

A regulation loop between Nrf1 α and MRTF-A controls migration and invasion in MDA-MB-231 breast cancer cells

YAO XU*, YING LUO*, CHEN LIANG*, WEIBING XING and TONGCUN ZHANG

Institute of Biology and Medicine, Wuhan University of Science and Technology, Wuhan, Hubei 430065, P.R. China

Received March 17, 2018; Accepted August 16, 2018

DOI: 10.3892/ijmm.2018.3816

Abstract. As a strong transactivator of promoters containing CarG boxes, myocardin-related transcription factor A (MRTF-A) is critical for the process of metastasis in tumor cells. Nuclear factor erythroid 2-like 1 (Nrf1) is well known as an important regulator of oxidative stress, which exists in multiple splicing forms with many unknown functions. The present study demonstrated a novel regulation loop between Nrf1 α (the longest splicing form of Nrf1) and MRTF-A that regulated the migration and invasion of breast cancer MDA-MB-231 cells. The underlying mechanism of this regulation loop was further investigated. In particular, Nrf1 α inhibited migration and invasion of breast cancer cells through inhibiting the expression of MRTF-A via miR-219. The current results revealed that miR-219 could bind to the MRTF-A 3'-UTR to directly regulate its expression. However, MRTF-A could reverse activate the Nrf1 α expression through binding to the CarG box in the Nrf1 α promoter. It can be speculated that this regulation loop may be a homeostasis mechanism in cells against tumorigenesis.

Introduction

Despite the increasing level of modern medical care, cancer remains difficult to be targeted effectively and cancer-related mortality remains high worldwide. Metastasis, one of the features of advanced cancer, is the predominant reason for the high mortality. Therefore, potential inhibition of metastasis and invasion of tumor cells has become the research direction and goal for many researchers. In recent years, more and more

transcription factors, long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) associated with tumor metastasis have been revealed. Among them, myocardin-related transcription factor A (MRTF-A) is a very important transcription factor which can promote tumor metastasis.

MRTF-A consists of 807 amino acid residues, and it belongs to the family of serum amyloid P-component serum amyloid P (SAP) proteins. MRTF-A can promote serum response factor (SRF) protein binding to the conserved cis regulatory element CC(A/T)6GG (known as CarG box), thus regulating the transcription of target genes. It has an important role in the growth and development of the organism (1-3). The activation of transforming growth factor (TGF) β -related signaling pathways can be very effective to induce MRTF-A translocation into the nucleus. Once there, MRTF-A promotes the transcription of epithelial-mesenchymal transition (EMT)-related molecules, such as α -smooth muscle actin (α -SMA), and enhances the migration and metastasis of tumor cells (4-10).

Other studies have reported that the activation of MRTF-A-mediated Rho associated coiled-coil containing protein kinase (ROCK) signaling pathway can regulate the phosphorylation of E-cadherin and decrease the adhesion ability of renal tubular epithelial cells, resulting in the occurrence of EMT (11). In the present study, while examining the role of MRTF-A in inducing the migration of breast cancer cells, it was observed that the transcription factor nuclear factor erythroid 2-like 1 (Nrf1) could regulate the above process. Nrf1 (also known as NFE2L1, LCRF1 or TCF11) belongs to the nuclear factor erythroid 2-related factor (NRF) family. It is ubiquitously expressed and essential for maintaining cellular homeostasis, organ integrity and oxidative stress during development and growth (12-17).

There are multiple splicing isoforms for Nrf1 in cells, such as the full-length Nrf1 α , as well as the LCR-F1/Nrf1 β , Nrf1 γ and Nrf1 δ isoforms. To date, the specific biological function of each isoform remains unclear. The present study aimed to explore the possible mechanisms of Nrf1 α and MRTF-A in regulating migration and invasion of MDA-MB-231 breast cancer cells.

Materials and methods

Cell culture. MDA-MB-231 cells used in the present study were purchased from American Type Culture Collection (cat. no. HTB-26; Manassas, VA, USA). The cells were

Correspondence to: Professor Tongcun Zhang or Mr. Weibing Xing, Institute of Biology and Medicine, Wuhan University of Science and Technology, 2 Huangjiahu West Road, Wuhan, Hubei 430065, P.R. China

E-mail: zhangtongcun@wust.edu.cn

E-mail: weibingxingwust@163.com

*Contributed equally

Key words: myocardin-related transcription factor A, nuclear factor erythroid 2-like 1, breast cancer, migration, invasion

seeded in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in humidified air with 5% CO₂. Cos-7 cells (American Type Culture Collection; cat. no. CRL-1651) and 293T cells (American Type Culture Collection; cat. no. CRL-3216) were cultured in DMEM containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml).

Plasmid construction and standard lentivirus production. Nrf1 α (Gene ID, 4779) and MRTF-A (Gene ID, 57591) were inserted into the lentivirus vector pCDH-CMV. The plasmids pCDH-Nrf1 α , pCDH-MRTF-A or pCDH-CMV (empty vector control) were cotransfected with the psPAX2 and pMG2.G into 293T cells (at ~70-80% confluency) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The medium was changed after 8 h, and 48 h later the supernatant was collected and filtered. The target short hairpin (sh) RNAs against Nrf1 α gene and MRTF-A gene were inserted into the pLKO.1 vector. As a negative control, a non-targeting sequence that had no significant homology to any mouse or human gene was inserted into pLKO.1. pCDH-Nrf1 α , shNrf1 α and their corresponding controls were a gift from Professor Jian Dong (North Carolina State University, Raleigh, NC, USA). The shMRTF-A plasmid has been previously described (18). shRNA lentiviral particles were produced by co-transfecting 293T cells using Lipofectamine 3000 with the lentivirus expression plasmids and packaging plasmids. Silencing efficiency was detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. miR-219 complementary sequence (AGAATTGCGTTTGGACAA TCA) was inserted into pcDNA3.1(-) vector to silence the function of miR-219 and pcDNA3.1(-) vector was used as a control.

Lentivirus transduction of MDA-MB-231 cells. MDA-MB-231 cells were cultured in high DMEM supplemented with 10% FBS and lentivirus was added at a multiplicity of infection (MOI) of 5. Following overnight incubation, the media containing the lentivirus was removed and fresh media was added.

