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MicroRNA-9-5p inhibits osteosarcoma cell promotion, metastasis and resistance to apoptosis *via* negatively targeting Grb2-associated binding protein 2

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The study explores the inhibition effects of MicroRNAs in osteosarcoma, as a means of suggesting it as treatment for bone cancer. MicroRNAs (miRNAs) are a sort of noncoding RNA molecules that regulates gene expression by targeting mRNAs and play critical roles in tumor development. This study probed the effect of miR-9-5p on osteosarcoma development. Human osteosarcoma cell lines U2-OS, 143B, MG63 and HOS and normal human osteoblast cell line hFOB were cultivated and expression of miR-9-5p and Grb2-associated binding protein 2 (Gab2) measured. The binding of miR-9-5p and Gab2 was confirmed using a bio-information program and dual luciferase reporter gene assay. Loss-of-functions of miR-9-5p and Gab2 were performed to measure their roles in osteosarcoma cell proliferation, invasion, migration and resistance to death. Result showed high miR-9-5p expression and low Gab2 expression in osteosarcoma cells, particularly in U2-OS cells. miR-9-5p directly bound to the 3′untranslated region of Gab2. Down-regulated miR-9-5p induced U2-OS cell proliferation, invasion and the resistance to death, while conversely, silenced Gab2 led to an opposite trend on U2-OS cell growth and metastasis. Moreover, co-effect of inhibited miR-9-5p and silenced Gab2 led to decreased cell proliferation but promoted cell apoptosis compared to inhibited miR-9-5p alone, while it led to enhanced cell proliferation and invasion, but reduced cell apoptosis compared to silenced Gab2 alone. To conclude, this study demonstrated that miR-9-5p could inhibit osteosarcoma cell proliferation, invasion, migration and resistance to death via negatively targeting Gab2.

Keywords: Luciferase, Malignant behaviours, Osteoblast, Osteosarcoma, Proliferation

Osteosarcoma is the most prevalent primary bone tumor and the third widespread tumor in children and young teenagers[1]. It is featured with quick development, great metastatic possibility, and unfavorable clinical prognosis[2]. To date, systemic chemotherapy and local control surgery remain the major treatments for osteosarcoma[3]. Despite advances in the treating options, the overall survival rate of osteosarcoma patients has not been considerably enhanced yet[4]. Nearly 30–40% of localized osteosarcoma patients develop recurrent or metastatic diseases, and in such cases, the average survival time will be reduced to no more than one year[5]. Thus, identifying novel molecules participating in tumor development is crucial to decrease the incidence and mortality of this malignant disease.

MicroRNAs (miRNAs) are a family of short noncoding RNAs molecules composed of 20-25 nucleotides that work as negative regulators of the expression of post-transcription gene *via* binding to the 3′untranslated region (3′UTR) of target mRNAs thus contributing to transcript decline[6,7]. They have emerged as prospective diagnostic and therapeutic options owing to their correlation with tumorigenesis, survival, and prognosis[8]. Abnormal expression of miRNAs has been documented in multiple pathological conditions including solid tumors indicating their participance in carcinogenesis[9]. Among them, miR-9-5p has been documented to work either as a tumor-inhibitor in several cancers including human Gastric cancer[10], colorectal carcinoma[11] and pancreatic cancer[12], or as a tumor-promoter such as in non-small cell lung cancer[13] and prostate cancer[14]. While the role of miR-9-5p in osteosarcoma remains unidentified. Gab2 has been frequently detected in several human cancers[15-17]. For instance, Gab2 overexpression was found in ovarian cancer, and its overexpression increased the formation of tumor sphere in ovarian cancer cells[18]. Importantly, a previous study suggested that Gab2 is over-expressed in human osteosarcoma cells[19]. Hence, this study hypothesized that miR-9-5p could directly target Gab2 and inhibit the osteosarcoma development.

Materials and Methods

Cell culture and transfection

Human osteosarcoma cell lines U2-OS, 143B, MG63 and HOS and normal human osteoblast cell

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line hFOB purchased from ATCC (Manassas, VA, USA) were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 U/mL penicillin in a 37°C incubator with 5% CO_2. Cells were passaged once the cell reached 80% of confluence. Next, the cells were randomly assigned into control, inhibitor negative control (NC), miR-9-5p inhibitor, small interfering-Gab2 (si-Gab2) group, miR-9-5p inhibitor + si-Gab2 groups after corresponding transfections. All the transfections were conducted as per the instructions of Lipofectamine RNAiMax Transfection Reagent (Invitrogen Inc., Carlsbad, CA, USA). The miR-inhibitor, miR-NC and siRNA plasmids were all purchased from Guangzhou RiboBio Co., Ltd (Guangzhou, Guangdong China). In brief, 50 nM plasmid was added into 100 μL serum-free Opti-MEM medium (Gibco Company, Grand Island, NY, USA) and further fixed with 1 μL RNAiMax. The mixture stood at room temperature (25°C) for 10 min and then seeded into 24-well plates, after which the cell suspension was added to the plates and cultured at 37°C with 5% CO_2 for 72 h, and then the cells were collected for following experiments.

