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Original Paper

miR-423-5p Inhibits Osteosarcoma Proliferation and Invasion Through Directly Targeting STMN1

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Key Words
Osteosarcoma • MicroRNAs • miR-423-5p • STMN1

Abstract
Background/Aims: Increasing evidences suggest that dysregulated expression of miRNAs contributes to the progression of various tumors. However, the underlying function of miR-423-5p in osteosarcoma remains unexplored. ***Methods:*** The expression of miR-423-5p and STMN1 were determined in osteosarcoma samples and cell lines via quantitative real-time PCR. Colony formation and Cell Counting Kit-8 (CCK-8) assays were performed to measure cell proliferation ability and transwell analysis was used to detect cell invasion, and dual luciferase reporter assay was perform to analysis the interaction between the miR-423-5p and STMN1. ***Results:*** The expression levels of miR-423-5p and STMN1 in the osteosarcoma tissues and cell lines were measured by qRT-PCR. Cell viability was determined using the clone formation and CCK-8 assays. A dual-luciferase reporter and Western blot were performed to stdudy the target gene of miR-423-5p. Here, we showed that miR-423-5p expression was downregulated in osteosarcoma tissues and cell lines. However, the expression of stathmin1 (STMN1) was downregulated in osteosarcoma tissues and cell lines. Moreover, STMN1 expression level was negatively correlated with the miR-423-5p expression in the osteosarcoma tissues. We identified STMN1 was a direct target gene of miR-423-5p in osteosarcoma cell. Overexpression of miR-423-5p inhibited osteosarcoma cell proliferation, colony formation and invasion. Furthermore, we demonstrated that STMN1 was involved in miR-423-5p-mediated cell behavior such as cell proliferation, colony formation and invasion in the osteosarcoma cell. ***Conclusion:*** Our present study indicated that miR-423-5p acted as a tumor suppressor gene in osteosarcoma partly through inhibiting STMN1 expression.

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Introduction

Osteosarcoma is the most prevalent malignant tumor of bone and is usually observed in young adults and adolescents [1-5]. Despite the recent advances in the treatment of osteosarcoma such as radiation therapy, adjuvant chemotherapy and surgery that have been achieved, the relative 5-year survival rate of osteosarcoma patients remains discontent [6-10]. Although various studies have investigated the development and mechanism of osteosarcoma, the molecular mechanism of osteosarcoma is still elusive [11-13]. Therefore, it is urgent to find novel therapeutic approaches and targets for treating osteosarcoma.

MicroRNAs (miRNAs) are an abundant class of small, endogenous, single-stranded and noncoding RNAs that modulate gene expression posttranscriptionally through binding to the 3'UTR (3'-untranslated region) of the target gene [14-17]. MiRNAs participate in many biological processes including cell apotosis, development, proliferation, differentiation, fat metabolism, migration and stress resistance [18-21]. Increasing evidences demonstrated that deregulation of miRNAs could be implicated in a lot of tumors such as hepatocellular carcinoma, glioblastoma, colorectal carcinoma, ovarian carcinoma, renal cell carcinoma and gastric cancer [22-27]. Especially, some miRNAs have been demonstrated to play crital roles in pathogenesis of osteosarcoma [28-30].

The present study aimed to investigate the role of miR-423-5p in osteosarcoma. We showed that miR-423-5p expression was decreased in osteosarcoma tissues and cell lines. We identified STMN1 as a direct target gene of miR-423-5p in the osteosarcoma cell. Overexpression of miR-423-5p suppressed the osteosarcoma cell proliferation, colony formation and invasion.

Materials and Methods

Tumor Specimens, cell lines and cell tranfection

Thirty paired osteosarcoma tissues and no-tumor specimens were collected from osteosarcoma patients at our hospital. These tissues were immediately kept in the liquid nitrogen until use. This study was approved with the Ethics Committee of Qingdao Central Hospital and human tissues for this research were collected with informed consent. The osteosarcoma cells (Saos-2, HOS, MG-63 and U2OS) and one normal osteoblast cell line were purchased from the Cell Source of the Academy of Sciences (Shanghai, China). These cells were cultured in the DMEM (Dulbecco's modified Eagle's medium, Invitrogen, USA) supplemented with FBS, penicillin and streptomycin. MiR-423-5p mimic and scramble were purchased from Ribobio (Guangzhou, China). Cell transfection was performed by using the Lipofectamine 2000 (Invitrogen, USA) in accordance to the instruction's information.

