miR-206 inhibits metastasis-relevant traits by degrading MRTF-A in anaplastic thyroid cancer

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Abstract. Thyroid cancer develops from follicular or parafollicular thyroid cells. A higher proportion of anaplastic thyroid cancer has an adverse prognosis. New drugs are being used in clinical treatment. However, for advanced thyroid malignant neoplasm such as anaplastic thyroid carcinoma, the major impediment to successful control of the disease is the absence of effective therapies. Elucidating molecular mechanism of the disease will help us to further understand the pathogenesis and progression of the disease and offer new targets for effective therapies. In this study, we found that MRTF-A expression was upregulated in metastatic anaplastic thyroid cancer tissues, compared with primary cancer tissues and it promoted metastasis-relevant traits *in vitro*. miR-206 was negatively associated with metastasis in anaplastic cancer and it degraded MRTF-A by targeting its 3'-UTR in ARO anaplastic thyroid cancer cells. In addition, miR-206 overexpression inhibited invasion and migration and silencing miR-206-promoted migration and invasion in the cells. Important, restoration of MRTF-A could abrogate miR-206-mediated migration and invasion regulation. Thus, we concluded that miR-206 inhibited invasion and metastasis by degrading MRTF-A in anaplastic thyroid cancer.

Introduction

The myocardin family members MRTF-A and MRTF-B (also known as megakaryocytic acute leukemia and megakaryoblastic leukemia-1/2) are well-established co-activators of serum response factor (SRF) (1,2). Myocardin expression is restricted to smooth and cardiac muscle lineages, whereas MRTF-A/B are expressed ubiquitously. The activity of MRTF-A/B is regulated by their translocation from the cytosol to the nucleus, which is triggered by the activation of Rho family proteins (2). Like myocardin, MRTF-A/B are reported to contribute to skeletal, cardiac and smooth muscle differentiation (3). Their importance in mammary myoepithelial differentiation (4,5) and the epithelial-mesenchymal transition (6) has been established, but their functions in cancer are absolutely different between myocardin and MRTF-A/B. Myocardin functions as an effective inducer of growth arrest and differentiation of some tumor and is frequently repressed during human malignant transformation (3,7). Interestingly, MRTF-A and MRTF-B have recently been implicated in tumor cell invasion and metastasis (8). Suppression of MRTF-A and -B decreased tumor cell motility in tumor xenografts in mice, while proliferation of the cells was unaffected. In addition, MRTF-depleted cancer cells failed to colonize the lungs after injection in the tail vein of mice, probably due to a defect in cell adhesion. Moreover, expression of an activated version of MRTF-A was able to increase lung colonization of poorly metastatic B16FO cells, leading to the conclusion that MRTF activity is a critical step for tumor cell metastasis. However, roles of MRTF-A and MRTF-B have not been reported in thyroid cancer up to now. In this study, we found that MRTF-A expression was upregulated in metastatic anaplastic thyroid cancer tissues, compared with primary tumor tissues. MRTF-A overexpression promoted migration, invasion and anoilds resistance in anaplastic thyroid cancer cells and silencing inhibited its motility, invasion and anoilds resistance.

MicroRNAs (miRNAs) are endogenous short non-coding RNA molecules that regulate gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (9-11). Abnormal miRNA expression has been linked to diseases, including cancer and it has been found implicated in a multitude of cellular processes including proliferation, differentiation, migration and apoptosis (11-17).

Many investigations have focused on the roles of miR-206 in cancer. miR-206 is downregulated and inhibits cell proliferation in breast cancer (18,19). It blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation (20). Downregulation of miR-206 promotes proliferation and invasion of laryngeal cancer by regulating VEGF expression (21) and it also targets notch3, activates apoptosis, and inhibits tumor cell migration and focus

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formation (22). Expression of the tumor suppressor miR-206 is associated with cellular proliferative inhibition and impairs invasion in ERα-positive endometrioid adenocarcinoma (23). miR-206 is associated with invasion and metastasis of lung cancer (24) and it can target c-*Met* (25). Overexpression of miR-206 decreases the expression of metabolic genes and dramatically impaired NADPH production, ribose synthesis, and *in vivo* tumor growth in mice (26). miR-206 inhibits gastric cancer proliferation in part by repressing cyclin D2 (27). Herein, we found that miR-206 was negatively associated with metastasis in anaplastic thyroid cancer and it degraded MRTF-A by targeting its 3'-UTR in ARO anaplastic thyroid cancer cells. In addition, miR-206 overexpression inhibited invasion, migration and anoilds resistance and silencing miR-206 promoted invasion, migration and anoilds resistance in anaplastic thyroid cancer. More important, restoration of MRTF-A abrogated pre-miR-206-mediated migration, invasion and anoilds resistance regulation. Thus, we concluded that miR-206 inhibited invasion, metastasis and anoilds-resistance by degrading MRTF-A.

Materials and methods

Thyroid cancer tissues, ARO/FRO cell lines. Nineteen thyroid cancer patients diagnosed with anaplastic thyroid cancer were recruited from the Second Artillery General Hospital of PLA and Tongji Hospital. The use of human tissue samples followed internationally recognised guidelines as well as local and national regulations. Research carried out on humans follow international and national regulations. Medical ethics committee approved the experiments undertaken. Informed consent was obtained from each individual. Thyroid cancer cell lines ARO and FRO was kindly donated by Dr Su Kai (Shanghai Cancer Center, China). Briefly, cells were maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

MRTF-A expressing plasmids/empty vectors, shMRTF-A/scramble, pre-miR-206/control miR, anti-miR-206/scramble and transfection experiments. MRTF-A expressing plasmid/empty vector and shMRTF-A plasmids/scramble were donated by Shao-Xin Huang (Boston University, Boston, MA, USA) and was as described previously (28). Pre-miR-206/control miR and anti-miR-206/scramble were purchased from Ambion, Inc. (Ambion, Austin, TX, USA). For transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluence for 24 h, and then transfected with transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 6 h, the medium was removed and replaced with normal culture medium for 48 h, unless otherwise specified.