Dual luciferase reporter gene assay

Dual luciferase reporter gene assay was performed as described by Ting *et al*. with adjustments[20]. The binding sites of miR-9-5p and Gab2 was initially predicted on an online program Target scan (http://www.targetscan.org), and the binding relation was further identified using dual luciferase reporter gene assay. The synthetic Gab2 (Gab2-WT) or Gab2 mutant-type (Gab2-Mut) sequences were and cloned to the upstream of firefly luciferase gene to produce pMIR-Gab2-WT and pMIR-Gab2-Mut luciferase reporter plasmids. Well-designed pMIR-based reporter plasmids along with either miRNA-9-3p mimic or mimic NC were co-transfected into HEK293T cells with pMIR-renilla luciferase used as an internal reference. Cells were lysed 48 h after transfections. Relative luciferase activity was evaluated with the dual-luciferase reporter assay system as per the kit's instructions (E1910, Promega Corp., Madison, Wisconsin, USA). In brief, 5 μL firefly luciferase buffer along with 5 μL substrate or 5 μL renilla luciferase buffer along with 5 μL substrate was added to detect the corresponding luciferase activities. The relative value of firefly luciferase activity/renilla luciferase activity was evaluated and analysed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

This was performed as described by Crookenden *et al.* with little modifications[21]. Total RNA from cells was extracted using Trizol (Invitrogen), and the RNA concentration was detected using Bio-Rad analyzer, after which 1 μg RNA was reversely transcribed to cDNA using a cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Next, real time-PCR was conducted applying a SYBR® Premix Ex Taq™ (TliRNase H Plus) assay kit (Takara Holdings Inc., Kyoto, Japan) on an ABI7500 qPCR kit (Thermo Fisher). The PCR condition (20 μL) was 10 min of pre-denaturation at 95°C, followed by 40 cycles of denaturation (95°C for 15 s), annealing (60°C for 60 s) and a final extension (72°C for 40 s). U6 was set as an internal reference for miR-9-5p while GAPDH was set as an internal reference for other genes. The $2^{-\Delta\Delta Ct}$ method was applied in which ΔΔCt refers to the ratio of the target gene expression between the experimental and the control groups (Table 1).

Western blot analysis

Western blot analysis was performed as described by Zhaowei *et al.* with slight modification[22]. Forty-eight hours after transfection, cells were lysed with Radio-Immunoprecipitation assay (RIPA) lysis buffer and then the total proteins were collected, and the protein concentration was evaluated *via* a BCA kit (P0012, Beyotime Biotechnology Co., Ltd, Shanghai, China). Extracted proteins were run on 10% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore Corp., Billerica, MA, USA). Next, the membranes were sealed with 5% bovine serum albumin (BSA) at room temperature for 1 h and washed with TBST (tris-buffered saline + 0.1% Tween 20), and then incubated with following primary antibodies at 4°C overnight: Gab2 rabbit antibody

Table 1 — Primer sequences of RT-qPCR

Gene	Primer sequence (5′-3′)
miR-9-5p	F: ACACTCCAGCTGGGAGTATGTCGATC
	R: TGGTGTCGTGGAGTCGTATTG
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
Gab2	F: GTGGGGGATCTGAATGTTTTTA
	R: GCCCCAGGGTAGAATGAAA
MMP-9	F: GGGGGAAGATGCTGCTGTT
	R: AGCGGTCCTGGCAGAAATAG
MMP-2	F: CTCAGATCCGTGGTGAGATCT
	R: CTTTGGTTCTCCAGCTTCAGG
GADPH	F: GAAGGTGAAGGTCGGAGTC
	R: GAAGATGGTATGGGATTTC

Note: F, forward; R, reverse

(1:1000, 3239, Cell Signaling Technology (CST), Beverly, MA, USA), matrix metalloproteinase-2 rabbit antibody (MMP-2, 1:1000, 40994, CST), MMP-9 rabbit antibody (1:1000, 13667, CST). Next, the membranes were washed in a shaker containing TBST for 3 times (10 min each time). Thereafter, the membranes were incubated with horseradish peroxidase-labelled goat-anti rabbit and goat-anti mouse secondary antibodies (1:10000, Jackson, USA) for 1 h at room temperature, and washed 3 times with TBST (15 min each time). After that, the membranes were taken out and the remaining liquid was absorbed by filter papers. Then, the membranes were added with Pro-light horseradish peroxidase chemoluminescence reagent (PA112, TianGen Biotech Co., Ltd. Beijing, China) and covered with cling films, after which they were exposed, visualized and fixed in the dark, and then the bands were analysed using an Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Cell-Counting Kit-8 (CCK8) method

The U2-OS cells in each group were trypsinized with the cell suspension adjusted to suitable concentration and seeded into 96-well plates at the density of 200 μL per well. Three duplicated wells were set for each group and another well added with medium only was set as control. The optical density (OD) values of cells were measured at 0 h, 24 h, 34 h and 72 h as per the instructions of the CCK-8 reagent (C0038, Beyotime Biotechnology Co., Ltd, Shanghai, China). In brief, the supernatant was discarded, and each well was added with 100 μL serum-free medium and 100 μL CCK-8 reagent. the OD 450 nm values of cells were measured following 1-4 h of incubation.