RT-qPCR. Total RNA, including miRNA, was extracted by using the miRNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA), according to the manufacturer's protocol. The samples were reverse-transcribed using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). qPCR was performed in an StepOne Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Fast SYBR Green Master Mix was obtained from Applied Biosystems (Thermo Fisher Scientific, Inc.). The relative expression levels of MRTF-A and Nrf1 α were normalized to GAPDH. The primers for the qPCR analysis are listed in Table I. Amplification of U6 small nuclear RNA served as an endogenous control to normalize miR-219 expression data. The primers for the miR-219 analysis are listed in Table II. Thermocycling conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec, then a melting curve

Table I. Sequences of primers used in reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Primer	Sequence (5'-3')
MRTF-A	Forward	AAGGAACCACCTGGCTATGA
	Reverse	CTCCGCTCTGAATGAGAATGT
Nrf1	Forward	GCTGGACACCATCCTGAATC
	Reverse	GTAGGGTCGTCCGTTCTCAT
GAPDH	Forward	TCAAGAAGGTGGTGAAGCAG
	Reverse	AGGTGGAGGAGTGGGTGTCC

MRTF-A, myocardin-related transcription factor A; Nrf1, nuclear factor erythroid 2-like 1.

analysis from 60 to 95°C every 0.2°C for 1.5 min was obtained. Each sample was analyzed in triplicate and quantified using the 2^{- $\Delta\Delta$ C_q} method (19).

Protein extraction and western blotting. For western blot analysis, protein samples were extracted from the cells with Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.). The concentrations of proteins were determined using a bicinchoninic acid quantification kit (Beyotime Institute of Biotechnology, Haimen, China). The proteins (20 μ g) were separated by SDS PAGE (10% gel) and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked using 5% non fat milk at 25°C for 1 h, and then incubated with primary antibodies overnight at 4°C. The antibodies used were as follows: Anti-human GAPDH antibody (cat. no. 97166; 1:2,000, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-human Nrf1 antibody (cat. no. 46743; 1:1,000, Cell Signaling Technology, Inc.), anti-human MRTF-A antibody (cat. no. ab49311; 1:1,000, Abcam, Cambridge, UK). Then, the membrane was incubated with IRDyeTM-800 conjugated anti-mouse or anti-rabbit secondary antibodies (cat. no. 115-005-146 and 115-005-144; 1:5,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 25°C for 1 h at room temperature. The protein signals were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). GAPDH expression was used as an internal control. The western blotting results were quantified using ImageJ software (version 2.0; National Institutes of Health, Bethesda, MD, USA).

Colony formation assay. Cells were transduced with Nrf1 α -expressing lentivirus or with shNrf1 α lentivirus or their corresponding controls, as indicated. Twenty-four hours later, transfected cells were trypsinized, counted and replated at a density of 200 cells per 6-cm dish. Ten days later, colonies resulting from the surviving cells were fixed with 3.7% methanol, and stained with 0.1% crystal violet. Following capturing photos, the crystal violet stain was washed with 33% acetic acid and the absorbance was measured at 570 nm. Each assay was performed in triplicate.

Wound healing assay. Cells were subcultured in 6-well plates at a density of 1x10⁵ cells/well. Upon >80% confluence, the

Table II. Sequences of primers used in reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Primer	Sequence (5'-3')
miR-219	Reverse transcription	CTCAACTGGTGTTCGTGGAGTCGGCAATTCAGTTGAGAGAATTGC
	Forward	ACACTCCAGCTGGGTGATTGTCCAAACGC
	Reverse	TGGTGTTCGTGGAGTTCG
U6	Reverse transcription	AACGCTTCACGAATTTGCGT
	Forward	CTCGCTTCGGCAGCACACA
	Reverse	AACGCTTCACGAATTTGCGT

cell monolayer was gently scraped with a yellow pipette tip to generate a linear wound and washed twice with serum-free medium to remove cell debris. Images were subsequently captured at 0 and 24 h. The closure of the wounds was quantified by the distance of cells moved into the wounded area. The experiment was repeated twice with triplicate measurements in each experiment. The results were quantified using ImageJ software (20).

Transwell invasion assay. The invasion assay was performed using transwell chambers (Corning Incorporated, Corning, NY, USA), that had Matrigel (50 μ l; BD Biosciences, San Jose, CA, USA) pre-coated polycarbonate membranes (8.0 μ m pore size). A total of 1×10^4 cells were suspended in 200 μ l FBS-free DMEM and added to the upper chamber. The lower chamber was filled with 500 μ l DMEM containing 10% FBS. Following incubation for 24 h, cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Cells in four random microscopic fields (magnification, x20) were counted in triplicates. After capturing photos, the crystal violet was washed with 33% acetic acid and the absorbance was measured at 570 nm.

Luciferase constructs, site-mutation, and luciferase assay. The human miR-219 promoter was fused to the coding sequence of the pGL-3 luciferase reporter vector (Promega Corporation). The mutant (mut)-miR-219 promoter was identical to the pGL-3-miR-219 promoter, except that the Nrf1 α binding antioxidant response element (ARE) site was cut down (the sequence was AGTGGAAAGC). The human Nrf1 promoter luciferase reporter plasmids were also constructed in the same way. The 3'-untranslated region (UTR) of the human MRTF-A was amplified from human genomic DNA and individually inserted into the pmiGLO vector (Promega Corporation). The primers for constructing the luciferase reporter plasmids are listed in Table III. Cells (2×10^5 /well) were plated in 24-well plates. Cos-7 cells were cotransfected with Nrf1 α expression plasmids (pcDNA3.1(-)-myocardin) or control vector (pcDNA3.1-) in combination with miR-219-luc or mut-miR-219-luc. The transfection of these plasmids was performed using Lipofectamine 3000 according to the manufacturer's protocols. Cells were harvested 24 h following transfection and luciferase activity was measured using the Dual luciferase Assay System (Promega Corporation). Results were expressed as a fold induction relative to the cells transfected with the control vector (pcDNA3.1-) after normalization to *Renilla* activity. In the results from the dual luciferase

assays, the columns represent the mean value of three independent experiments and the error bars represent the standard deviation.

Chromatin immunoprecipitation (ChIP) assay. A ChIP Assay kit (Merck KGaA, Darmstadt, Germany) was used, following the manufacturer's instructions. Following treatments as indicated, each experimental group was incubated with 1% formaldehyde to cross-link DNA-protein complexes. After washing with ice-cold PBS for three times, cells were lysed in SDS lysis buffer. Then, the lysate was sonicated to shear DNA to 200-1,000 bp fragments. Anti-human Nrf1 antibody (cat. no. 46743; Cell Signaling Technology, Inc.) or anti-human MRTF-A antibody (cat. no. ab49311; Abcam) were used to immunoprecipitate the cross-linked proteins at 4°C overnight. Immunoglobulin G (cat. no. ab172730; Abcam) acted as the negative control. The DNA was used as a template for PCR, where the myocardin binding sites were utilized. The PCR products were separated on 1% agarose gel. The PCR primer sequences are listed in Table IV.