Western blot analysis. Western blot analysis was performed as described before (29). In brief, after incubation with primary antibody anti-MRTF-A (1:500; Abcam, Cambridge, MA, USA) and anti-β-actin (1:500; Abcam) overnight at 4˚C, IRDye™-800 conjugated anti-rabbit secondary antibodies (Li-COR, Biosciences, Lincoln, NE, USA) were used for 30 min at room temperature. The specific proteins were visualized by Odyssey™ Infrared Imaging System (Gene Co., Lincoln, NE, USA).

Migration and invasion assay. For transwell migration assays, $2.5x10^4$-$5.3x10^4$ cells were plated in the top chamber with the non-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences, San Jose, CA, USA). For invasion assays, $1.25x10^5$ cells were plated in the top chamber with Matrigel-coated membrane (24-well insert; pore size, 8 mm; BD Biosciences). In both assays, cells were plated in medium without serum or growth factors, and medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h and cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with the Diff-Quick Staining Set (Dade) and counted.

Wound healing assay. Cells ($5x10^5$) were seeded onto each 35-mm glass bottom dish (MatTek Co., Ashland, MA, USA) and cultured at 37˚C with 5% CO_2 for 24 h. The confluent monolayer of cells was wounded. Monolayers of cells were wounded with pipette tips. After washing with warm PBS, the cells were incubated in fresh culture medium. The wounded areas were photographed at the beginning (0 h, top panels) and the end (10 h, bottom panels) of the assay with Nikon inverted microscope (Eclipse TE-2000U, Nikon, Japan) equipped with a video camera (DS-U1, Nikon, Japan).

3-(4,5-Dimethylthiazol-2-yl), 3,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells seeded on 96-well plates, were stained at indicated time point with 100 ml sterile MTT dye (0.5 mg/ml, Sigma, St. Louis, MO, USA) for 4 h at 37˚C, followed by removal of the culture medium and addition of 150 ml of dimethyl sulphoxide (DMSO) (Sigma). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength.

miRNA microarray. Total RNA from cultured cells, with efficient recovery of small RNAs, was isolated using the mirVana miRNA Isolation kit (Ambion). cRNA for each sample was synthesized by using 3' IVT Express kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocols. The purified cRNA was fragmented by incubation in fragmentation buffer (provided in the 3'IVT express kit) at 95˚C for 35 min and chilled on ice. The fragmented labeled cRNA was applied to MicroRNA2.0 array (Affymetrix) and hybridized in Genechip hybridization oven 640 (Affymetrix) at 45˚C for 18 h. After washing and staining in Genechip fluidics station 450 (Affymetrix), the arrays were scanned by using Genechip scanner 3000 (Affymetrix). The gene expression levels of samples were normalized and compared by using Partek GS 6.5 (Partek, Inc., St. Louis, MO, USA). Average-linkage hierarchical clustering of the data was applied by using the Cluster (Eisen *et al*, Stanford, Stanford University, CA, USA; http://rana.lbl.gov) and the results were displayed by using TreeView (Eisen *et al*, Stanford, Stanford University, CA, USA; http://rana.lbl.gov).

Methods of bioinformatics. The analysis of potential microRNA target sites were performed using common prediction algorithms - TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/).

Immunofluorescence analyses. For immunofluorescence analyses of cells were plated on glass coverslips in 6-well plates and transfected with 30 nM pre-miR-206 or control miR. At 36 h after transfection, coverslips were stained with the mentioned anti-MRTF-A antibodies. Alexa Fluor 488 goat anti-rabbit IgG antibody was used as secondary antibody (Invitrogen). Coverslips were counterstained with DAPI (Invitrogen-Molecular Probes, Eugene, OR, USA) for visualization of nuclei. Microscopic analysis was performed with a confocal laser-scanning microscope (Leica Microsystems, Bensheim, Germany). Fluorescence intensities were measured in a few viewing areas for 200-300 cells per coverslip and analyzed using ImageJ 1.37v software (http://rsb.info.nih.gov/ij/index.html).

Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) for MRTF-A. Total RNA was isolated from cells using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from the total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random hexamer primers (Sangon, Shanghai, China). The thermal cycle profile was as follows: denaturation for 30 sec at 95˚C, annealing for 45 sec at 52-58˚C depending on the primers used, and extension for 45 sec at 73˚C. PCR products were visualized on 2% agarose gels stained with ethidium bromide under UV transillumination. qRT-PCR was performed with a Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. The primer sequences for MRTF-A: forward primer 5'-ATGGAGCTGGTGGAGAAGAATATC-3' and reverse 5'-GAAGGAGGAACTGTCTGCTACC-3'.

Real-time PCR for miRNA. Total RNA from cultured cells, with efficient recovery of small RNAs, was isolated using the mirVana miRNA Isolation kit (Ambion). Detection of the mature form of miRNAs was performed using the mirVana qRT-PCR miRNA Detection kit, according to the manufacturer's instructions (Ambion). The U6 small nuclear RNA was used as an internal control.

Luciferase reporter assay. The 3'-untranslated region (3'-UTR) of human MRTF-A mRNA was cloned in pRL-TK (Promega) using PCR-generated fragment. Site-directed mutagenesis of the predicted target-site of miR-206 in the MRTF-A-3'-UTR was carried out using Quik change-mutagenesis kit (Stratagene, Heidelberg, Germany), with MRTF-A-WT-luc as a template. For reporter assays, cells was transiently transfected with WT or mutant reporter plasmid and microRNA or anti-microRNA using Lipofectamine 2000 (Invitrogen). Reporter assays were performed 36 h post-transfection using the Dual-luciferaseassay-system (Promega), and normalized for transfection efficiency by cotransfected Renilla-luciferase.

