Original Paper

LncRNA GAS5 Represses Osteosarcoma Cells Growth and Metastasis via Sponging MiR-203a

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Key Words

Osteosarcoma • LncRNA GAS5 • MiR-203a • TIMP2 • PI3K/AKT/GSK3 β signaling • NF- κ B signaling

Abstract

Background/Aims: LncRNA GAS5, a growth suppressor, has been reported to exert anti-tumor actions in various cancers, whereas the exact mechanism underlying the anti-tumor action is still unclear. This study was aimed to investigate the effect of lncRNA GAS5 on osteosarcoma and tried to explore the underlying mechanisms. **Methods:** Expressions of lncRNA GAS5 in MG-63 cells were silenced by shRNA transfection, while were overexpressed by vector transfection. Cell viability, migration, invasion and apoptosis were respectively assessed by MTT, Transwell assay and flow cytometry. Regulations between lncRNA GAS5 and miR-203a, as well as between miR-203a and TIMP2 were detected by qPCR, western blot and dual luciferase activity assay. **Results:** LncRNA GAS5 was down-regulated in MG-63 and OS-732 cells compared with FOB1.19 cells. Silence of lncRNA GAS5 significantly promoted MG-63 cells viability, migration and invasion, and up-regulated Cyclin D1, Cyclin B1, CDK1 and CDK4 expression. miR-203a was negatively regulated by lncRNA GAS5. The promoting activities of lncRNA GAS5 on MG-63 cells growth and metastasis were reversed by miR-203a silencing. TIMP2 was a target of miR-203a and the anti-growth and anti-metastasis actions of miR-203a were reversed by TIMP2 silence. Further, lncRNA GAS5 silence, miR-203a overexpression, and TIMP2 silence could activate PI3K/AKT/GSK3 β signaling while block NF- κ B signaling. **Conclusion:** LncRNA GAS5 might be a tumor suppressor in osteosarcoma via sponging miR-203a, sequestering miR-203a away from TIMP2.

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Introduction

Osteosarcoma, also known as osteogenic sarcoma, is a primary osteogenic tumor with a high degree of malignancy, characterizing by tumor cells directly form bone-like tissue [1]. Osteosarcoma is most prevalent in young people under the age of 20. Surgery is one of the main treatments for patients with osteosarcoma. Despite the success of surgery for

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osteosarcoma, the 5-year overall survival rate of patients with metastatic or recurrent osteosarcoma remains dismally poor [2]. This calls for a better understanding of the pathogenesis and molecular mechanisms of osteosarcoma, which will help in the clinical treatment and prognosis of this disease.

Long non-coding RNAs (lncRNAs) are a category of non-protein encoding RNAs with longer than 200 nucleotides. lncRNAs are previously considered as “dark matter” of the transcription [3], but recently most of the known lncRNAs have been reported to be critical in a number of biological and pathological processes, like cell proliferation, migration, invasion, apoptosis and differentiation. The functions of lncRNAs in regulating gene expression in human cancers have been gradually uncovered. lncRNA growth arrest-specific transcript 5 (GAS5), a suspensor in cell growth, has been reported to participate in the development of multiple cancers, including colorectal cancer [4], non-small-cell lung cancer [5] and breast cancer [6]. Specifically, in cervical cancer, decreased expression of lncRNA GAS5 is correlated with a poor prognosis [7]. An *in vitro* investigation revealed that silencing of lncRNA GAS5 induced a significant increase in pancreatic cancer cells proliferation [8]. The previous studies demonstrated lncRNA GAS5 as a tumor suppressive gene; however, the role of lncRNA GAS5 in osteosarcoma is remain unclear.

It has been acceptable that lncRNAs implicate in the pathogenesis through interacting miRNAs [9]. miRNAs are another kind of non-coding RNA that are associated with organism development and various human diseases. The relationship between lncRNA and miRNA is not entirely clear yet [10], but some studies have indicated that lncRNAs have sponge function to titrate miRNAs [11], sequester miRNAs away from their target mRNAs by binding effect, thereby exerting tumor-promoting or tumor-suppressive activities in multiple kind of cancers [12]. The lncRNA-miRNA-mRNA interactions may be conducive to understand the mechanism and pathogenesis of cancer.

In this study, we aimed to investigate the functional effect of lncRNA GAS5 on osteosarcoma cells growth and metastasis. Additionally, we focused on the interaction between lncRNA GAS5 and miR-203a. We explored the relationship between miR-203a and a miR-203a target gene TIMP2. The findings in this study will be helpful for enlarging our insight into the pathogenesis of osteosarcoma.