Statistical analysis. Quantitative data are expressed as mean \pm standard error of the mean. Statistical analysis of differences between two groups was performed by Student's t-test. A one-way analysis of variance followed by Tukey's test was used for comparing differences among multiple groups. Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Nrf1 α inhibits migration and invasion in MDA-MB-231 breast cancer cells. To investigate the effect of Nrf1 α in breast cancer cells, the MDA-MB-231 breast cancer cell line was transduced to overexpress Nrf1 α (Nrf1 α group), or to knockdown the expression of Nrf1 α (shNrf1 α group). Western blot analysis was used to evaluate the expression of Nrf1 α in each group (Fig. 1A and B). Then, by using colony formation, wound healing and transwell invasion assays, the growth, migration and invasion capacities were measured in each cell group, respectively. Notably, when Nrf1 α was overexpressed, the proliferation, migration and invasion ability of tumor cells was significantly decreased compared with the control group (Fig. 1C, E and G). By contrast, when endogenous Nrf1 α was silenced by shRNA, the tumor cells exhibited increased proliferation, migration and invasion

Table III. Primers used to generate the luciferase reporter plasmids by polymerase chain reaction.

Plasmid name	Primer	Sequence (5'-3')
miR219 promoter-WT	F	GGGACTCGAGTTGCCAGTCCATCTTGTTGTGTT
	R	AAGTCTCGAGTTTGAATAACGCCACGGGGCCATCA
miR219 promoter cut down	F	TAGGCTCGAGGCTCCAGAGGCCTTTGGTTTCCATG
	R	AAGTCTCGAGTTTGAATAACGCCACGGGGCCATCA
Nrf1 promoter-WT	F	GCGCGTAGCAATTCCATGAGTGGTTTGCTG
	R	ATTAGCTAGCGCTGCCTCCACAGCAGGCC
Nrf1 promoter-cut CarG 1	F	GTGCTAGCCCGGGCTCGAGCGCAAGCACAAAATGGACTCG
	R	ACTTAGATCGCAGATCTCGAGCACAGCAGGCCCTAAGCCC
Nrf1 promoter-cut CarG 1,2	F	GTGCTAGCCCGGGCTCGAGGGGGTCTTTGGGCTGTTTC
	R	ACTTAGATCGCAGATCTCGAGGAGCTCGGAGCCTCCGCTTA
MRTF-A 3'UTR-WT	F	ATTCGCTAGCAAGACGGGGTGGGGAAGGG
	R	GGGGTCTAGACAGCTGCTCTCTCTGCCCTG
MRTF-A 3'UTR-MUT	F	TCCACATGGT TGTGAGTCTTTGGGGGGCA GCCCCTGCTT TTTCCC
	R	GGGAAAAGCAGGGGCTGCCCCCAAAGACTCACACCATGTGGA

F, forward; R, reverse; Nrf1, nuclear factor erythroid 2-like 1; MRTF-A, myocardin-related transcription factor A; UTR, untranslated region; WT, wild-type; MUT, mutant.

Table IV. Sequences of primers used in chromatin immunoprecipitation assay.

Target region	Primer	Sequence (5'-3')
miR-219 promoter ARE	F	TTCAGCATGGTCTTCTCAG
	R	AACCAAAGGCCTCTGGAG
Nrf1 promoter CarG box1	F	GTACTTAATCTGCAAACC
	R	TGAGTCATTAGTCCCTGT
Nrf1 promoter CarG box2	F	AGATGGGACTGGAGAAAT
	R	GTAGAAACAGCCCCAAAGG

F, forward; R, reverse; ARE, antioxidant response element; Nrf1, nuclear factor erythroid 2-like 1.

compared with the control group (Fig. 1D, F and H). These findings suggested that Nrf1 α was negatively associated with the migration and invasion of MDA-MB-231 cells. Nrf1 α expression could inhibit migration and invasion of MDA-MB-231 breast cancer cells.

Nrf1 α inhibits migration and invasion by suppressing endogenous MRTF-A expression. Previous studies had demonstrated that MRTF-A promotes the migration and invasion of tumor cells, including breast cancer (21-23). As an important transcription factor associated with tumor metastasis, MRTF-A was a focus of study in our laboratory. While exploring the potential molecular mechanisms by which Nrf1 α regulates the function of tumor cells, a regulatory relationship between Nrf1 α and MRTF-A was discovered. As presented in Fig. 2A-D, the expression of endogenous MRTF-A was negatively associated with the expression of Nrf1 α , at both the mRNA and protein level. Subsequently, the endogenous MRTF-A expression was silenced by shRNA (Fig. 2E and F),

and then Nrf1 α was overexpressed or knocked down on this setting. The results demonstrated that, upon MRTF-A silencing, Nrf1 α lost the ability of regulating MDA-MB-231 cell migration and invasion (Fig. 2G and H).

Nrf1 α inhibits the expression of MRTF-A via miR-219. The aforementioned results demonstrated that Nrf1 α inhibited the expression of MRTF-A to regulate migration and invasion in breast cancer cells. However, the mechanism by which Nrf1 α regulated the expression of MRTF-A was unclear. It was hypothesized that miRNAs may have participated in this process. Through miRBase and TargetScan prediction programs analysis (24,25), miR-219 was selected. Analysis of miR-219 expression levels demonstrated that miR-219 was upregulated when Nrf1 α overexpressed (Fig. 3A), while when Nrf1 α was silenced, miR-219 levels were decreased (Fig. 3B). Next, the miR-219 complementary sequence was used to silence the function of miR-219 in the MDA-MB-231 cells, and then Nrf1 α was overexpressed or knocked down. RT-qPCR