Anoikis assays. Anoikis resistance was evaluated by seeding $7.5x10^4$ cells in ultralow attachment plates (Corning). After 24 h of anchorage-independent culture, cells were transfected as indicated and resuspended in 0.4% trypan blue (Sigma) and cell viability was assessed.

Statistical analysis. Data are presented as mean ± SEM Student's t-test (two-tailed) was used to compare two groups ($P<0.05$ was considered significant), unless otherwise indicated (χ^2 test).

Results

MRTF-A expression was upregulated in metastatic anaplastic thyroid cancer. In an attempt to identify MRTF-A expression between thyroid cancer tissues and adjacent normal tissues, we performed western blotting in cancer tissues versus normal tissues. Protein was isolated from 6 pairs of thyroid cancer tissues and normal tissues (patients no. 1-6). However, we did not detect any difference between cancer tissues and adjacent normal tissues (Fig. 1A). Because MRTF-A was recently implicated in tumor invasion and metastasis, we hypothesized that MRTF-A was also associated with invasion and metastasis in thyroid cancer (8). Thus, using western blot assay, we detected MRTF-A expression between primary thyroid cancer tissues and metastatic thyroid cancer tissues obtained from further 6 patients (patients no. 7-12). Cancer metastasis has occurred in all the 6 patients. Although we did not detect any difference between cancer tissues and normal tissues, we found that MRTF-A protein was significantly increased in metastatic thyroid cancer tissues, compared with primary thyroid cancer (Fig. 1B). All the results implied that MRTF-A was associated with metastasis of anaplastic thyroid cancer.

MRTF-A promotes metastasis-relevant traits in thyroid cancer cells. In an attempt to identify the role of MRTF-A in regulating migration and invasion of ARO cells, the cells were transfected with MRTF-A expressing plasmids. After stable transfection, MRTF-A protein expression was detected by western blotting and the results showed that MRTF-A protein was increased by MRTF-A expressing plasmids in the cells (Fig. 2A). Next, we performed migration and invasion assay to detect migration and invasion of ARO cells transfected with MRTF-A expressing plasmids and empty vectors. Ectopic MRTF-A promoted motility and invasion by ~4-fold in the cells (Fig. 2B). To confirm the results, wound-healing assay was performed. Wound-healing assay showed that MRTF-A significantly promoted motility in the cells (Fig. 2C). To further show the effects of MRTF-A on metastasis, we performed anoikis assays to analyze its effects on anoikis resistance. Also, MRTF-A-transfected cells exhibited ~150% increased resistance to anoikis-mediated cell death (Fig. 2D). Having demonstrated that MRTF-A overexpression promoted migration and invasion in ARO cells, to provide further evidence that the roles of MRTF-A were involved in migration and invasion, we studied the effects of shMRTF-A, an inhibitor of MRTF-A. After stable transfection, MRTF-A expression was detected by western blotting. The results showed that shMRTF-A significantly downregulated MRTF-A expression in ARO cells (Fig. 2E). Next, we performed migration and invasion assay to detect migration and invasion of ARO cells transfected with shMRTF-A plasmids and scramble.

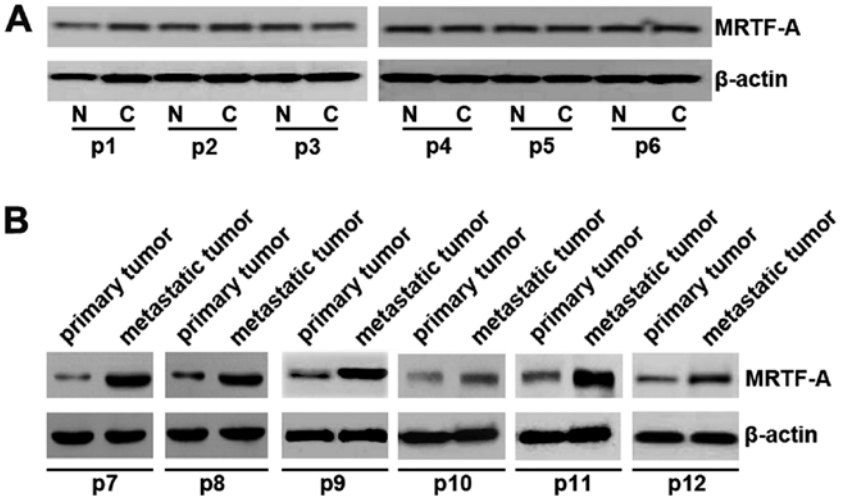

Figure 1. MRTF-A is upregulated in metastatic anaplastic thyroid cancer tissues. (A) Western blotting for MRTF-A in primary thyroid cancer tissues (C) and adjacent normal tissues (N). Patients were numbered as 1-6. All the 6 patients were diagnosed as anaplastic thyroid cancer. β-actin was a loading control. n=6. (B) Western blotting for MRTF-A in primary thyroid cancer tissues and metastatic thyroid cancer tissues. Patients were numbered as 7-12. All the 6 patients were diagnosed as metastatic anaplastic thyroid cancer. β-actin was a loading control. n=6.

Silencing MRTF-A inhibited motility and invasion by ~0.6-fold (Fig. 2F). To further confirm the roles of shMRTF-A on metastasis, we performed anoikis assays to analyze its effects on anoikis resistance. Contrary to MRTF-A overexpression, shMRTF-A-transfected cells exhibited ~30% decreased resistance to anoikis-mediated cell death (Fig. 2G).

We also performed MTT assay to detect whether it affected proliferation in ARO cells. Although MRTF-A was associated with metastasis in thyroid cancer patients and promoted migration and invasion in thyroid cancer ARO cells, neither overexpressing MRTF-A nor silencing it affected the cell viability (Fig. 2H and I).