Flow cytometry

Flow cytometry was performed as described by Danylovych *et al.*[23]. Forty-eight hours after transfection, cells in each group were successively detached with ethylene diamine tetraacetic acid-free trypsin, washed with phosphate buffer saline (PBS) for twice, centrifuged at 1000 g for 5 min and then collected for follow-up experiments. Cell apoptosis was measured as per the instructions of the AnnexinV-fluorescein isothiocyanate (FITC) assay kit (GK3603, Beijing Dingguo Changsheng Biotechnology Co., Ltd, Beijing, China). The cells were resuspended with PBS and counted, and then approximately 10^5 cells were harvested and suspended with 500 μL binding buffer, which was followed by mixing 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) and 10 min of reaction at room temperature in dark condition. Next, the cell apoptosis was measured using a flow cytometer. The green fluorescence of Annexin V-FITC was observed in channel FL1 while the red fluorescence of PI was observed in channel FL3. The cell cycle assay kit (C1052) was purchased from Beyotime. Collected cells were washed twice with ice-cold PBS, fixed with 1 mL cold 75% ethanol (−20°C), and incubated at 4°C for 2 h or overnight. After that, the cells were rinsed twice with cold PBS and added with 500 μL PI staining solution (prepared according to the instructions). Then the cells were resuspended and warm-bathed at 37°C for 30 min in dark condition, after which the cell cycle was measured using the flow cytometer at 4°C in the dark. Each experiment was performed in triplicate.

Trans-well assay

Seventy-two hours after transfection, the U2-OS cells were starved for 12 h, detached with trypsin, washed with PBS for twice, adjusted to 1×10^6 cells/mL and resuspended. Matrigel (BD Biosciences, San Jose, CA, USA) was dissolved at 4℃ overnight and diluted at 1:10 in serum-free DMEM on ice. Each well in the apical chamber was coated with 100 μL diluted Matrigel and allowed to stand for 2 h at 37℃, and further washed twice with serum-free DMEM for following experiments. The resuspended cells (50 μL) were added into the apical chamber wells, while 600 μL complete medium supplemented with 10% FBS was added to the basolateral chamber and the Trans-well kits were cultured in at 37℃ with 5% CO_2 for 24 h. Then the Transwell kits were taken out and the cells on the inner side of the apical chamber were wiped away using cotton swabs, after which the invaded cells were fixed with formaldehyde for 30 min at room temperature subjected to 20 min of crystal violet staining. Next, cells in each group were washed with clean water for 3 times and observed under a microscope with 6 fields randomly selected, and the average cell volume in each field was calculated. Cell migration assay was conducted at the same way as the invasion assay but rather than coated with Matrigel, the apical chamber was added with 100 μL cell suspension instead.

Statistical analysis

Data were analyzed using SPSS 21.0 (IBM Corp. Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. Differences among multiple groups were compared using one-way analysis of variance (ANOVA) and Bonferroni correction was

applied for pair comparisons after ANOVA. *P* was obtained from two-tailed test and *P* <0.05 was regarded as statistically significant.

Results

miRNA-9-5p was down-regulated while Gab2 was up-regulated in osteosarcoma cells

The miR-9-5p expression in normal human osteoblast cell hFOB and osteosarcoma cell lines U2-OS, 143B, MG63 and HOS was detected using RT-qPCR, which suggested that compared to the hFOB cell line, miR-9-5p presented a lowest expression in U2-OS cells (*P* <0.05) (Fig. 1A). Meanwhile, Gab2 expression in each group of cells was evaluated using Western blot analysis, which indicated that Gab2 expression was significantly elevated in U2-OS cells (*P* <0.05) (Fig. 1B).

miRNA-9-3p negatively targeted Gab2

The computer-based program (http://www.targetscan.org) predicted that miR-9-3p could bind to the 3′ UTR of Gab2 (Fig. 2A). Correspondingly, compared to the NC group, cells co-transfected with miR-9-5p and Gab2-WT presented significantly decreased luciferase activity (*P* <0.05), while those co-transfected with miR-9-5p and Gab2-Mut presented no major difference (*P* >0.05) (Fig 2B). To further identify if miR-9-5p targets Gab2, U2-0S cells were delivered with miR-9-5p inhibitor and si-Gab2, after which the miR-9-5p expression was measured using RT-qPCR (Fig. 2C). The results showed that compared to the control group, the miR-9-5p expression showed no significant difference between the inhibitor NC and the si-Gab2 groups (*P* >0.05), while the miR-9-5p expression in the miR-9-5p inhibitor group was obviously reduced as compared to the inhibitor NC group (*P* <0.05). Moreover, compared to the si-Gab2 group, the expression of miR-9-5p in the miR-9-5p inhibitor + si-Gab2 group was down-regulated (*P* <0.05). Meanwhile, the Gab2 protein level showed little difference between the control and inhibitor NC groups (*P* >0.05), while compared to the inhibitor NC group, the Gab2 protein level was markedly enhanced in the miR-9-5p inhibitor group but down-regulated in the si-Gab2