Materials and Methods

Cell culture

Human osteosarcoma cell lines MG-63 and OS-732, and a human osteoblast cell line hFOB1.19 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 U/ml streptomycin (GIBCO, Grand Island, NY, USA). The cells were all pre-cultured in a humidified incubator at 37°C with 5% CO₂ until confluence was reached. The subculture was collected for use in this study.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA from each cell line was extracted by using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). The purity of RNA was evaluated by modified formaldehyde agarose gel electrophoresis. OD₂₆₀/OD₂₈₀ absorbance ratio was detected by using a spectrophotometer (Genova Nano, BIBBY, UK). The One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China) was used for qPCR analysis to test the expression levels of *GAS5* and *TIMP2*. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used for testing the expression of *miR-203a*. The primers were synthesized by GenePharma (Shanghai, China). The expressions of *GAS5* and *TIMP2* were normalized to *GAPDH*, and *miR-203a* expression was normalized to *U6* snRNA. Fold changes were calculated by 2^{-ΔΔCt} method. The data were analyzed by Real-Time StatMiner (Integromics, Granada, Spain) [13].

Cell transfection

The specific short-hairpin RNA (shRNA) against human lncRNA GAS5 was cloned into pENTR™/U6 plasmid (GenePharma, Shanghai, China), and referred as sh-GAS5. A non-targeting shRNA (shNC, GenePharma) was used as a negative control. The full-length of lncRNA GAS5 sequence was transfected into pcDNA™ 3.1 vector (ThermoFisher, Scientific, China) and referred as pc-GAS5. The empty pcDNA3.1 vector was used as a negative control. miR-203a mimic, inhibitor or their respective scramble controls were synthesized by RiboBio (Guangzhou, China). TIMP2 specific siRNA (si-TIMP2) and siRNA negative control were purchased from Santa Cruz (Santa Cruz Biotechnology, USA). For TIMP2 overexpressing, the full-length of TIMP2 sequence was transfected into pcDNA™ 3.1 vector (ThermoFisher, Scientific, China) and referred as pc-TIMP2. All transfections were performed by using Lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. The stably transfected MG-63 cells were selected by using the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA). The stable transfected cells were selected for the subsequent experiments.

Cell viability assay

After transfection, cell viability of stable transfected MG-63 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Transfected cells were seeded in 96-well plates at the density of 2×10^3 cells/well. After 1 day of culturing, MTT (Sigma-Aldrich, St. Louis, MO, USA) was added into each well and incubated for another 4 h at 37°C. Then, 150 µl dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was added into the plates were shaken for 10 min. The absorbance was measured at 570 nm (OD_{570}) using 680 microplate enzym-linked immunosorbent assay (ELISA) reader (Bio-Rad Laboratories, Hemel Hempstead, UK). Cell viability was expressed as a ratio of the control group and calculated as the absorbance_{treated} divided by absorbance_{control}.

Migration and invasion assay

Cell migration was determined by using a modified Boyden chamber migration assay chamber with a pore size of 8 µm polycarbonate membrane (Corning, New York, USA). In brief, MG-63 cells after transfection were suspended in 200 µl serum-free medium, and were seeded on the upper compartment of 24-well chamber. 600 µl of complete medium was added into the lower compartment. After 12 h of incubation at 37°C, cells were fixed with 100% ethanol (NIST, USA) for 30 min. Non-traversed cells were removed from the upper surface of the filter chamber using a cotton swab. Traversed cells on the lower chamber were stained with 0.1% crystal violet (Amresco, USA). To quantify migration, the cells in per well were counted from five independent fields under a microscope (Leica Microsystems, Wetzlar, Germany) at 40 × magnification.

The invasion of MG-63 cells after transfection was determined by using a BD BioCoat™ Matrigel™ Invasion Chamber (8 µm polycarbonate membrane) supplemented with matrigel. Briefly, cells were treated for induction condition, and 5×10^4 cells in 200 µl serum-free medium were seeded on the upper compartment of 24-well plates, while complete medium containing 10% FBS was added into the lower chamber. The invasion chambers were incubated for 48 h in the incubator with 5% CO₂ at 37°C. The invaded cells were removed with a cotton swab carefully, and invaded cells were fixed with 100% ethanol (NIST, USA) and stained by crystal violet solution (Amresco, USA). To quantify invasion, the cells in per well were counted from five independent fields under the microscope (Leica Microsystems, Wetzlar, Germany) at 40 × magnification.