The consequences of MRTF-A expression were not unique to ARO cells: MRTF-A protein was not only increased by MRTF-A expressing plasmids (Fig. 3A), but also promoted invasion (Fig. 3B), motility (Fig. 3B), and anoikis resistance (Fig. 3C), yet did not affect proliferation (Fig. 3D), in FRO human thyroid cancer cells. Hence, MRTF-A promotes *in vitro* surrogates of metastatic ability in thyroid cancer.

miR-206 degrades MRTF-A in thyroid cancer cells. Having demonstrated that MRTF-A expression is specifically upregulated in metastatic thyroid cancer and it promotes metastasis-relevant traits *in vitro*, MRTF-A expression in metastatic thyroid cancer was studied for clarifying the mechanisms promoting MRTF-A expression in the disease. MicroRNAs (miRNAs) are a new class of small (~22 nucleotide) noncoding RNAs and negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition (9-11). Downregulation of specific miRNA can contribute to oncogene overexpression (30). Thus we evaluated whether MRTF-A was upregulated by defection of specific miRNA in metastatic thyroid cancer.

In an attempt to identify the level of miRNA expression in primary thyroid cancer and metastatic thyroid tissues, we performed miRNA profiling. RNAs isolated from 3 pairs of primary tumors and metastatic tumors were hybridized to a custom miRNA microarray platform. After hybridization, quantification, and normalization, we found that miR-206, miR-31 and miR-96 were significantly decreased in the metastatic tumors compared with primary tumors >100-fold (Fig. 4A).

As further confirmation, we used two common prediction algorithms - TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/) to analyze 3'-UTR of MRTF-A. All 2 algorithms predicted that miR-206 could target 3'-UTR of MRTF-A (Fig. 4B). In order to further study whether that miR-206 expression was associated with metastasis of thyroid cancer, we performed real-time PCR to detect miR-206 expression in 5 metastatic tumors and 14 primary tumors. Consistent with the results of miRNA microarray, real-time PCR demonstrated that miR-206 expression was significantly downregulated in metastatic tumors (Fig. 4C). Target sites on 3'-UTR of MRTF-A are shown in Fig. 4D. We reasoned that miR-206 could downregulate MRTF-A expression by targeting its 3'-UTR in thyroid cancer and that MRTF-A was overexpressed in thyroid cancer, due to lack of miR-206.

In an attempt to identify the role of miR-206 in regulating MRTF-A expression in ARO cells, cells were transfected with pre-miR-206 and control miR. After transfection, miR-206 expression was detected by real-time PCR and the results showed that miR-206 was increased by the pre-miR-206 in the cells (Fig. 4E). To confirm the reason, we performed immunofluorescence analyses in ARO cells transfected with pre-miR-206 or control miR. The results showed that MRTF-A protein was evidently suppressed in the cells transfected with pre-miR-206 (Fig. 4F). We next performed RT-PCR and western blotting to detect MRTF-A expression in ARO cells transfected with pre-miR-206 or control miR. The results showed that MRTF-A protein (Fig. 4G) and mRNA (Fig. 4H) were significantly downregulated in the cells transfected with pre-miR-206. Consistent with the results of RT-PCR, real-time