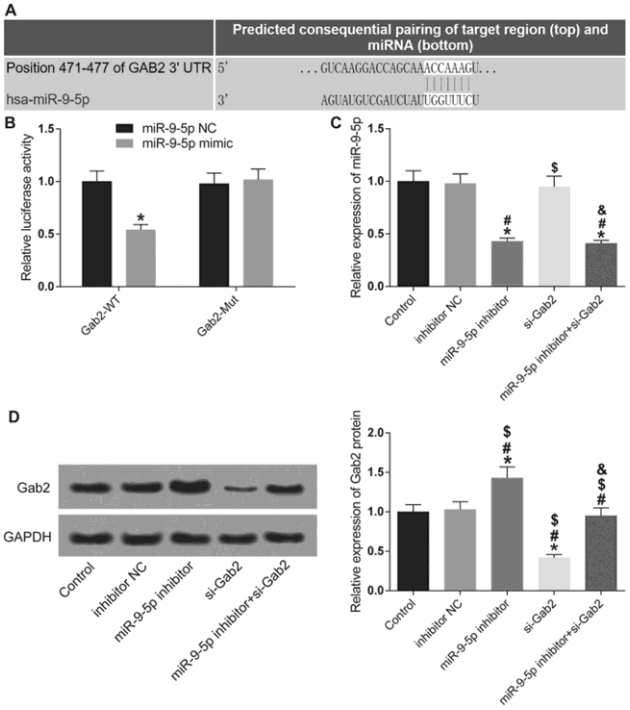

Fig. 2 — miR-9-3p negatively targeted Gab2. Note: A-B, binding relation of miR-9-5p and Gab2 predicted using a computer-based bio-information system (http://www.targetscan.org) (A) and identified using dual luciferase reporter gene assay (B) *, compared to the miR-9-5p group, *P* <0.05; C, miR-9-5p expression in U2-0S cells assessed using RT-qPCR; D, Gab protein level in U2-0S cells detected using Western blot analysis; In panel C and D, *, compared to the control group, *P* <0.05, #, compared to the inhibitor NC group, *P* <0.05; $, compared to the miR-9-5p inhibitor group, *P* <0.05, &, compared to the si-Gab2 group, *P* <0.05

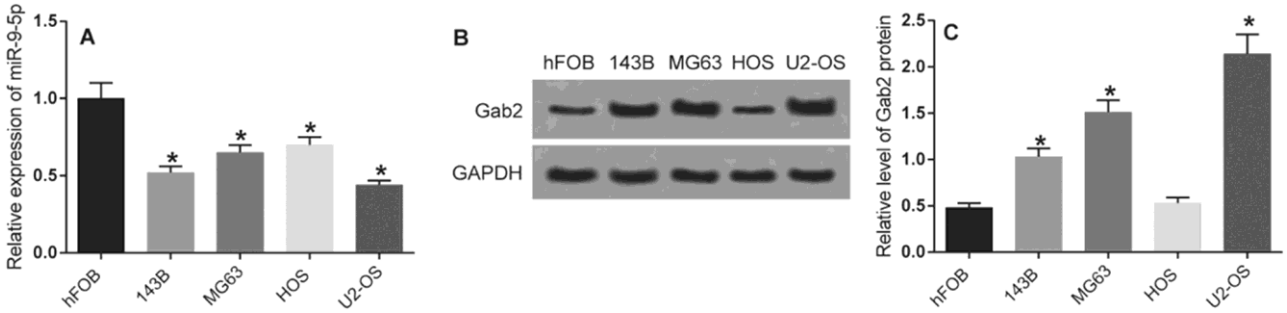

Fig. 1 — miR-9-5p was down-regulated while Gab2 was up-regulated in osteosarcoma cells. Note: A, miR-9-5p expression in osteoblast and osteosarcoma cells measured using RT-qPCR; B, protein level of Gab2 in osteoblast and osteosarcoma cells detected using Western blot analysis; *, compared to the hFOB cell line, *P* <0.05