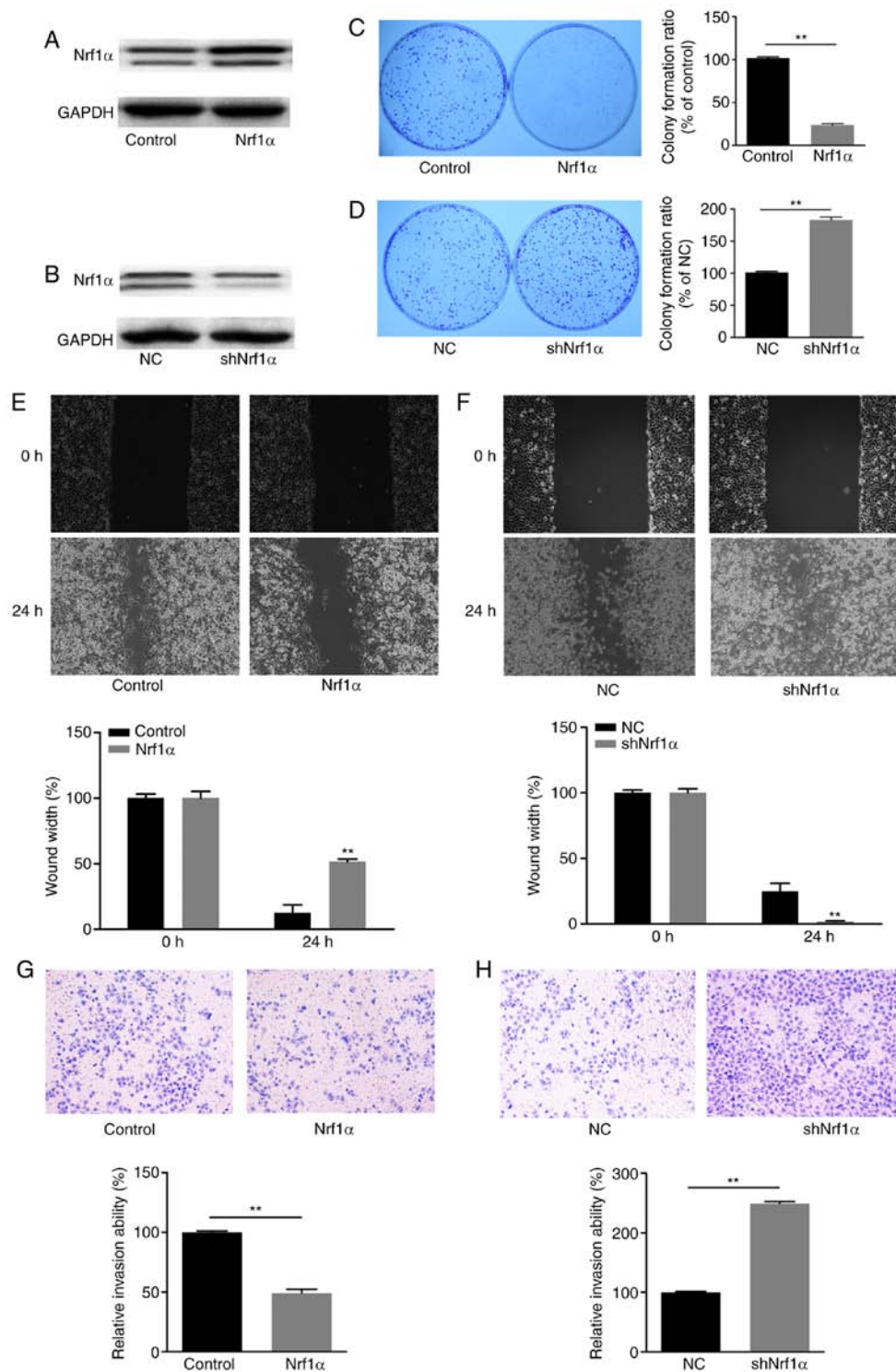


Figure 1. Nrf1 α inhibits migration and invasion in MDA-MB-231 cells. (A) Western blotting assays were performed to quantitatively measure the protein levels of Nrf1 α following overexpression or (B) shRNA knockdown. GAPDH expression was used as an internal control to show equal loading of the protein samples. (C) Colony formation assay was performed to determine the proliferation of MDA-MB-231 cells following Nrf1 α overexpression or (D) shRNA knockdown. (E) Cell migration was detected by wound healing assay following Nrf1 α overexpression or (F) shRNA knockdown. (G) Transwell invasion assay for Nrf1 α or (H) shNrf1 α -transduced MDA-MB-231 cells. $n \geq 3$. ** $P < 0.01$ compared with control. Nrf1 α , nuclear factor erythroid 2-like 1; sh, short hairpin; NC, negative control.

and western blot assays were used to detect the expression of endogenous MRTF-A. The results demonstrated that Nrf1 α lost its function to regulate MRTF-A expression, following miR-219 silencing (Fig. 3C and D). To further investigate the molecular mechanism by which Nrf1 α regulates miR-219,

luciferase assays were used to directly examine the effect of Nrf1 α on the transcriptional activity of the miR-219 promoter, which contains a predicted ARE site. As illustrated in Fig. 3E, Nrf1 α could bind to the ARE site to activate the transcriptional activity of the miR-219 promoter. In addition, CHIP assay