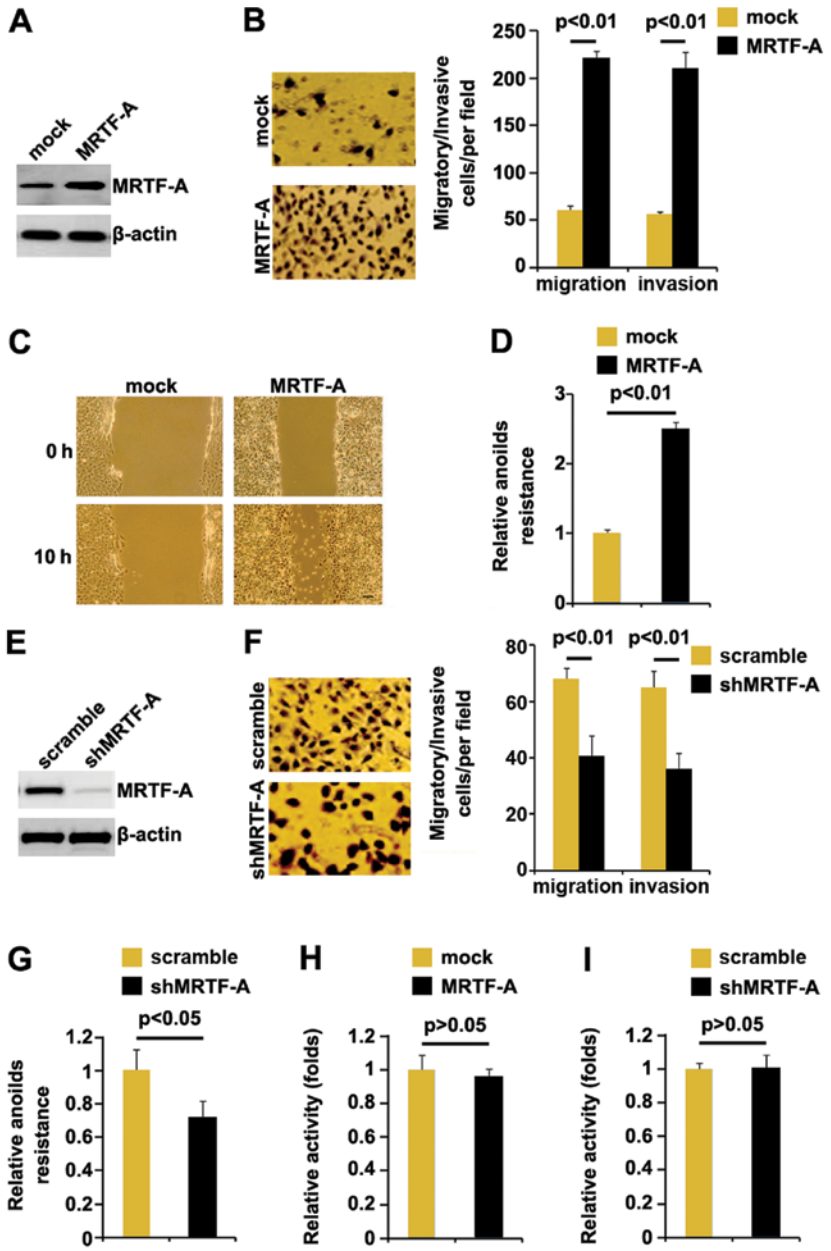

Figure 2. MRTF-A promotes metastasis-relevant traits in thyroid cancer cells. (A) Western blotting for MRTF-A in ARO thyroid cancer cells transfected with MRTF-A expressing plasmids. Mock groups were transfected with empty vector. β-actin was a loading control. n=3. (B) Matrigel invasion assay and Transwell invasion assay for ARO cells transfected with MRTF-A and empty vector (mock). n=3. (C) Wound-healing assays for ARO cells transfected with MRTF-A and empty vector (mock). n=3. (D) Anoikis assays for ARO cells infected as indicated. n=3. (E) Western blotting for MRTF-A in thyroid cancer cells transfected as indicated. β-actin was a loading control. n=3. (F) Matrigel invasion assay and Transwell invasion assay for ARO cells transfected with shMRTF-A or scramble. n=3. (G) Anoikis assays for ARO cells infected as indicated. n=3. (H) MTT assay for ARO cells infected as indicated. n=3. (I) MTT assay for ARO cells infected as indicated. n=3.

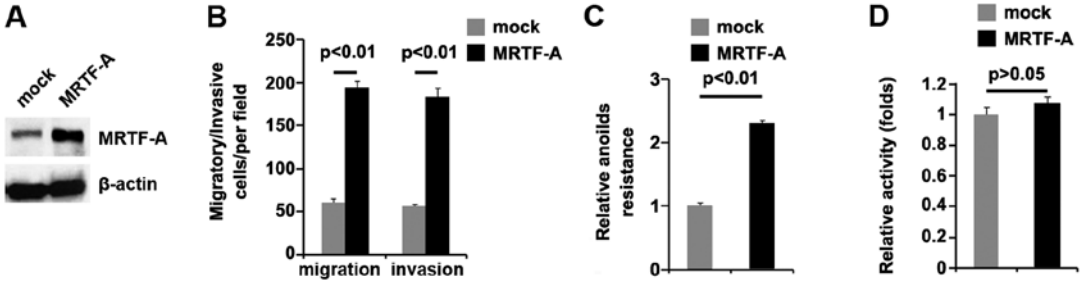

Figure 3. MRTF-A promotes migration, invasion and anoikis resistance in FRO thyroid cancer cells. (A) Western blotting for MRTF-A in FRO thyroid cancer cells transfected with MRTF-A expressing plasmids. Mock groups were transfected with empty vector. β-actin was a loading control. n=3. (B) Matrigel invasion assay and Transwell invasion assay for FRO cells transfected as indicated. n=3. (C) Anoikis assays for FRO cells infected as indicated. n=3. (D) MTT assay for FRO cells infected as indicated. n=3.