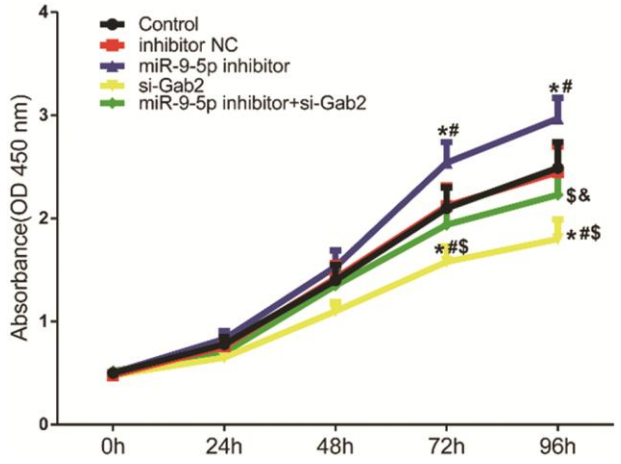

Fig. 3 — Down-regulated miR-9-5p promoted U2-OS proliferation but silenced Gab2 inhibited U2-OS proliferation. Note: A, cell proliferation in each group measured using CCK-8 method; *, compared to the control group, P <0.05, $^{\#}$, compared to the inhibitor NC group, P <0.05; $^{\$}$, compared to the miR-9-5p inhibitor group, P <0.05, $^{\&}$, compared to the si-Gab2 group, P <0.05

group (all P <0.05). Compared to the si-Gab2 group, the Gab2 protein expression was notably increased in the miR-9-5p inhibitor + si-Gab2 group (P <0.05) (Fig. 2D). These results demonstrated that all the transfections were successfully performed and miR-9-5p could negatively mediate Gab2 expression.

Down-regulated miRNA-9-5p promoted U2-OS proliferation but silenced Gab2 inhibited U2-OS proliferation

Proliferation of U2-OS was measured *via* CCK-8 method (Fig. 3). The results suggested that after 96 h of culture, cell proliferation was markedly increased in the miR-9-5p but declined in the si-Gab2 group compared to that in the inhibitor NC group (all P <0.05). Moreover, cell proliferation in the miR-9-5p inhibitor + si-Gab group was significantly reduced compared to the miR-9-5p inhibitor group but elevated compared to the si-Gab2 group (all P <0.05). These findings revealed that inhibited miR-9-5p could promote U2-OS cell proliferation but silenced Gab2 could suppress this proliferation.

Down-regulated miRNA-9-5p inhibited cell cycle arrest and apoptosis of U2-OS cells while silenced Gab2 led to an opposite trend

The flow cytometry results (Fig. 4A) suggested that the U2-OS cell apoptosis showed no notable difference between the control and inhibitor NC group (P >0.05). While compared to the inhibitor NC group, the apoptosis of U2-OS cell in the miR-9-5p inhibitor group was notably reduced but that in the si-Gab2 group was markedly enhanced (all P <0.05). Meanwhile, U2-OS cell apoptosis in the miR-9-5p inhibitor + si-Gab group was significantly increased compared to the miR-9-5p inhibitor group but decreased compared to the si-Gab2 group (all P <0.05). Regarding the cell cycle of U2-OS cells, the flow cytometry results (Fig. 4B) demonstrated that the U2-OS cell cycle showed little significant difference between the control and inhibitor NC group (P >0.05). Compared to the inhibitor NC group, the ratio of U2-OS cells in the G0/G1 phase in the miR-9-5p inhibitor group was obviously reduced while that in the si-Gab2 group was markedly enhanced (all P <0.05). Meanwhile, U2-OS cell apoptosis in the miR-9-5p inhibitor + si-Gab group was significantly increased compared to the miR-9-5p inhibitor group but decreased compared to the si-Gab2 group (all P <0.05). These findings demonstrated that lowly-expressed miR-9-5p could reduce the ratio of cells in the G0/G1 phase and inhibit apoptosis of U2-OS cells, while silenced Gab2 could lead to an opposite trend.

Down-regulated miRNA-9-5p promoted U2-OS invasion and migration but silenced Gab2 reversed this trend

Cell invasion and migration was measured using Trans-well assays (Fig. 5), and the results showed that cell invasion and migration presented little difference between the control and inhibitor NC groups (P >0.05). Compared to the inhibitor NC group, the invasive and migratory abilities of U2-OS cells were markedly enhanced in the miR-9-5p inhibitor group but decreased in the si-Gab2 group (all P <0.05). While the invasive and migratory abilities of U2-OS cells in the miR-9-5p inhibitor + si-Gab group were markedly reduced compared to the miR-9-5p inhibitor group but enhanced compared to the si-Gab2 group (all P <0.05).

Down-regulated miRNA-9-5p elevated the expression of invasion- and migration-related factors

MMP-2 and MMP-9 are well-known tumor invasion promoters. Here, our study explored the expression of these two factors in each group (Fig. 6A & B). The MMP-2 and MMP-9 expression showed no notable difference between the control and the inhibitor NC groups (all P >0.05), which was consistence with the Transwell assay results. Meanwhile, compared to the inhibitor NC group, the MMP-2 and MMP-9 expression was significantly elevated in the miR-9-5p inhibitor group while decreased in the si-Gab2 group (all P <0.05). Likewise, the MMP-2 and MMP-9 expression in the miR-9-5p inhibitor + si-Gab group was markedly reduced compared to the miR-9-5p inhibitor group but enhanced compared to the si-Gab2 group (all P <0.05).