Early apoptosis assay

After transfection, early apoptosis was detected by using Annexin V- PE Apoptosis Detection Kit (South Biotech, USA). MG-63 cells in culture medium were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA), then washed twice again and stained in propidium iodide (PI)/ fluorescein isothiocyanate (FITC) conjugated Annexin V with the presence of 50 µg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA). The samples were incubated in the dark for 1 h at room temperature. The early apoptotic cells (Annexin-V positive and PI negative) were detected by using FACScan (BD Biosciences, USA). All the measurement was performed in triplicate and data was analyzed by FlowJo Version 10.0 software (FlowJo LLC, USA).

Dual luciferase activity assay

The sequence of TIMP2 3'-UTR containing the predicted miR-203a binding site was cloned into pGL2-Basic Vector (E1641, Promega, WI, USA) as the reporter vector TIMP2-wild-type (TIMP2-wt). Mutated TIMP2 3'-UTR was used as a negative control, namely TIMP2-mutated-type (TIMP2-mt). Each vector was co-transfected with miR-203a mimic into HEK 293T cells. The cells were collected 48 h later, and the Firefly and Renilla luciferase activity were measured by using the Dual-luciferase reporter system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Western blot assay

The protein samples were extracted by using RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland) and quantified with the Bio-Rad Protein Assay Kit (Pierce, Appleton, WI, USA). Western blot system was established using a Bio-Rad Tris Gel system according to the manufacturer's instructions. All blots were transferred onto polyvinylidene difluoride (PVDF) membrane and incubated with primary antibodies overnight at 4°C. The primary antibodies with the dilution of 1:1000 in this step were as follows: TIMP2 (sc21735), Bcl-2 (sc509), Bax (sc20067), Caspase-3 (sc7272), Caspase-9 (sc17784), nuclear factor- κ B (p65) (sc101713), p65 (sc109) and phosphorylated p65 (p-p65, sc101749) (Santa Cruz Biotechnology, Dallas, TX, USA), Cyclin D1 (ab32053), Cyclin-dependent kinase 1 (CKD1, ab133327), CKD4 (ab13357), cleaved-Caspase-3 (ab2302), cleaved-Caspase-9 (ab2324), PI3K (ab86714), phosphorylated PI3K (p-PI3K, ab32651), AKT (ab8805), phosphorylated AKT (p-AKT, ab38449), Glycogen synthase kinase 3 β (ab32391), phosphorylated GSK3 β (p-GSK3 β , ab75745) and GAPDH (ab8245) (Abcam, USA). Then the membranes were washed and incubated with secondary antibodies marked by horseradish peroxidase (HRP, 1:5000; Santa Cruz) for 1 h at room temperature. The membranes were transferred into E-chemiDoc™ XRS system (Bio-Rad, USA), and 200 μ l Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, MA, USA) was added. The signals were captured and the intensity of the bands was quantified by using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were repeated three times. The results of multiple experiments were presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed by using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). *P*-values were calculated using a one-way analysis of variance (ANOVA) for multiple groups and two-tailed Student's *t*-test between two groups [14]. *P* < 0.05 was considered to indicate statistically significant.

Results

lncRNA GAS5 promoted osteosarcoma cells growth and metastasis

lncRNA GAS5 expression levels in different cell lines were determined by qPCR. The results in Fig. 1A showed that lncRNA GAS5 expression levels were significantly suppressed in human osteosarcoma cell lines MG-63 and OS-732 compared with human osteoblast cell line hFOB1.19 (*P* < 0.01). Since MG-63 cells have the sharpest decrease in lncRNA GAS5 expression, MG-63 cell line was selected for use in the subsequent experiments.

Then, the transfection efficiency of MG-63 cells transfected with lncRNA GAS5 shRNA was determined by qPCR. The results in Fig. 1B showed that the expression of lncRNA GAS5 was clearly inhibited compared with shNC control (*P* < 0.001). MTT assay results (Fig. 1C) suggested that cell viability was improved by transfection with sh-GAS5 compared with transfection with shNC (*P* < 0.05 or *P* < 0.001). Moreover, the expressions of Cyclin D1, Cyclin B1, CDK1 and CDK4 were all up-regulated in sh-GAS5 group than in shNC group (Fig. 1D and 1E). Transwell detection results in Fig. 2A-2D showed that, MG-63 cells migration and invasion were both enhanced after sh-GAS5 transfection (*P* < 0.05).