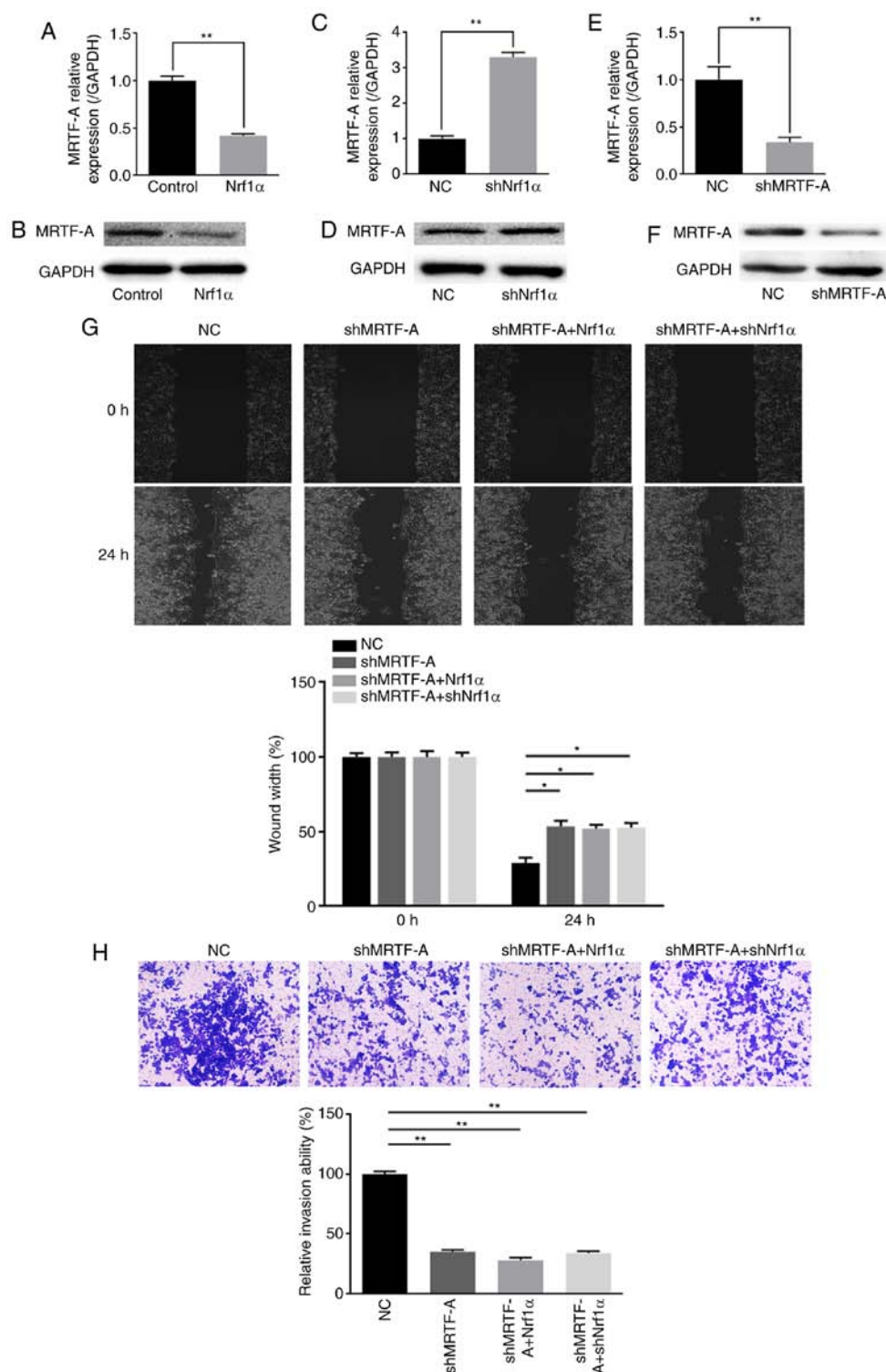


Figure 2. MRTF-A affects the function of Nrf1 α in MDA-MB-231 cells. (A) mRNA and (B) protein expression levels of MRTF-A (relative to GAPDH) following Nrf1 α overexpression for 48 h. (C) mRNA and (D) protein expression levels of MRTF-A (relative to GAPDH) following Nrf1 α knockdown by shRNA for 48 h. (E) Confirmation of the knockdown effect of shMRTF-A knockdown at the mRNA and (F) protein level. (G) Wound healing assay was used to detect the effect of MRTF-A following overexpression or knockdown of Nrf1 α on MDA-MB-231 cell migration. (H) Transwell invasion assay was used to detect the effect of MRTF-A following overexpression or knockdown of Nrf1 α on MDA-MB-231 cell invasion. $n \geq 3$. * $P < 0.05$ and ** $P < 0.01$, with comparisons indicated by lines. MRTF-A, myocardin-related transcription factor A; Nrf1 α , nuclear factor erythroid 2-like 1; sh, short hairpin; NC, negative control.

confirmed the direct binding of Nrf1 to the miR-219 promoter (Fig. 3F).

It is well known that the most common method by which miRNAs regulate gene expression is to act on their 3'-UTR and degrade their mRNA. As illustrated in Fig. 3G, binding sites

of miR-219 were predicted to exist in the 3'-UTR of MRTF-A. Therefore, the effect of miR-219 on the MRTF-A 3'-UTR was examined by luciferase assay. The results confirmed that miR-219 could bind to the 3'-UTR region of MRTF-A directly, reducing the mRNA levels of MRTF-A (Fig. 3H).

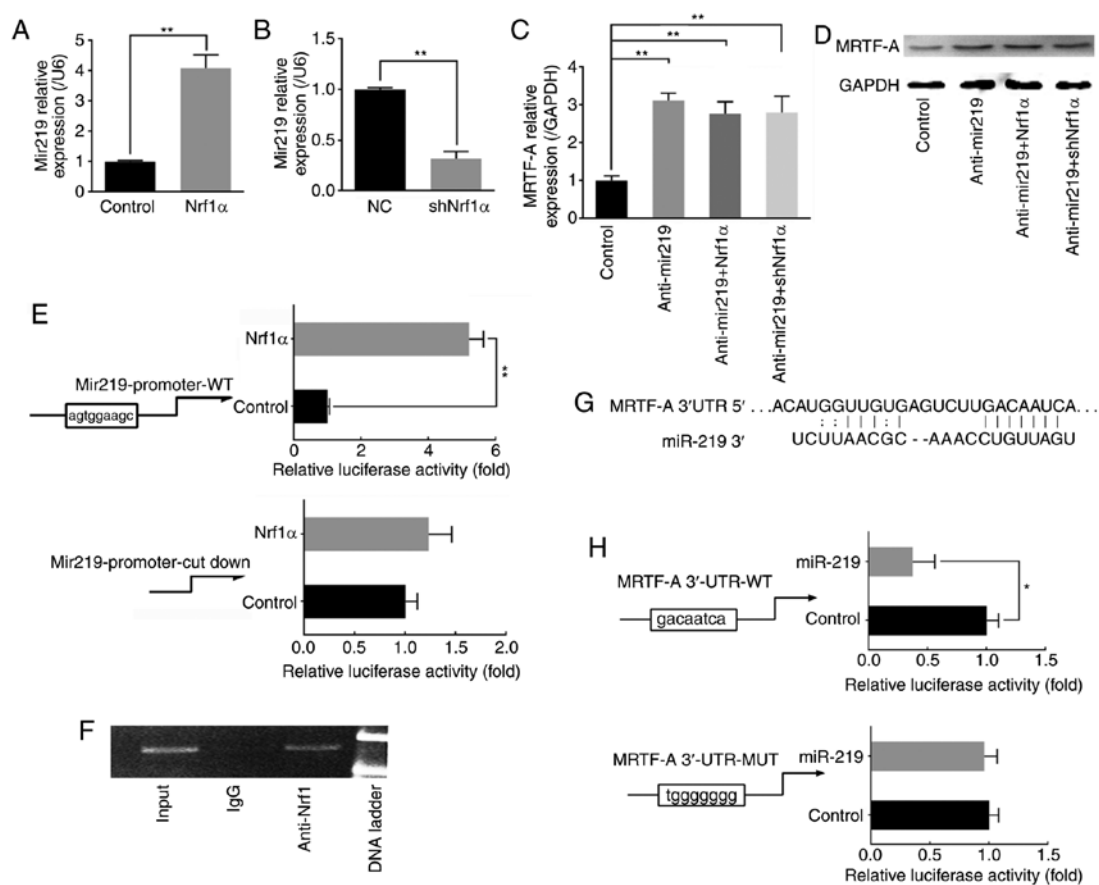


Figure 3. Nrf1 α inhibits the expression of MRTF-A via miR-219. (A) MDA-MB-231 cells were transfected with Nrf1 α or control empty vector for 24 h, and then miR-219 levels were detected. (B) Endogenous Nrf1 α in MDA-MB-231 cells, the effect of Nrf1 α on mRNA levels and (D) protein expression levels of MRTF-A was examined. (E) Cos-7 cells were transfected with 0.8 μ g Nrf1 α and 0.2 μ g miR219-luc or mut-miR219-luc expression plasmids, and then a luciferase assay was performed. Empty vector pcDNA3.1 plasmid was used as a negative control. (F) Chromatin immunoprecipitation assay was used to determine the sites on the miR-219 promoter that Nrf1 α directly bound in MDA-MB-231 cells. (G) A predicted binding site for miR-219 was observed on the 3'-UTR of MRTF-A. (H) Stable transfection of miR-219 into Cos-7 cells resulted in decreased luciferase activities of the MRTF-A 3'-UTR. $n \geq 3$. * $P < 0.05$ and ** $P < 0.01$, with comparisons indicated by lines. Nrf1 α , nuclear factor erythroid 2-like 1; MRTF-A, myocardin-related transcription factor A; sh, short hairpin; UTR, untranslated region; NC, negative control; WT, wild-type; MUT, mutant.