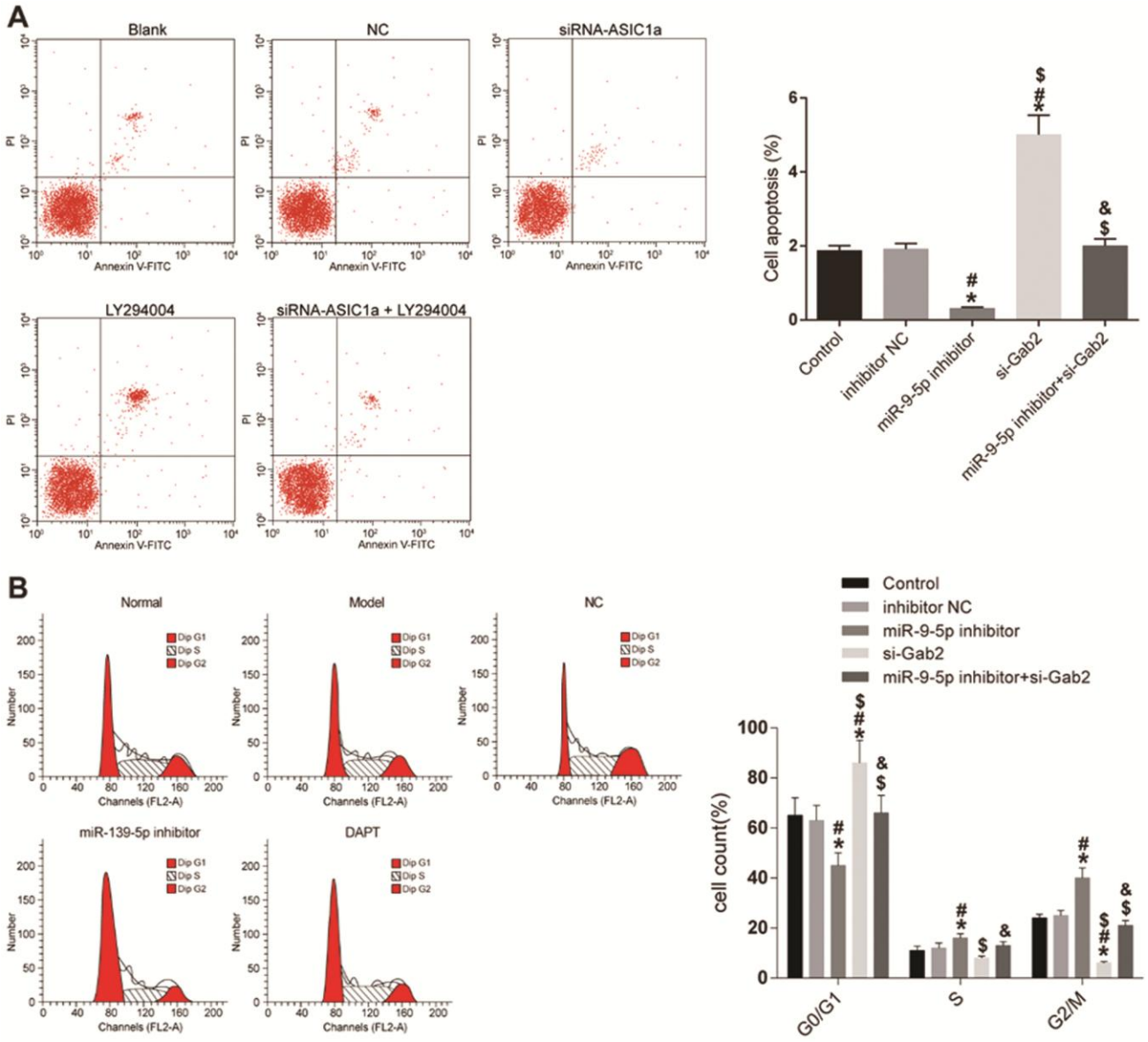

Fig. 4 — Down-regulated miR-9-5p inhibits cell cycle arrest and apoptosis of U2-OS cells while silenced Gab2 promoted cell apoptosis. Note: A, U2-OS cell apoptosis detected using flow cytometry; B, U2-OS cell cycle measured using flow cytometry; *, compared to the control group, P <0.05, #, compared to the inhibitor NC group, P <0.05; $, compared to the miR-9-5p inhibitor group, P <0.05, &, compared to the si-Gab2 group, P <0.05

Discussion

Osteosarcoma is the most prevalent bone primary malignancy in young people[24]. An intensive understanding of the initiation and development mechanisms of osteosarcoma is required for developing novel therapeutic options for osteosarcoma patients to enhance overall survival. To date, multiple miRNAs have been documented to be involved in osteosarcoma development[25]. Also, miRNA has been reported to potentially inhibit proliferation in some cancers *via* targeting GRB2[26,27]. Here, the current study investigated the role of miRNA-9-5p in osteosarcoma and found that it could inhibit the development of osteosarcoma cells including proliferation, invasion and resistance to death by negatively targeting Gab2.