The impacts of lncRNA GAS5 overexpression on MG-63 cells growth and metastasis were also detected. In Fig. 3A, lncRNA GAS5 expression was significantly increased by transfection

Fig. 1. Effect of lncRNA GAS5 silence on MG-63 cells proliferation. (A) The expression of lncRNA GAS5 in human osteosarcoma cell lines (MG-63 and OS-732) and a human osteoblast cell line hFOB1.19 were detected by qPCR. (B) The RNA level expression of lncRNA GAS5 in MG-63 cells after transfection with sh-GAS5 and shNC were measured by qPCR. (C) Cell viability after transfection was detected by MTT assay. (D) Quantitative analysis of proliferation-related protein expressions based on western blot analysis. (E) Representative image of western blot analysis. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ compared to hFOB1.19 cells or shNC group.

Fig. 2. Effects of lncRNA GAS5 silence on MG-63 cells migration and invasion. (A-B) Transwell assay was performed to measure migration after MG-63 cells were transfected with sh-GAS5 or shNC. (C-D) Transwell assay with Matrigel was used to measure invasion. *, $P < 0.05$ compared to the control group.

with pc-GAS5 (a GAS5 expressing vector) when compared with its negative control pcDNA3.1 ($P < 0.001$). Cell viability was significantly decreased ($P < 0.05$ or $P < 0.001$, Fig. 3B), and Cyclin D1, Cyclin B1, CDK1 and CDK4 expressions were remarkably down-regulated by pc-

Fig. 3. Effects of lncRNA GAS5 overexpression on MG-63 cells proliferation, migration and invasion. (A) The RNA level of lncRNA GAS5 was tested by qPCR in MG-63 cells after transfection with pc-GAS5 or pcDNA3.1. (B) Cell viability was detected by MTT assay. (C) Expression changes of proliferation-related protein were measured by western blot. (D) Transwell assay was performed to measure cell migration. (E) Transwell assay with matrigel was used to measure cell invasion. *, $P < 0.05$ and ***, $P < 0.001$ compared to pcDNA3.1 group.

AS5 transfection (Fig. 3C). Meanwhile, as shown in Fig. 3D and 3E, cell migration and invasion were both decreased in the pc-GAS5 group compared with the pcDNA3.1 group ($P < 0.05$).

lncRNA GAS5 affects MG-63 cells growth and metastasis via regulating miR-203a expression

We firstly detected miR-203a expression in sh-GAS5 transfected cells by qPCR. As shown in Fig. 4A, sh-GAS5 transfection significantly increased the expression of miR-203a compared with the negative control group ($P < 0.001$). Then, the expression of miR-203a in MG-63 cells was increased by inhibitor transfection to see the involvement of miR-203a in MG-63 cells viability and migration. Transfection efficiency shown in Fig. 4B indicated that miR-203a expression was clearly down-regulated in miR-203a inhibitor transfected group ($P < 0.01$). Data in Fig. 4C-4G showed that, miR-203a inhibitor resulted in significant increases in cell viability, migration, invasion ($P < 0.05$ or $P < 0.01$) and a significant increase in mitotic cell rate ($P < 0.001$). More interestingly, sh-GAS5 induced the increases of cell viability, migration, invasion, the up-regulations of Cyclin D1, Cyclin B1, CDK1, and CDK4, and the inhibition of cell apoptosis were all abolished or attenuated by miR-203a inhibitor.

Overexpression of TIMP2 decreased MG-63 cells growth and metastasis

Next, TIMP2 was overexpressed in MG-63 cells by vector expression to explore the role of TIMP2 in osteosarcoma. In Fig. 5A and 5B, up-regulations of TIMP2 at mRNA and protein levels were observed in MG-63 cells after transfection with pc-TIMP2 ($P < 0.01$). Cell viability, migration and invasion were reduced, while cell apoptosis was significantly increased in pc-TIMP2 group compared with negative control group (Fig. 5C-5F, $P < 0.05$ or $P < 0.01$ or $P < 0.001$). Then, the apoptosis-related protein expressions were measured by western blot assay. As shown in Fig. 5G, the expression of Bcl-2 was obviously decreased, while Bax, cleaved caspase-3 and cleaved caspase-9 were increased when TIMP2 overexpression.

Fig. 4. Involvement of miR-203a in LncRNA GAS5-modulated cell growth and metastasis of MG-63 cells. (A) The RNA levels of miR-203a in cells transfected with sh-GAS5 or shNC. (B) The RNA levels of miR-203a in cells transfected with miR-203a inhibitor or inhibitor control. RNA levels of miR-230 were measured by qPCR. (C) Cell viability was detected by MTT assay in MG-63 cells after transfection with sh-GAS5, miR-203a inhibitor, sh-GAS5 plus miR-203a inhibitor or negative controls. *, $P < 0.05$ and **, $P < 0.01$ comparison between sh-GAS5 and shNC + siNC group. #, $P < 0.05$ and ##, $P < 0.01$ comparison between sh-GAS5 + miR-203a inhibitor and sh-GAS5 + siNC group. (D) Expression changes of proliferation-related proteins were measured by western blot. (E) Transwell assay was performed to measure cell migration. (F) Transwell assay with matrigel was used to measure invasion. (G) Apoptotic cells were measured by flow cytometry. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ compared to the indicated group.