MRTF-A affects Nrf1 α expression through binding to the CarG box in the Nrf1 α promoter. Previous studies have demonstrated that Nrf1 α could inhibit the migration and invasion of breast cancer cells via MRTF-A. The present study revealed the potential molecular interplay between these two factors in this process. Notably, MRTF-A was demonstrated to negatively regulate the expression of Nrf1 α . To further explore the relationship between Nrf1 α and MRTF-A, a MRTF-A-overexpressing MDA-MB-231 line was established (Fig. 4A and B). As presented in Fig. 4C and E, the results of RT-qPCR analysis indicated that the mRNA levels of Nrf1 α presented a positive correlation with the mRNA levels of MRTF-A, whether MRTF-A was overexpressed or knocked down. The western blot assay results also demonstrated that the protein expression of these two factors was similar to the mRNA expression (Fig. 4D and F). As a strong drive factor of containing the CarG locus genes, MRTF-A may activate Nrf1 α through this pathway. Two potential CarG boxes were observed on the Nrf1 α promoter region. The results from the luciferase assay indicated that the transcriptional activity of Nrf1 α promoter could be upregulated by MRTF-A (Fig. 4G). However, the

Nrf1 α transcriptional activity was not affected when the far CarG box (CarG 1) was removed, or when both CarG boxes were removed (Fig. 4G). These results might indicate that MRTF-A affected the Nrf1 α transcriptional activity through binding to the far CarG box (CarG 1). To further explore the mechanism of this regulation, ChIP was used. The results demonstrated that MRTF-A was bound to the far CarG box (CarG 1), but not the near CarG box (CarG 2), which was consistent with the results from the luciferase assay (Fig. 4H).

Discussion

The present study demonstrated that Nrf1 α regulated migration and invasion of breast cancer cells by inhibiting the expression of MRTF-A. Notably, MRTF-A can reverse activate the expression of Nrf1 α by forming a complex with SRF binding to the CarG box in the promoter of Nrf1 α . Thus, a regulation loop exists between the two factors in the breast cancer cell line MDA-MB-231 (Fig. 5).

The transcription factor Nrf1 has an important role in upregulating the antioxidant response by increasing

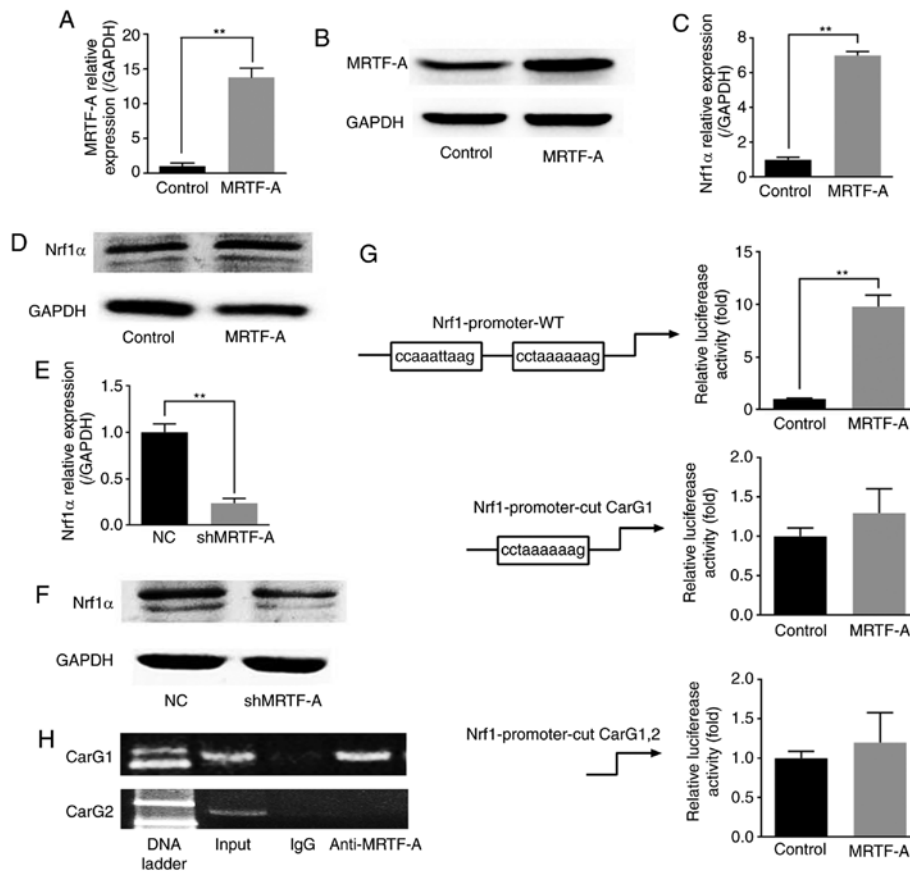


Figure 4. MRTF-A affects Nrf1 α expression through binding to the CarG box in the Nrf1 α promoter. (A) mRNA and (B) protein expression levels of MRTF-A following transduction of MDA-MB-231 cells with a MRTF-A-overexpressing lentivirus. (C) mRNA and (D) protein expression levels of Nrf1 α in MRTF-A-overexpressing MDA-MB-231 cells. (E) mRNA and (F) protein expression levels of Nrf1 α in MDA-MB-231 cells following MRTF-A knock down by shRNA. (G) Cos-7 cells were transfected with MRTF-A and Nrf1-luc or mutant Nrf1-luc plasmids, and then luciferase assays were performed. The empty vector pcDNA3.1 plasmid was used as a negative control. (H) Chromatin immunoprecipitation assay was used to determine the sites on the Nrf1 promoter that the MRTF-A/SRF complex binds in MDA-MB-231 cells. $n \geq 3$. $**P < 0.01$, with comparisons indicated by lines. MRTF-A, myocardin-related transcription factor A; Nrf1 α , nuclear factor erythroid 2-like 1; sh, short hairpin; NC, negative control; WT, wild-type.

