagram of the *in vivo* experiments. **B,** The influence of shrimp miR-S8 on solid tumors in mice. The melanoma stem cells or non-stem cells were injected into nude mice, followed by treatment with miR-S8 or miR-S8-scrambled. Forty-five days later after the first miRNA injection, the mice were sacrificed and the tumor sizes were examined. **C,** The effects of miR-S8 on tumor volume. The tumor volume of miR-S8-treated mice was examined every week. The mean of 5 mice is indicated. **, $P < 0.01$. **D,** The impact of miR-S8 on tumor weight. The data are the means of 5 mice. **, $P < 0.01$. **E,** The expression level of miR-S8 in solid tumors. Total RNAs extracted from the tumors of miR-S8- or miR-S8-scrambled-treated mice were analyzed using Northern blot analysis with DIG-labeled probes. U6 was used as a control. **F,** The YB-1 protein level in solid tumors of mice treated with miR-S8 or miR-S8-scrambled. Western blot analysis (left) was conducted to examine the YB-1 protein level (right). **, $P < 0.01$. In Western blot analysis, β-tubulin was used as a control. **G,** Immunohistochemical analysis of YB-1 protein in solid tumors of mice treated with miR-S8 or miR-S8-scrambled-treated mice. YB-1 protein was detected with YB-1-specific antibody (yellow). The nuclei were stained with hematoxylin (blue). Scale bar, 50 μm.

treated with miR-S8 compared with those in mice treated with the control (miR-S8-scrambled; Fig. 5B), whereas non-stem cells did not generate any tumors in mice, showing that the shrimp miR-S8 suppressed tumor development *in vivo*. The data on tumor volume and weight revealed that miR-S8 led to significant decreases of tumor volume and weight compared with the control (Fig. 5C and D). These findings indicated that shrimp miR-S8 could inhibit tumorigenesis of melanoma stem cells *in vivo*.

To evaluate whether the suppression of tumorigenesis resulted from the targeting of YB-1 by miR-S8, the expression levels of miR-S8 and YB-1 in solid tumors of mice treated with miR-S8 or miR-S8-scrambled were examined. The results of Northern blots demonstrated that miR-S8 could be detected in tumors of miR-S8-treated mice (Fig. 5E). Western blot analysis data revealed that the YB-1 expression was significantly reduced by miR-S8 compared with the control (miR-S8-scrambled; Fig. 5F), showing that the targeting of YB-1 by miR-S8 led to the inhibition of tumorigenesis in mice *in vivo*. Immunohistochemical staining results showed that the YB-1 protein level in solid tumors of mice treated with miR-S8 was significantly lower than that of the control (miR-S8-scrambled; Fig. 5G).

The above findings indicated that shrimp miR-S8 could suppress tumorigenesis of melanoma stem cells by targeting the *YB-1* gene *in vivo*.

Discussion

A current hypothesis posits that tumor cells after anticancer therapies have a distinct molecular phenotype that confers resistance to treatment (18). These remaining tumor cells may be cancer stem cells. Therefore, eliminating cancer stem cells may improve the outcome of treatment for cancers (19). The ideal strategy would be specific targeting of cancer stem cells by abrogating some important factors that are required for the self-renewal activity of cancer stem cells. At present, a number of stemness genes (such as *Oct-3/4*, *Nanog*, *ALDH1*, and *ABCG2*) have been identified, which are required and associated with the stemness of cancer stem cells (17, 20–22). As an important regulatory mechanism of gene expression, transcription factors have critical implications for embryogenesis, cellular differentiation, proliferation, and apoptosis (23). Many transcription factors, such as the pluripotency and neurodevelopmental factor SRY-related HMG box-2 (SOX2), POU class 3 homeobox 2 (POU3F2), spalt-like transcription factor 2 (SALL2), and oligodendrocyte transcription factor 2 (OLIG2) are responsible for the gene expression regulation of stem cell and cancer stem cell differentiation (10, 24–26). These transcription factors bind and activate *cis* regulatory elements that modulate transcription and thereby directly control gene expression programs in cancer stem cells. However, the regulation of transcription factor gene expression in cancer stem cells has not been extensively explored. As important regulatory factors of gene expression, miRNAs can target multiple genes, suggesting that individual miRNAs may regulate the expressions of different genes from different species. In this investigation, the findings showed that a shrimp miRNA (miR-S8) could inhibit the expression of the YB-1 transcription factor in a cross-species manner, leading to the suppression of tumorigenesis of melanoma stem cells. Our study indicated that natural animal miRNAs might be important sources for antitumor drugs.