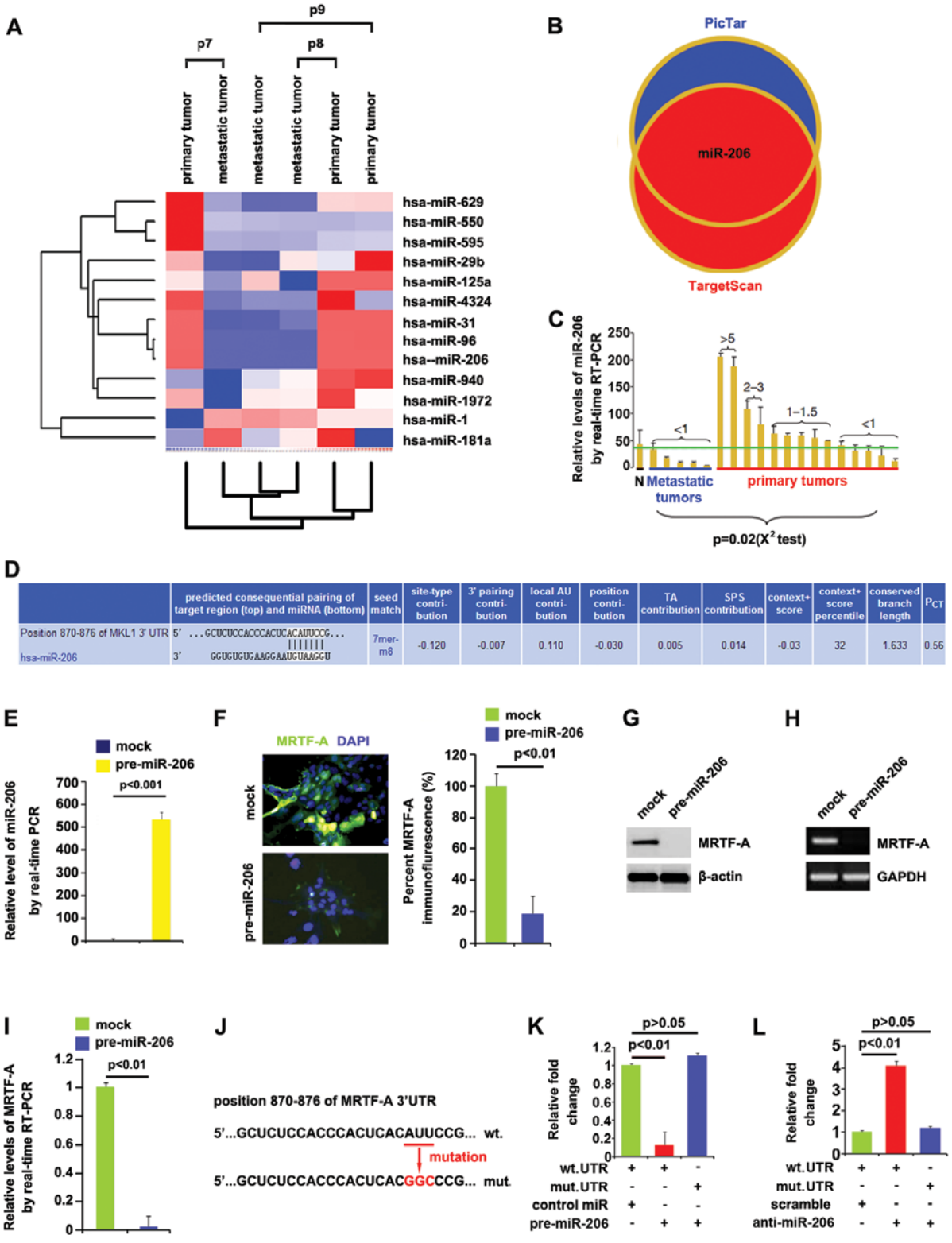

Figure 4. miR-206 is downregulated in metastatic thyroid cancer tissues and degrades MRTF-A in ARO cells. (A) Partial heat map of miRNA microarray analysis of primary thyroid cancer tissues and metastatic cancer tissues of 3 patients. n=3. (B) Vin Diagram showing the predicted microRNA targeting 3'-UTR of MRTF-A mRNA from databases (TargetSan and PicTar). (C) Real-time RT-PCR for miR-206 in 19 thyroid cancer tissues from patients with indicated status of metastasis. N, normal breast tissue. Error bars indicate SEM of triplicate experiments. n=19. (D) Schematic of predicted miR-206-binding sites in the 3'-UTR of MRTF-A mRNA by TargetSan. (E) Real-time PCR for miR-206 in ARO cells. ARO cells were infected with pre-miR-206 or control miR (mock). n=3. (F) Immunofluorescence analyses for ARO cells transfected with pre-miR-206 and control miR (mock). Left panel shows microscopic images of immunofluorescence staining of one representative experiment (x100 magnifications). Right panel shows graphic presentation of mean fluorescence intensities. n=3. (G) Western blotting for MRTF-A protein in ARO cells infected as indicated. β-actin was a loading control. n=3. (H) RT-PCR for MRTF-A mRNA in ARO cells infected as indicated. GAPDH was a loading control. n=3. (I) Real-time PCR for MRTF-A in ARO cells infected with pre-miR-206 or control miR (mock). U6 was a loading control. n=3. (J) Diagram of MRTF-A-3'-UTR-containing reporter constructs. MUT, contains 3-base-mutation at the miR-206-target region, abolishing its binding. (K) Reporter assay, with cotransfection of 500 ng WT- or MUT-reporter and 50 nM control-miR (mock), or pre-miR-206 as indicated. n=3. (L) Reporter assay, with cotransfection of 500 ng WT- or MUT-reporter and 50 nM scramble, or anti-miR-206 as indicated. n=3.