Initially, down-regulated miRNA-9-5p while up-regulated Gab2 was found in osteosarcoma cells. In previous study, downregulated miR-9-5p by TGF-β1 led to HSC activation, including cell proliferation, α-SMA and collagen expression, and this is indicative in many carcinomas[28]. Thus, its upregulation is critical to

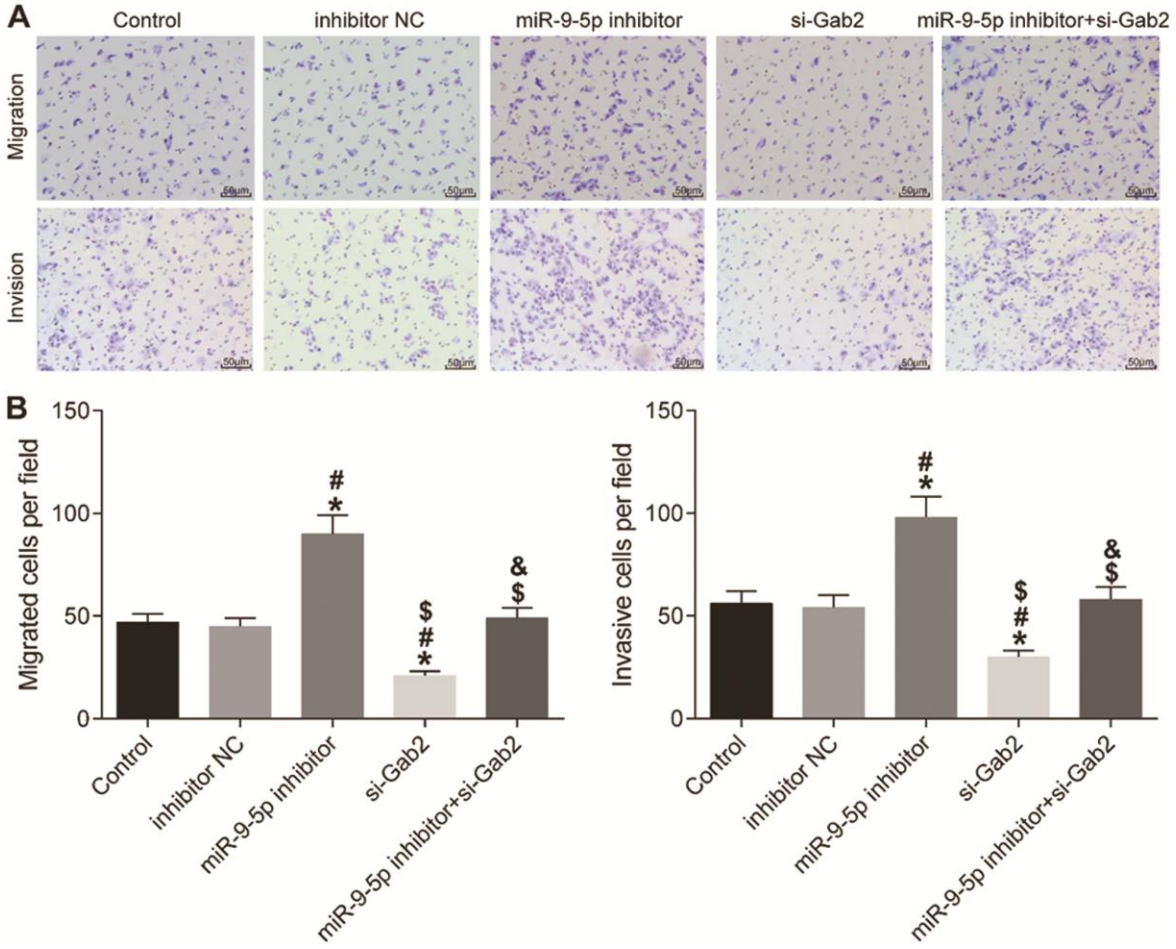

Fig. 5 — Down-regulated miR-9-5p promoted U2-OS cell invasion and migration but silenced Gab2 reversed this trend. Note: A, cell invasion and migration detected using Trans-well assays; B, analysis of the invasion and migration of each group of U2-OS cells; *, compared to the control group, P <0.05, $^{\#}$, compared to the inhibitor NC group, P <0.05; $^{\$}$, compared to the miR-9-5p inhibitor group, P <0.05, $^{\&}$, compared to the si-Gab2 group, P <0.05