As a DNA/RNA-binding protein, YB-1 is involved in multiple cellular processes including proliferation, differentiation, and stress response (27–29). YB-1 protein can shift from the cytoplasm to the nucleus and bind to the promoters of its target genes (30, 31), such as the genes encoding EGF receptor, multidrug resistance proteins, and cell division cycle proteins (29, 32). The expression level of YB-1 is frequently increased in tumors of different origins, including malignant tumors (33, 34). As previously reported, YB-1 may play important pro-oncogenic roles in malignant transformation, cell invasion, and drug resistance in various cancers (35). Therefore, YB-1 is regarded as a marker of tumor resistance to chemotherapy in many types of cancers (36). Despite of the involvement of YB-1 in tumorigenesis, the role of YB-1 in cancers, as well as in cancer stem cells, has not been investigated. Our results revealed that YB-1 had major effects on the stemness of cancer stem cells and that the expression of YB-1 could be regulated by a natural animal miRNA for the first time. Our study showed that shrimp miR-S8 inhibited YB-1 expression by mediating the degradation of YB-1 mRNA. In animals, target mRNA degradation by miRNAs is a major contribution to target gene silencing (37, 38). As reported previously, miRNAs can directly induce 5′-to-3′ exonucleolytic digestion of complementary mRNAs at matching regions (39–41). During the process of exonucleolytic digestion, mRNAs are first deadenylated by the CAF1 (CCR4-NOT transcription complex subunit 8)-CCR4 (C-C motif chemokine receptor 4)-NOT deadenylase complex, followed by decapping with the enzyme DCP2 (decapping mRNA 2; ref. 42). DCP2 exerts its decapping function with the aid of other cofactors for full activity or stability, such as DCP1 (decapping mRNA 1), EDC4 (enhancer of mRNA decapping 4), and the DEAD-box protein RCK (also known as Me31B). In this context, shrimp miR-S8 triggered the degradation of YB-1 mRNA in melanoma stem cells in a cross-species manner, leading to the suppression of tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F. Yang, X. Zhang
Development of methodology: F. Yang, X. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Yang, J. Wei, S. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Yang, X. Zhang
Writing, review, and/or revision of the manuscript: F. Yang, X. Zhang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhang
Study supervision: X. Zhang