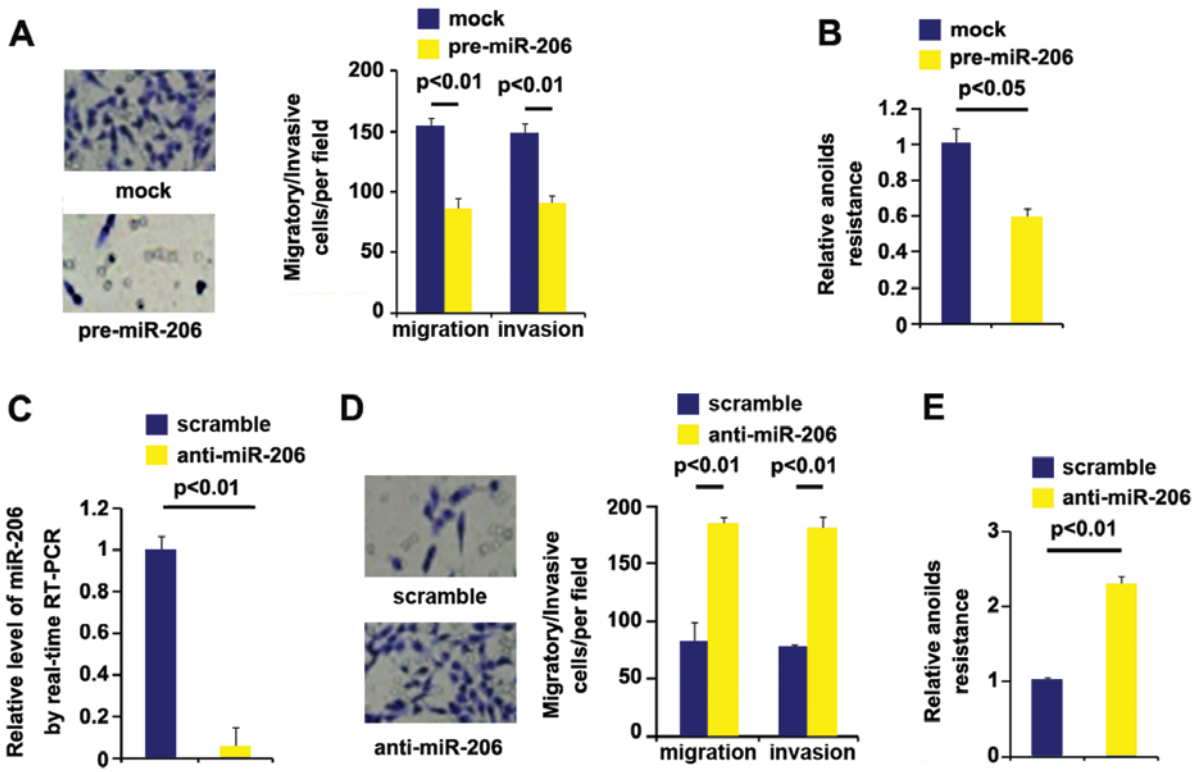

Figure 5. miR-206 inhibited metastasis-relevant traits in ARO cells. (A) Invasion and migration assays for ARO cells transfected with the pre-miR-206 or control miR (mock). n=3. (B) Anoikis assays for ARO cells infected as indicated. n=3. (C) Real-time PCR for miR-206 in ARO cells. ARO cells infected with anti-miR-206 or scramble. n=3. (D) Invasion and migration assays for ARO cells transfected with the anti-miR-206 or scramble. n=3. (E) Anoikis assays for ARO cells infected as indicated. n=3.

PCR demonstrated that MRTF-A mRNA was reduced in ARO cells transfected with pre-miR-206, compared with control miR-transfected groups (Fig. 4I).

To further demonstrate the direct regulation of MRTF-A by miR-206, we constructed luciferase reporters with the targeting sequences of wild-type (MRTF-A-WT-luc) and mutated MRTF-A 3'-UTRs (MRTF-A-MUT-luc) (Fig. 4J). Both the wild-type and mutant reporters were introduced into ARO cells.

Luciferase reporter assay showed that the luciferase activities of MRTF-A-WT-luc plasmids were significantly suppressed in the cells transfected with pre-miR-206, implying that miR-206 targeted 3'-UTR of MRTF-A mRNA (Fig. 4K). In order to further identify that miR-206 targeted 3'-UTR of MRTF-A by the predicted sites, we mutated 3 bases in the predicted sites (Fig. 4J). In addition, mutant reporters were introduced into ARO cells, as expected the luciferase activities of MRTF-A-MUT-luc were not suppressed by miR-206 in ARO cells (Fig. 4K).

Having demonstrated that miR-206 overexpression inhibited MRTF-A-WT-luc plasmids by the predicted sites, we next studied whether silencing miR-206 could affect activity of the MRTF-A-WT-luc plasmids. Thus luciferase reporters assay was performed again and the results showed that contrary to pre-miR-206, anti-miR-206 significantly promoted luciferase activity of MRTF-A-WT-luc in ARO cells (Fig. 4L). Moreover, mutant reporters were also introduced into ARO cells, but the luciferase activities of MRTF-A-MUT-luc were not affected by anti-miR-206 in ARO cells (Fig. 4L). All the data illustrated that miR-206 degraded MRTF-A by targeting the specific sites predicted *in silico* in anaplastic thyroid cancer cells.

miR-206 overexpression inhibits invasion and migration and silencing miR-206 promotes migration and invasion in thyroid cancer. Having demonstrated that miR-206 was decreased in the metastatic tumors compared with primary tumors and it could degrade metastasis-relevant MRTF-A expression, we reasoned that it was also associated with metastasis-relevant traits in thyroid cancer cells. It was confirmed that miR-206 could be increased by pre-miR-206 (Fig. 4E). Next, we performed migration and invasion assay to detect migration and invasion of ARO cells transfected with pre-miR-206 and control miR. Ectopic miR-206 inhibited motility and invasion by about 2-fold (Fig. 5A). To further show the effects of miR-206 on metastasis, we performed anoikis assays to analyze its effects on anoikis resistance. Also, miR-206 overexpressing cells exhibited 40% decreased resistance to anoikis-mediated cell death (Fig. 5B).

Having demonstrated that miR-206 overexpression inhibits metastasis-relevant traits in ARO cells, to provide further evidence that miR-206 was involved in ARO cell migration and invasion, we studied the effects of anti-miR-206, an inhibitor of miR-206. After stable transfection, miR-206 expression was detected by real-time PCR. The results showed that anti-miR-206 significantly downregulated miR-206 expression in ARO cells (Fig. 5C). Next, we performed migration and invasion assay to detect migration and invasion of ARO cells transfected with anti-miR-206 and