TIMP2 was a target gene of miR-203a

MG-63 cells were transfected with miR-203a mimic, inhibitor and negative controls. The mRNA and protein expression levels of *TIMP2* were detected. As

shown in Fig. 4A and 6B, miR-203a mimic transfection decreased the expression of *TIMP2* in both mRNA ($P < 0.05$) and protein levels, whereas miR-203a suppression significantly increased *TIMP2* expression ($P < 0.01$). Dual luciferase activity assay results in Fig. 6C showed that miR-203a significantly inhibited the activity of *TIMP2*-wt group ($P < 0.05$), indicating *TIMP2* might be a target gene of miR-203a.

The involvement of *TIMP2* in miR-203a-modulated growth and metastasis in MG-63 cells were detected then. The results of Fig. 7A suggested that miR-203a mimic transfection suppressed the expression of *TIMP2* ($P < 0.05$), while miR-203a and *TIMP2* simultaneous overexpression significantly reversed this trend ($P < 0.01$). MTT assay results in Fig. 7B showed that miR-203a mimic improved cell viability ($P < 0.05$), but co-transfection with miR-203a mimic and pc-*TIMP2* significantly inhibited cell viability ($P < 0.05$ or $P < 0.01$). The similar effects were found in cell migration and invasion. The results suggested that cell migration and invasion were enhanced by miR-203a mimic, whereas were reduced by miR-203a and *TIMP2* simultaneous overexpression ($P < 0.05$, Fig. 7C and 7D). The apoptosis

Fig. 5. Effects of TIMP2 overexpression on MG-63 cells growth and metastasis. (A) The mRNA and (B) protein levels of TIMP2 were detected by qPCR and western blot respectively in MG-63 cells after transfection with pc-TIMP2 and pcDNA3.1. (C) Cell viability was measured by MTT assay. (D) Transwell assay was performed to measure cell migration. (E) Transwell assay with matrigel was used to measure invasion. (F) Apoptotic cells were measured by flow cytometry. (G) Expression changes of apoptosis-related proteins were measured by western blot. *, $P < 0.05$, ** $P < 0.01$ and ***, $P < 0.001$ compared to pcDNA3.1 group.

Fig. 6. Regulatory role of miR-203a in TIMP2 expression. (A) The mRNA and (B) protein levels of TIMP2 in MG-63 cells were detected by qPCR and western blot respectively after transfection with miR-203a mimic, inhibitor or negative controls. (C) Luciferase activity assay was performed after transfection with TIMP2 3'UTR wild-type (wt) and miR-203a mimic, or TIMP2 3'UTR mutant-type (mt) and miR-203a mimic. *, $P < 0.05$ and **, $P < 0.01$ compared to the indicated group.

assay results (Fig. 7E) showed that miR-203a mimic plus pc-TIMP2 significantly increased the rate of apoptotic cells compared with miR-203a mimic alone ($P < 0.01$). From Fig. 7F, we found that the expression of Bcl-2 was decreased, and Bax, cleaved caspase-3, cleaved caspase-9 were increased after miR-203a inhibitor transfection. However, co-transfection with miR-203a inhibitor and si-TIMP2 up-regulated Bcl-2, and down-regulated Bax, cleaved caspase-3, and cleaved caspase-9.

Further, the interaction between TIMP2 and lncRNA GAS5 were detected by qPCR and western blot. As shown in Fig. 7G and 7H, the mRNA and protein expression levels of TIMP2 were up-regulated by pc-GAS5 transfection, on the contrary, sh-GAS5 down-regulated TIMP2 expression ($P < 0.05$ or $P < 0.01$).

Regulatory role of lncRNA GAS5, miR-203a and TIMP2 in PI3K/AKT/GSK3 β and NF- κ B signaling pathways

The expressions of major factors related with PI3K/AKT/GSK3 β and NF- κ B signaling pathways were detected by western blot after cells were transfected with miR-203a mimic, inhibitor, sh-GAS5, pc-TIMP2, respectively, as shown in Fig. 8A, the phosphorylated PI3K, AKT and GSK3 β expressions were increased after sh-GAS5 or miR-203a mimic, and decreased by miR-203a inhibitor. The down-regulations of phosphorylated PI3K, AKT and GSK3 β were reversed by si-TIMP2 transfection. From Fig. 8B, the phosphorylated I κ B α and p65 expressions were decreased after sh-GAS5 or miR-203a mimic transfection, and were increased by miR-203a inhibitor + si-TIMP2 group compared with miR-203a inhibitor group.