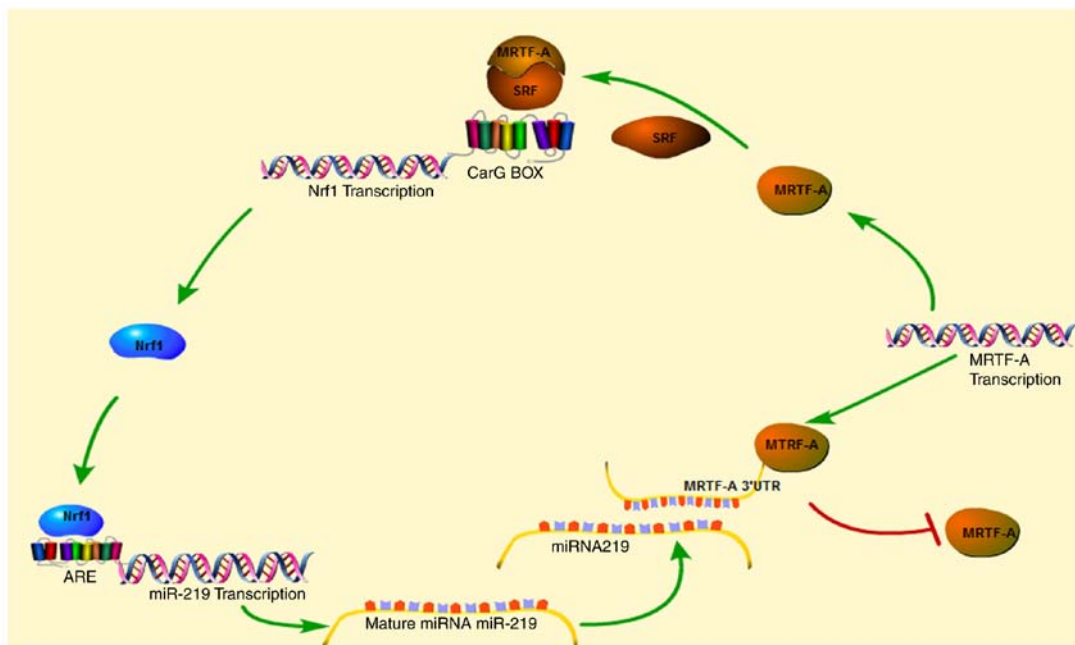


Figure 5. Nrf1 and MRTF-A interaction diagram. Nrf1 α contributes to the regulation of migration and invasion by inhibiting the expression of MRTF-A, via miR-219. In addition, MRTF-A can reverse activate the expression of Nrf1 α by forming a complex with SRF and by binding to the CarG box in the promoter of Nrf1 α . Nrf1 α , nuclear factor erythroid 2-like 1; MRTF-A, myocardin-related transcription factor A; SRF, serum response factor; ARE, antioxidant response element; UTR, untranslated region.

glutathione biosynthesis (13,14,16,26). Nrf1 is also known to regulate a variety of antioxidant genes through the combination with ARE (27). In the endoplasmic reticulum (ER), Nrf1 is cleaved into many forms, such as Nrf1 α , LCR-F1/Nrf1 β , Nrf1 γ and Nrf1 δ , and translocated from the ER to the nucleus in response to ER stress (28,29). To date, the specific biological function of each subtype remains unclear. Therefore, it is helpful to explore the functional differences of each subtype. In the present study, it was demonstrated that the migration and invasion of breast cancer cells were inhibited following overexpression of Nrf1 α . By contrast, the ability of breast cancer cells to migrate and invade was improved when the expression of Nrf1 α was silenced. Similar findings have been previously reported in HepG2 cells following Nrf1 α knock down (30). This may suggest that the effect of Nrf1 α in inhibiting migration and invasion may be common in multiple types of cancer. Subsequently, the present study attempted to reveal the molecular mechanism by which Nrf1 α inhibited migration and invasion in breast cancer cells.

MRTF-A is an important transcription factor associated with tumor migration and invasion. The present study explored the hypothesis that a regulatory relationship may exist between the two factors. Nrf1 α could indeed inhibit the expression of MRTF-A. Nrf1 α is known to positively regulate genes through ARE sites. Therefore, it was speculated that miRNAs may exist that have a role in the Nrf1 α /MRTF-A regulation loop. Bioinformatics analysis was used to discover potential miRNAs that may regulate MRTF-A expression. In addition, the promoter regions of these miRNAs were examined for the presence of ARE sites. Following these criteria, miR-219 was identified as a potential target. Results from luciferase and ChIP assays demonstrated that Nrf1 α indeed regulated MRTF-A expression via miR-219, which could directly bind to the 3'-UTR of MRTF-A.

In addition, MRTF-A was demonstrated to directly upregulate the expression of Nrf1 α by forming a complex with SRF binding to the CarG box. Previous studies have also demonstrated that MRTF-A is associated with cancer-related processes by the SRF/MRTF-A signaling for the induction of target genes (31-35).

In conclusion, the present study demonstrated that a regulation loop exists between Nrf1 α and MRTF-A, and that this loop controls the process of breast cancer cell migration and invasion. Furthermore, the potential underlying mechanism was explored. The present findings may provide a theoretical reference for the clinical inhibition of tumor metastasis. Further confirmation of these results with mouse models or patient tissues will be required in future studies.

Acknowledgements

Not applicable.

Funding

This study was financially supported by the National Natural Science Foundation of China (grant nos. 31471282 and 31570764).

Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YX, WX and TZ designed the experiments. YX, YL and CL performed the experiments, analyzed and interpreted the data. YX and YL were major contributors in writing the manuscript. The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Liao XH, Wang N, Liu QX, Qin T, Cao B, Cao DS and Zhang TC: Myocardin-related transcription factor-A induces cardiomyocyte hypertrophy. *IUBMB Life* 63: 54-61, 2011.
- Parmacek MS: Myocardin-related transcription factor-a: Mending a broken heart. *Circ Res* 107: 168-170, 2007.
- Small EM, Thatcher JE, Sutherland LB, Kinoshita H, Gerard RD, Richardson JA, Dimaio JM, Sadek H, Kuwahara K and Olson EN: Myocardin-related transcription factor-a controls myofibroblast activation and fibrosis in response to myocardial infarction. *Circ Res* 107: 294-304, 2010.
- Fan L, Sebe A, Péterfi Z, Masszi A, Thirone AC, Rotstein OD, Nakano H, McCulloch CA, Szászi K, Mucsi I and Kapus A: Cell contact-dependent regulation of epithelial-myofibroblast transition via the Rho-Rho kinase-phospho-myosin pathway. *Mol Biol Cell* 18: 1083-1097, 2007.
- Miano JM, Long X and Fujiwara K: Serum response factor: Master regulator of the actin cytoskeleton and contractile apparatus. *Am J Physiol Cell Physiol* 292: C70-C81, 2007.
- Sebe A, Masszi A, Zulys M, Yeung T, Speight P, Rotstein O, Nakano H, Mucsi I, Szászi K and Kapus A: Rac, PAK and p38 regulate cell contact-dependent nuclear translocation of myocardin-related transcription factor. *FEBS Lett* 582: 291-298, 2008.
- Zhao XH, Laschinger C, Arora P, Szászi K, Kapus A and McCulloch CA: Force activates smooth muscle α -actin promoter activity through the Rho signaling pathway. *J Cell Sci* 120: 1801-1809, 2007.
- O'Connor JW, Riley PN, Nalluri SM, Ashar PK and Gomez EW: Matrix rigidity mediates TGF β 1-induced epithelial-myofibroblast transition by controlling cytoskeletal organization and MRTF-A localization. *J Cell Physiol* 230: 1829-1839, 2015.
- Fan WH and Karnovsky MJ: Increased MMP-2 expression in connective tissue growth factor over-expression vascular smooth muscle cells. *J Biol Chem* 277: 9800-9805, 2002.
- Hinson JS, Medlin MD, Lockman K, Taylor JM and Mack CP: Smooth muscle cell-specific transcription is regulated by nuclear localization of the myocardin-related transcription factors. *Am J Physiol Heart Circ Physiol* 292: H1170-H1180, 2007.
- Tian YC, Fraser D, Attisano L and Phillips AO: TGF- β 1-mediated alterations of renal proximal tubular epithelial cell phenotype. *Am J Physiol Renal Physiol* 285: F130-F142, 2007.