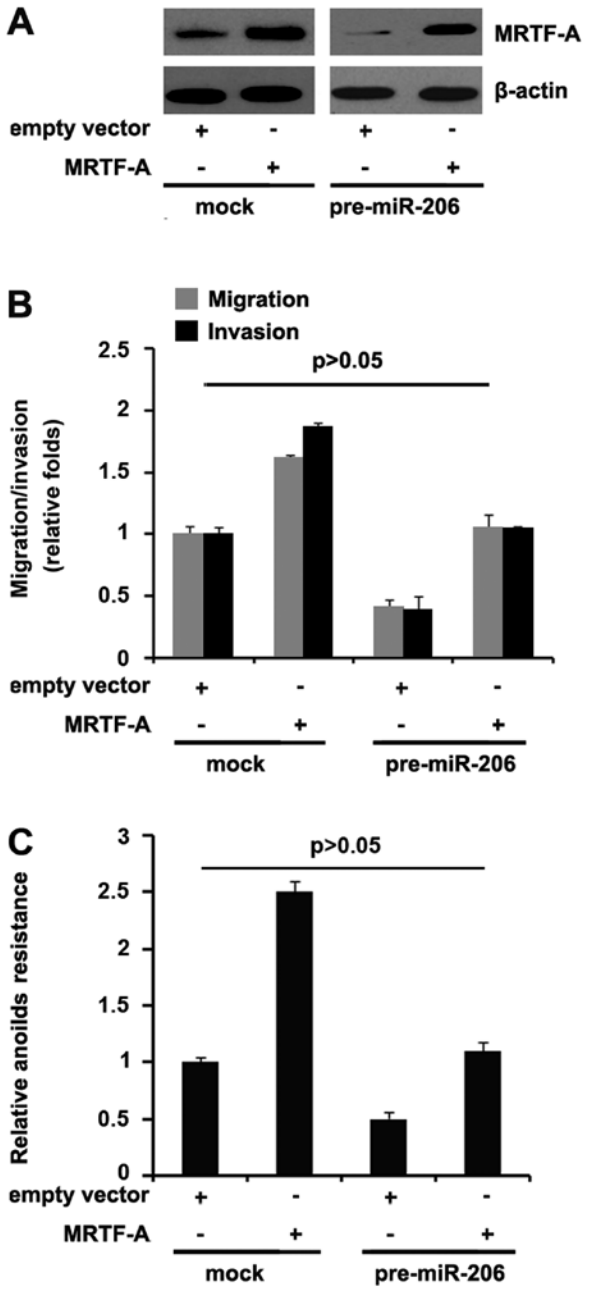

Figure 6. MRTF-A abrogates miR-206-mediated migration, invasion and anoikis resistence regulation. (A) Western blotting for MRTF-A protein in control miR and pre-miR-206-treated ARO cells infected as indicated. β-actin was a loading control. n=3. (B) Invasion and migration assays in control miR and pre-miR-206-treated ARO cells infected as indicated. n=3. (C) Anoikis assays for ARO cells infected as indicated. n=3.

scramble. Silencing miR-206 promotes motility and invasion by more than 2-fold (Fig. 5D). To further confirm that the roles of anti-miR-206 on migration and invasion, we performed anoikis assays to analyze its effects on anoikis resistance. Contrary to miR-206 overexpression, anti-miR-206-transfected cells exhibited more than 2-fold increased resistance to anoikis-mediated cell death (Fig. 5E).

MRTF-A abrogates the regulation of pre-miR-206-mediated migration and invasion. Because miR-206 directly degraded MRTF-A through its 3'-UTR, we reasoned that ectopic expression of MRTF-A by transfection of the cDNA that did not contain the predicted target of 3'-UTR should escape the regulation of miR-206 and thus attenuate or eliminate miR-206 function. To this end, we transfected MRTF-A expressing plasmids or empty vector (mock) into control miR or pre-miR-206-treated ARO cells. Immunoblot analysis revealed that transfection of MRTF-A expressing plasmids eliminated the effect of miR-206 on MRTF-A protein (Fig. 6A).

Because overexpression of miR-206 in ARO cells inhibited motility and invasion, and in order to identify whether MRTF-A could abrogate or attenuate the roles of miR-206 on motility and invasion, control miR or pre-miR-206-treated ARO cells were transfected with either MRTF-A expressing plasmids or empty vectors (mock), we performed migration and invasion assay and then found that pre-miR-206-treated ARO cells displayed >50% decrease in migration and invasion compared to control miR-treated cells (Fig. 6B). Restoration of MRTF-A sufficed to reverse the loss of migration and invasion (Fig. 6B) as well as resistance to anoikis-mediated cell death (Fig. 6C) observed in pre-miR-206-treated cells. Thus, we concluded that miR-206 inhibited migration and invasion via degrading MRTF-A expression.

Discussion

Gene expression is fine-tuned and tightly regulated through complex transcriptional signaling networks involving interactions of miRNA and target genes (31-36). Megakaryoblastic leukemia 1 (MKL1) is a member of the myocardin-related transcription factor (MRTF) family and functions as a co-activator for serum response factor (SRF), which regulates essential biological processes ranging from gastrulation and development to cell survival and apoptosis (37-39).

Myocardin-related transcription factor-A (MRTF-A), also termed megakaryocytic acute leukemia (MAL), basic, SAP and coiled-coil (BSAC), and MKL1, was originally identified as a chromosome 22 encoded fusion partner of the t(1;22)(p13;q13) translocation causing acute megakaryoblastic leukemia (AMKL) in infants and children (40,41). As a result of the translocation, the MRTF-A gene is fused to the RNA-binding motif protein 15 (RBM15), also known as OTT gene, on chromosome 1 (40,41). This fusion gene encodes the deregulated protein RBM15-MKL1 with potential oncogenic properties (42,43). MRTF-A was also identified in a screen for genes that protect against tumor necrosis factor-induced cell death, and named BSAC (44). However, its roles still keep emerging in thyroid cancer. Consistent with previous reports that MRTF-A promoted tumor cell invasion and metastasis (8,45,46), we found that MRTF-A expression was not only upregulated in metastatic thyroid cancer tissues, but its overexpression promoted metastasis-relevant traits in thyroid cancer cells. To further indentify that MRTF-A also promotes the metastasis-relevant traits *in vivo* will strengthen the conclution. First steps in the development of novel pharmacological tools targeting transcriptional responses of MRTF-A signaling in cancer were made with the recent development of compounds that selectively target the Rho/MKL1 signaling and inhibit the invasion of prostate cancer cells in a Matrigel model of metastasis (47). Given the incontestable involvement of MRTF-A in tumorigenesis and the effects it exerts on tumor cell invasion and metastasis, it will be important to assess how

MRTF-A is integrated within the major signaling pathways that communicate extracellular signals and mechanical cues to the transcriptional machinery to alter the motility of cancer cells.

Elucidating the mechanism by which miR-206 inhibits metastasis-relevant traits in anaplastic thyroid cancer by downregulating MRTF-A will help us to better understand the mechanism of invasion and metastasis. We found miR-206 was significantly downregulated in metastatic thyroid cancer and had potential to target 3'-UTR of MRTF-A mRNA. Following studies confirmed that miR-206 degraded MRTF-A by targeting its 3'-UTR. Thus, restoration of miR-206 may represent a promising therapeutic way to suppress MRTF-A-mediated metastasis. However, the roles of miR-206 also need to be further confirmed *in vivo*.

Recognition of the differential regulation and function of MRTF-A in tumors will ultimately provide a better understanding of the signaling pathways that can be therapeutically modulated.

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