Fig. 7. Involvement of TIMP2 in miR-203a-modulated cell growth and metastasis of MG-63 cells. (A) The mRNA expression level of TIMP2 in MG-63 cells was detected by qPCR after transfection with miR-203a mimic or miR-203a mimic plus pc-TIMP2. (B) Cell viability was detected by MTT. *, $P < 0.05$ comparison between miR-203a mimic and NC group. #, $P < 0.05$ and ##, $P < 0.01$ comparison between miR-203a mimic and miR-203a mimic + pc-TIMP2 group. (C) Transwell assay was performed to measure cell migration. (D) Transwell assay with matrigel was used to measure invasion. (E) Apoptotic cells were measured by flow cytometry. (F) The protein expression levels of apoptosis-related factors were measured by western blot. (G) The mRNA and (H) protein levels of TIMP2 were respectively measured by qPCR and western blot after transfection with pc-GAS5, sh-GAS5 or negative controls. *, $P < 0.05$ and **, $P < 0.01$ compared to the indicated group.

Fig. 8. Regulatory roles of lncRNA GAS5, miR-203a and TIMP2 in PI3K/AKT/GSK3 β and NF- κ B signaling pathways. (A) Western blot analytical results demonstrating the phosphorylated levels of PI3K, AKT and GSK3 β in MG-63 cells after transfection with sh-GAS5, sh-NC, Scramble, miR-203a mimic, miR-203a inhibitor, miR-203a inhibitor plus si-TIMP2, or negative controls. (B) Western blot analytical results demonstrating the phosphorylated levels of I κ B α and p-65 after transfection.

Discussion

In recent decades, the treatment of osteosarcoma has been greatly improved benefit from novel adjuvant therapies, such as neo-adjuvant chemotherapy [15]. However, tumor metastasis is still plaguing patients with osteosarcoma [1]. In-depth study on the pathogenesis and molecular mechanisms of osteosarcoma will help to improve the current dilemma of osteosarcoma treatment. LncRNAs have gained widespread interest in recent years [16]. They are thought to be involved in many human diseases, particularly in the development and progression of human malignancies [17]. In the present study, we found that lncRNA GAS5 was down-regulated in osteosarcoma cell lines. Silence of lncRNA GAS5 in osteosarcoma cells significantly improved cell viability, migration and invasion *in vitro*. On the contrary, lncRNA GAS5 overexpression inhibited cell viability, migration, invasion and increased cell apoptosis. These results suggested a tumor suppressive role of lncRNA GAS5 in osteosarcoma cells.

It is well-known that lncRNA GAS5 is a growth suppressor, that it is up-regulated in T cells when cell growth is inhibited by starvation or rapamycin treatment [18, 19]. LncRNA GAS5 also exerts tumor suppressive functions by controlling of cell proliferation, cell-cycle, and apoptosis in multiple cancers including non-small cell lung cancer [20], breast cancer [6] and stomach cancer [21]. Moreover, overexpression of lncRNA GAS5 in human colorectal cancer significantly repressed the proliferation both *in vitro* and *in vivo* [22]. Consistence with the current understanding of lncRNA GAS5 in cancer cells, our study found that silence of lncRNA GAS5 promoted MG-63 cells viability, migration and invasion. The findings could enrich the knowledge regarding the roles of lncRNA GAS5 in osteosarcoma cells and provide new insights on lncRNA GAS5 as a novel tumor suppressor in tumorigenesis.

Accounting evidence displays that lncRNAs could regulate gene expression at transcriptional, post-transcriptional and epigenetic levels [16, 23]. In order to investigate the mechanism of lncRNA GAS5 in osteosarcoma cells growth and metastasis, the interaction

between lncRNA GAS5 and its target miR-203a was detected in this study. We found that miR-203a was negatively regulated by lncRNA GAS5, that silence of lncRNA GAS5 increased expression level of miR-203a. Inhibition of miR-203a in MG-63 cells reversed the effect of lncRNA GAS5 silence on cells growth and metastasis, suggesting the anti-tumor actions of lncRNA GAS5 in MG-63 cells possibly by repressing the expression of miR-203a.