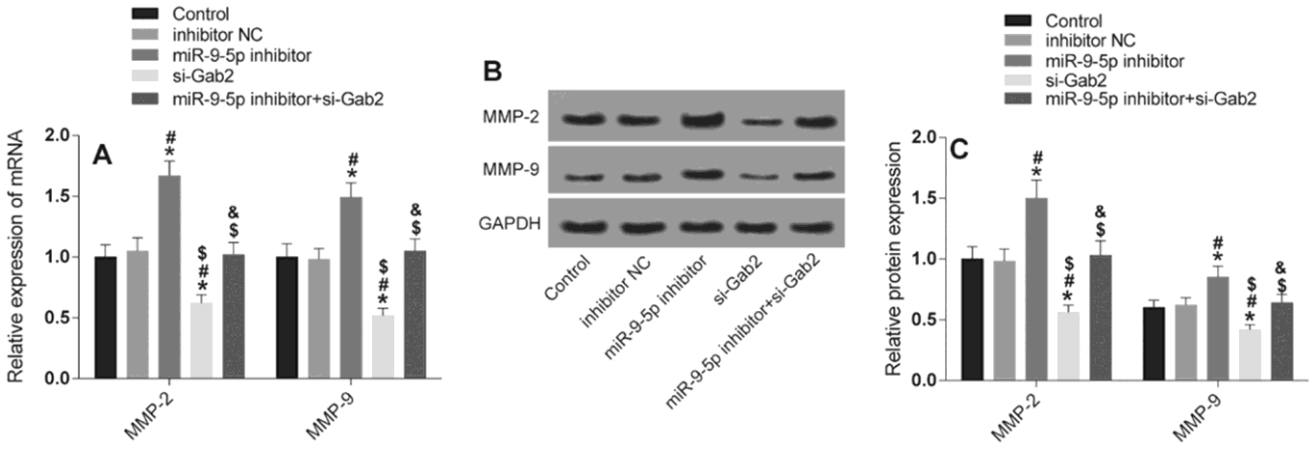

Fig. 6 — Down-regulated miR-9-5p elevated the MMP-2 and MMP-9 expression in U2-OS cells. Note: A, MMP-2 and MMP-9 mRNA expression detected using RT-qPCR; MMP-2 and MMP-9 protein levels determined using Western blot analysis; *, compared to the control group, P <0.05, $^{\#}$, compared to the inhibitor NC group, P <0.05; $^{\$}$, compared to the miR-9-5p inhibitor group, P <0.05, $^{\&}$, compared to the si-Gab2 group, P <0.05

prevent and inhibit certain cancer cells. miRNA-9-5p has been reported as a tumor inhibitor in several cancers including osteosarcoma[6]. Meanwhile, miRNA-9-5p directly bound to the 3′UTR of Gab2 according to bio-information software prediction and dual luciferase reporter gene assay. Gab2 is a well-known tumor-related gene and it has been found highly expressed in osteosarcoma cells[19].

Findings from this study showed that down-regulated miRNA-9-5p led to enhanced U2-OS cell proliferation, invasion, migration and resistance to death, while silenced Gab2 led to a contrary tendency. It has been suggested that miRNA-9-5p suppresses growth and development of pancreatic cancer by targeting GOT1[29]. Meanwhile, miRNA-9-5p also has shown the tumor-suppressive effect on papillary thyroid cancer *via* inhibiting cell proliferation and promoting cell apoptosis[30]. Likewise, in osteosarcoma, up-regulated miR-9-5p has been suggested to inhibit cell promotion and growth but promote cell cycle arrest in G0/G1 period and cell apoptosis[6]. This also agrees with the findings of Yu *et al.*[28], who reported that upregulated miR-9-5p effectively prevented liver fibrosis. Regarding Gab2, inhibited Gab2 expression has been found to inhibit cell proliferation, and invasion of glioma[31], glioblastoma[16] and breast cancer[32]. Similarly, studies by Wang *et al.* showed that silenced Gab2 was identified to decrease osteosarcoma cell invasion and migration[19], and this agrees with the findings of our study. Moreover, our study showed that down-regulated miRNA-9-5p enhanced MMP-2 and MMP-9 expression in osteosarcoma cells. MMPs are well-known metastasis-related factors. Up-regulation of miRNA-9-5p has been documented to reduce MMPs levels in osteoarthritis[33]. While Gab2 has been suggested to facilitate the metastasis and epithelial to mesenchymal transition by activating MMPs in colorectal cancer[34]. These results further provided evidence that down-regulated miRNA-9-5p could lead to osteosarcoma cell invasion and migration, while silenced Gab2 could lead to an opposite trend.

Conclusion

To sum up, it can be concluded that miRNA-9-5p could inhibit osteosarcoma development by suppressing its malignant behaviors such as proliferation, invasion, migration and resistance to apoptosis *via* negatively targeting Gab2 expression. Thus, miRNA-9-5p might serve as a novel treating target for osteosarcoma. However, there might be several target genes of miRNA-9-5p while only Gab2 was included in the current study. More studies would be performed in this field to validate our findings and to develop more therapeutic options for osteosarcoma.

Conflict of interest

All authors declare no conflict of interest.

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