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
2. Hollander PD, Savage MI, Brown PH. Targeted therapy for breast cancer prevention. AM J Pathol 2013;183:1096–112.
3. Gucalp A, Traina TA. Triple-negative breast cancer: adjuvant therapeutic options. Chemother Res Pract 2010;2011:2090–107.
4. Buss EC, Ho AD. Leukemia stem cells. Int J Cancer 2011;129:2328–36.
5. Pinto CA, Widodo E, Waltham M, Thompson EW. Breast cancer stem cells and epithelial mesenchymal plasticity-Implications for chemoresistance. Cancer Lett 2013;341:56–62.
6. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275–84.
7. Hurt EM, Kawasaki BT, Klarmann GJ, Thomas SB, Farrar WL. CD44|[+]|CD24|[−]| prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis. Br J Cancer 2008; 98:756–65.
8. Alvero A, Chen RH, Montagna M, Montagna M, Schwartz PE, Rutherford T, et al. Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. Cell Cycle 2009;8: 158–66.
9. Lundberg IV, Edin S, Eklöf V, Å Ö, Palmqvist R, Wikberg ML. SOX2 expression is associated with a cancer stem cell state and down-regulation of CDX2 in colorectal cancer. BMC Cancer 2016;16:471–82.
10. Suvà ML, Rheinbay E, Gillespie SM, Patel AP, Wakimoto H, Rabkin SD, et al. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. Cell 2014;157:580–94.
11. Holdt LM, Stahringer A, Sass K, Pichler G, Kulak NA, Wilfert W, et al. Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. Nat Commun 2016;7:1–14.
12. de Almeida RA, Fraczek MG, Parker S, Delneri D, O'Keefe RT. Non-coding RNAs and disease: the classical ncRNAs make a comeback. Biochem Soc Trans 2016;44:1073–8.
13. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215–33.
14. Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, et al. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 2009;138:592–603.
15. He Y, Zhang X. Comprehensive characterization of viral miRNAs involved in white spot syndrome virus (WSSV) infection. RNA Biol 2012;9: 1019–29.
16. Yang G, Yang L, Zhao Z, Wang J, Zhang X. Signature miRNAs involved in the innate immunity of invertebrates. PLoS One 2012;7:e39015.
17. Ginestier C, Min HH, Charafejauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2011;1: 555–67.
18. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8:755–68.
19. Vaillant F, Asselin-Labat ML, Shackleton M, Lindeman GJ, Visvader JE. The emerging picture of the mouse mammary stem cell. Stem Cell Rev Rep 2007;3:114–23.
20. Imaizumi A, Onishi H, Yamasaki A, Kawamoto M, Umebayashi M, Morisaki T, et al. Hypoxic conditions promote gemcitabine sensitivity in a pancreatic cancer stem cell line. Anticancer Res 2016;36:653–7.
21. Jin C, Zhang X, Sun M, Zhang Y, Zhang G, Wang B. Clinical implications of the coexpression of SRC1 and NANOG in HER-2-overexpressing breast cancers. Onco Targets Ther 2016;9:5483–8.
22. Al-Ejeh F, Smart CE, Morrison BJ, Chenevix-Trench G, López JA, Lakhani SR, et al. Breast cancer stem cells: treatment resistance and therapeutic opportunities. Carcinogenesis 2011;32:650–8.
23. Morris SA, Daley GQ. A blueprint for engineering cell fate: current technologies to reprogram cell identity. Cell Res 2013;23:33–48.
24. Hanna JH, Saha K, Jaenisch R. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell 2010;143:508–25.
25. Chang KS, Yim H. Roles of SALL2 in tumorigenesis. Arch Pharm Res 2016;40:146–51.
26. Chen HY, Lee YH, Chen HY, Yeh CA, Chueh PJ, Lin YM. Capsaicin inhibited aggressive phenotypes through downregulation of tumor-associated NADH oxidase (tNOX) by POU domain transcription factor POU3F2. Molecules 2016;21:733–45.
27. El-Naggar AM, Veinotte CJ, Cheng H, Grunewald TG, Negri GL, Somasekharan SP, et al. Translational activation of HIF1α by YB-1 promotes sarcoma metastasis. Cancer Cell 2015;27:682–97.
28. Jürchott K, Bergmann S, Stein U, Walther W, Janz M, Manni I, et al. YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. J Biol Chem 2003;278:27988–96.
29. Homer C, Knight DA, Hananeia L, Sheard P, Risk J, Lasham A, et al. Y-box factor YB1 controls p53 apoptotic function. Oncogene 2005;24:8314–25.
30. Gens GP, Stromskaia TP, Kalita OV, Vaĭman AV, Rybalkina EIu, Ovchinnikov LP, et al. Studies of YB-1 protein in breast tumors. Klin Lab Diagn 2009;4:21–4.
31. Shinkai K, Nakano K, Cui L, Mizuuchi Y, Onishi H, Oda Y, et al. Nuclear expression of Y-box binding protein-1 is associated with poor prognosis in patients with pancreatic cancer and its knockdown inhibits tumor growth and metastasis in mice tumor models. Int J Cancer 2016;139:433–45.
32. Eliseeva IA, Kim ER, Guryanov SG, Ovchinnikov LP, Lyabin DN. Y-box-binding protein 1 (YB-1) and its functions. Biochemistry 2011;76: 1402–33.
33. Harada M, Kotake Y, Ohhata T, Kitagawa K, Niida H, Matsuura S, et al. YB-1 promotes transcription of cyclin D1 in human non-small-cell lung cancers. Genes Cells 2014;19:504–16.
34. Evdokimova V, Tognon C, Ng T, Ruzanov P, Melnyk N, Fink D, et al. Translational activation of snail1 and other developmentally regulated transcription factors by YB-1 promotes an epithelial-mesenchymal transition. Cancer Cell 2009;15:402–15.
35. Fraser DJ, Phillips AO, Zhang X, van Roeyen CR, Muehlenberg P, En-Nia A, et al. Y-box protein-1 controls transforming growth factor-beta1 translation in proximal tubular cells. Kidney Int 2008;73:724–32.
36. Kosnopfel C, Sinnberg T, Schittek B. Y-box binding protein 1—a prognostic marker and target in tumour therapy. Eur J Cell Biol 2014;93:61–70.
37. Wu L, Belasco JG. Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. Mol Cell 2008;29:1–7.
38. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009;136:642–55.
39. Yekta S, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science 2004;304:594–6.
40. Rehwinkel J, Behm-Ansmant I, Gatfield D, Izaurralde E. A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA 2005;11:1640–7.
41. Wu L, Fan J, Belasco JG. MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci 2006;103:4034–9.
42. Eulalio A, Huntzinger E, Nishihara T, Rehwinkel J, Fauser M, Izaurralde E. Deadenylation is a widespread effect of miRNA regulation. RNA 2009;15: 21–32.