Studies have proved that miRNAs act as oncogenes or suppressors to regulate the expressions of human cancer genes [12]. We further studied the target gene of miR-203a in MG-63 cells. We found that miR-203a directly targeted TIMP2 and regulated the expression of TIMP2. It has been reported that TIMP2 inhibited the most lethal cancer cells metastasis, thereby alleviating the pain caused by surgery, radiotherapy and chemotherapy in the treatment of osteosarcoma [24]. Furthermore, TIMP2 has been proved to be involved in brain metastasis of breast cancer [25] and invasive behavior of pancreatic cancer [26]. In this study, we found that TIMP2 was a target of miR-203a, and overexpression of TIMP2 had inhibitory effects on MG-63 cells growth and metastasis. Above data demonstrated that TIMP2 might act as a tumor suppressor, and the interaction between miR-203a and TIMP2 was related with osteosarcoma cells growth and metastasis.

To further understand the role of lncRNA GAS5 in regulating osteosarcoma cells growth and metastasis, the potential related molecular mechanism had been investigated. We observed that lncRNA GAS5 silence and miR-203a overexpression activated PI3K/AKT/GSK3 β signaling pathway while blocked NF- κ B signaling pathway. The deactivation of PI3K/AKT/GSK3 β signaling pathway and the activation of NF- κ B signaling pathway induced by miR-203a suppression were reversed by TIMP2 silence. GSK3 β is a downstream target molecule of PI3K/AKT pathway that induces apoptosis through regulation of apoptosis-related proteins [27]. GSK-3 β was phosphorylated in the signaling pathway of PI3K/AKT is activated. NF- κ B is found to be involved in cellular processes and to control DNA transcription in almost all animal cell types [28]. Overregulated NF- κ B is linked to cancer, inflammation, autoimmune disease, and aging [29, 30]. Herein, our results indicated that lncRNA GAS5 acted as a tumor suppressor in osteosarcoma cells triggered an “anti-tumor pathway” involving PI3K/AKT/GSK3 β and NF- κ B signaling.

In this study, *in vitro* experiments suggested that lncRNA GAS5 might act as a tumor suppressor in osteosarcoma cells by regulating tumor cells growth and metastasis. One possible mechanism underlies the anti-tumor actions is that lncRNA GAS5 works as a sponge for miR-203a, sequestering miR-203a away from TIMP2, and thus deactivating PI3K/AKT/GSK3 β signaling and activating NF- κ B signaling. There still lacks *in vivo* data to cross-check the function of lncRNA GAS5 in osteosarcoma. More effort is required for clinical application of lncRNA GAS5 in osteosarcoma treatment.

Disclosure Statement

No conflict of interests exists.

References

- 1 Picci P: Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis 2007;2:6.
- 2 Abarrategi A, Tornin J, Martinez-Cruzado L, Hamilton A, Martinez-Campos E, Rodrigo JP, Gonzalez MV, Baldini N, Garcia-Castro J, Rodriguez R: Osteosarcoma: Cells-of-Origin, Cancer Stem Cells, and Targeted Therapies. Stem Cells Int 2016;2016:3631764.
- 3 Gibb EA, Brown CJ, Lam WL: The functional role of long non-coding RNA in human carcinomas. Mol Cancer 2011;10:38.
- 4 Lei Y, Jing-jing L, Ke-nan Z, Qing-zhong T, Jin L: A tumor suppressive role of lncRNA GAS5 in human colorectal cancer. Open Life Sci 2016;11:105-109.
- 5 Shi X, Sun M, Liu H, Yao Y, Kong R, Chen F, Song Y: A critical role for the long non-coding RNA GAS5 in proliferation and apoptosis in non-small-cell lung cancer. Mol Carcinog 2015;54 Suppl 1:E1-E12.