12. Farmer SC, Sun CW, Winnier GE, Hogan BL and Townes TM: The bZIP transcription factor LCR-F1 is essential for mesoderm formation in mouse development. *Genes Dev* 11: 786-798, 1997.
13. Chan JY, Kwong M, Lu R, Chang J, Wang B, Yen TB and Kan YW: Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. *EMBO J* 17: 1779-1787, 1998.
14. Kwong M, Kan YW and Chan JY: The CNC basic leucine zipper factor, nrf1, is essential for cell survival in response to oxidative stress-inducing agents role for nrf1 in gamma-gcs(l) and gss expression in mouse fibroblasts. *J Biol Chem* 274: 37491-37498, 1999.
15. Xu Z, Chen L, Leung L, Yen TB, Lee C and Chan JY: Liver-specific inactivation of the Nrf1 gene in adult mouse leads to nonalcoholic steatohepatitis and hepatic neoplasia. *Proc Natl Acad Sci USA* 102: 4120-4125, 2005.
16. Ohtsui M, Katsuoka F, Kobayashi A, Aburatani H, Hayes JD and Yamamoto M: Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. *J Biol Chem* 283: 33554-33562, 2008.
17. Tsujita T, Peirce V, Baird L, Matsuyama Y, Takaku M, Walsh SV, Griffin JL, Uruno A, Yamamoto M and Hayes JD: Transcription factor Nrf1 negatively regulates the cystine/glutamate transporter and lipid-metabolizing enzymes. *Mol Cell Biol* 34: 3800-3816, 2014.
18. Xu Y, Luo Y, Wang ZY, Li X, Zheng P and Zhang TC: MRTF-A can activate Nrf2 to increase the resistance to doxorubicin. *Oncotarget* 8: 8436-8446, 2017.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
20. Schneider CA, Rasband WS and Eliceiri KW: NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9: 671-675, 2012.
21. Hermann MR, Jakobson M, Colo GP, Rognoni E, Jakobson M, Kupatt C, Posern G and Fässler R: Integrins synergise to induce expression of the MRTF-A-SRF target gene ISG15 for promoting cancer cell invasion. *J Cell Sci* 129: 1391-1403, 2016.
22. Eisenach PA, Schikora F and Posern G: Inhibition of arginyl-transferase 1 induces transcriptional activity of myocardin-related transcription factor A (MRTF-A) and promotes directional migration. *J Biol Chem* 289: 35376-35387, 2014.
23. Zhang WL, Lv W, Sun SZ, Wu XZ and Zhang JH: miR-206 inhibits metastasis-relevant traits by degrading MRTF-A in anaplastic thyroid cancer. *Int J Oncol* 47: 133-142, 2015.
24. Griffiths-Jones S, Saini HK, Van Dongen S and Enright AJ: miRBase: Tools for microRNA genomics. *Nucleic Acids Res* 36: D154-D158, 2007.
25. Edris B: A comparison of the oligomap and targetscan algorithms for miRNA target analysis. *BMI* 231, 2011.
26. Chen L, Kwong M, Lu R, Ginzinger D, Lee C, Leung L and Chan JY: Nrf1 is critical for redox balance and survival of liver cells during development. *Mol Cell Biol* 23: 4673-4686, 2003.
27. Gęgotek A and Skrzydlewska E: CNC proteins in physiology and pathology. *Postępy Hig Med Dosw (Online)* 69: 729-743, 2015 (In Polish).
28. Zhang Q, Pi J, Woods CG and Andersen ME: A systems biology perspective on Nrf2-mediated antioxidant response. *Toxicol Appl Pharmacol* 244: 84-97, 2010.
29. Zhang Y, Li S, Xiang Y, Qiu L, Zhao H and Hayes JD: The selective post-translational processing of transcription factor Nrf1 yields distinct isoforms that dictate its ability to differentially regulate gene expression. *Sci Rep* 5: 12983, 2015.
30. Ren Y, Qiu L, Lü F, Ru X, Li S, Xiang Y, Yu S and Zhang Y: TALENs-directed knockout of the full-length transcription factor Nrf1 α that represses malignant behaviour of human hepatocellular carcinoma (HepG2) cells. *Sci Rep* 6: 23775, 2016.
31. Scharenberg MA, Chiquet-Ehrismann R and Asparuhova MB: Megakaryoblastic leukemia protein-1 (MKL1): Increasing evidence for an involvement in cancer progression and metastasis. *Int J Biochem Cell Biol* 42: 1911-1914, 2010.
32. Medjkane S, Perez-Sanchez C, Gaggioli C, Sahai E and Treisman R: Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. *Nat Cell Biol* 11: 257-268, 2009.
33. Descot A, Hoffmann R, Shaposhnikov D, Reschke M, Ullrich A and Posern G: Negative regulation of the EGFR-MAPK cascade by actin-MAL-mediated Mig6/Errfi-1 induction. *Mol Cell* 35: 291-304, 2009.
34. Leitner L, Shaposhnikov D, Descot A, Hoffmann R and Posern G: Epithelial protein lost in neoplasm α (Eplin-alpha) is transcriptionally regulated by G-actin and MAL/MRTF coactivators. *Mol Cancer* 9: 60, 2010.
35. Yoshio T, Morita T, Tsujii M, Hayashi N and Sobue K: MRTF-A/B suppress the oncogenic properties of v-ras- and v-src-mediated transformants. *Carcinogenesis* 31: 1185-1193, 2010.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.