- 6 Pickard MR, Williams GT: Regulation of apoptosis by long non-coding RNA GAS5 in breast cancer cells: implications for chemotherapy. *Breast Cancer Res Treat* 2014;145:359-370.
- 7 Cao S, Liu W, Li F, Zhao W, Qin C: Decreased expression of lncRNA GAS5 predicts a poor prognosis in cervical cancer. *Int J Clin Exp Pathol* 2014;7:6776-6783.
- 8 Lu X, Fang Y, Wang Z, Xie J, Zhan Q, Deng X, Chen H, Jin J, Peng C, Li H, Shen B: Downregulation of gas5 increases pancreatic cancer cell proliferation by regulating CDK6. *Cell Tissue Res* 2013;354:891-896.
- 9 Cao MX, Jiang YP, Tang YL, Liang XH: The crosstalk between lncRNA and microRNA in cancer metastasis: orchestrating the epithelial-mesenchymal plasticity. *Oncotarget* 2017;8:12472-12483.
- 10 Nd DG, Matkovich SJ: Menage a Trois: intimate relationship among a microRNA, long noncoding RNA, mRNA. *Circ Res* 2014;114:1362-1365.
- 11 Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni M: Long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Nat Commun* 2011;147:358-369.
- 12 Shenouda SK, Alahari SK: MicroRNA function in cancer: Oncogene or a tumor suppressor? *Cancer Metastasis Rev* 2009;28:369-378.
- 13 Livak KJ, Schmittgen TD: Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2^{-ΔΔC_T} Method. *Methods* 2001;25:402-408.
- 14 Shin SS, Keshavjee S, Gelmanova IY, Atwood S, Franke MF, Mishusheva AV, Anandav YG, Pasechnikov AD, Barnashov A, Tonkel TP, Cohen T: Development of extensively drug-resistant tuberculosis during multidrug-resistant tuberculosis treatment. *Am J Respir Crit Care Med* 2015;182:426-432.
- 15 Bacci G, Longhi A, Versari M, Mercuri M, Briccoli A, Picci P: Prognostic factors for osteosarcoma of the extremity treated with neoadjuvant chemotherapy: a single institution experience of 99 patients treated at a single institution. *Cancer* 2006;106:1154-1161.
- 16 Rinn JL, Chang HY: Genome regulation by long non-coding RNAs. *Annu Rev Biochem* 2012;81:145-166.
- 17 Esteller M: Non-coding RNAs in human disease. *Nat Rev Genet* 2011;12:861-874.
- 18 Mourtadamaarabouni M, Hasan AM, Farzaneh F, Willemze R: Inhibition of human T-cell proliferation by mammalian target of rapamycin (mTOR) inhibitors requires noncoding RNA growth-arrest-specific transcript 5 (GAS5). *Mol Pharmacol* 2011;79:127-138.
- 19 Qiao HP, Gao WS, Huo JX, Yang Y: lncRNA GAS5 functions as a tumor suppressor in renal cell carcinoma. *Asian Pac J Cancer Prev* 2013;14:1077-1082.
- 20 Shi X, Sun M, Liu H, Yao Y, Kong X, Zhang Z, Zhang Y: A critical role for the long non-coding RNA GAS5 in proliferation and apoptosis in non-small cell lung cancer. *Mol Carcinogenesis* 2015;54:E1-E12.
- 21 Liu Y, Jing Z, Zhang W, Gong H, Hu C, Huang G, Ying Z: lncRNA GAS5 enhances G1 cell cycle arrest via binding to YBX1 to regulate p21 in stomach cancer. *Sci Rep* 2015;5:10159.
- 22 Yin D, He X, Zhang Z, Kong R, De W, Zhang Z: Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Med Oncol* 2014;31:1-8.
- 23 Vadaiee M, Morris G: Long antisense non-coding RNAs and the epigenetic regulation of gene expression. *Biochem Concepts* 2014;4:411-415.
- 24 Yin D, He X, Zhang Z, Kong R, De W, Zhang Z: Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Med Oncol* 2014;31:1-8.
- 25 Mendes O, Kim HT, Lungu G, Stoica G: MMP2 role in breast cancer brain metastasis development and its regulation by TIMP2 and ERK1/2. *Clin Exp Metastasis* 2007;24:341-351.
- 26 Rigg AS, Lemoine NR: Adenoviral delivery of TIMP1 or TIMP2 can modify the invasive behavior of pancreatic cancer and can have a significant antitumor effect *in vivo*. *Cancer Gene Ther* 2001;8:869-878.
- 27 Hermida MA, Dinesh Kumar J, Leslie NR: GSK3 and its interactions with the PI3K/AKT/mTOR signalling network. *Adv Biol Regul* 2017;65:5-15.
- 28 Panday A, Inda ME, Bagam P, Sahoo MK, Osorio D, Batra S: Transcription Factor NF-kappaB: An Update on Intervention Strategies. *Arch Immunol Ther Exp (Warsz)* 2016;64:463-483.
- 29 Zhou R, Hu G, Gong AY, Chen XM: Binding of NF-kappaB p65 subunit to the promoter elements is involved in LPS-induced transactivation of miRNA genes in human biliary epithelial cells. *Nucleic Acids Res* 2010;38:3222-3232.
- 30 Haddad JJ, Abdelkarim NE: NF-kB cellular and molecular regulatory mechanisms and pathways: therapeutic pattern or pseudoregulation? *Cell Immunol* 2011;271:5-14.