qRT-PCR

Total RNAs from cells or tissues were isolated with Trizol reagent in accordance with manufacturer's instruction. For miR-423-5p detection, qRT-PCR was performed by using the SYBR green PCR mix according to the manufacturer's instructions. The miR-423-5p and STMN1 expression level were normalized to the expression of U6 and GAPDH respectively. The relative mRNA expression was determined by the comparative Ct method. The primers were used as following: STMN1, Forward: 5'-GTACTTCTGGACTCACGGGC-3'; Reverse: 5'-AAGGCAAGAGTGGTCTGCTC-3'; GAPDH, Forward: 5'-TGAAGGTCGGAGTCAACGGA-3' Reverse: 5'-CCTGGAAGATGGTGATGGGAT-3'.

Western blot analysis

Total protein was extracted from cultured cell or tissue by using PIPA (200 m NaVO4, 2 X lysis buffer, 0.5 M EDTA, 200 mM NaF, 10 X sodiumdeoxycholate, 25 X protease inhibitor) according to instructions. Total protein was separated by 12% SDS gel electrophoresis and then tranferred to the PVDF membranes (BioRad, USA). The membrane was probed the antibodies overnight (STMN1 and GAPDH, Abcam), then

incubated with secondary antibodies. The signal of protein was detected with the chemiluminescence (Amersham Life Science, UK) following to the manufacturer's instructions. GAPDH was detected as the internal control protein.

Cell Proliferation Assay, invasion and colony formation assay

Cell Proliferation was accessed using the CCK-8 assay (Cell Counting Kit-8, Dojindo, Japan). Cells were cultured in the 96-well plate after transfection. The number of cells was determined by measuring absorbance at the 450 nm on the microplate reader. Cell invasion was determined by using Transwell (Millipore Corporation, USA) coated with Matrigel (Life Technologies, USA). The MG-63 cells were cultured on the upper chamber in the DMEM with no-serum and 10% FBS was added to the lower chamber. The invasive cells on the lower surface were fixed with methanol and stained with crystal violet. For cell colony formation, cells were kept on the 6-well plate (300 cells/well) and cultured for 2 weeks in the DMEM supplemented with 10% FBS. Cell colony was stained with crystal violet.

Luciferase reporter assays

The fragment of the wild type STMN1 3'UTR (WT 3'UTR) containing miR-423-5p target site was generated using PCR and the mutant STMN1 3'UTR (Mut 3'UTR) was amplified by PCR method. The fragments including the Mut 3'UTR or WT 3'UTR region of STMN1 were cloned into psiCHECK-2 vector (Promega, USA), which included firefly and renilla luciferase reporter gene. Cells were transfected with miR-423-5p mimic and scramble and Mut 3'UTR or WT 3'UTR vector. Luciferase activity was determined by using the Dual-Luciferase Reporter analysis kit (Promega Corporation, USA) following to the instructions.

Statistical analysis

Data were presented as mean ± SD (standard deviation). The difference between two groups was analyzed by Student's t-test and one-way analysis of variance was done to determine the difference when there were more than two groups. $P< 0.05$ was considered significantly different.

Results

miR-423-5p expression was decreased in osteosarcoma tissues and cell lines

We firstly detected the miR-423-5p expression in osteosarcoma tissues. As shown in the Fig. 1A, miR-423-5p expression was downregulated in 29 cases (29/40; 72.5 %) of osteosarcoma tissues compared with adjacent tissues. The expression of miR-423-5p was lower in osteosarcoma tissues than in the non-tumor specimens (Fig. 1B). We also showed that miR-423-5p expression was downregulated in osteosarcoma cell lines (U2OS, SOSP-9607, MG-63 and SAOS-2) and one normal osteoblast cell line (hFOB) (Fig. 1C).

STMN1 expression was increased in osteosarcoma tissues and cell lines

We next detected the STMN1 expression in the osteosarcoma tissues. As shown in the Fig. 2A, STMN1 expression was increased in 31 cases (31/40; 77.5 %) of osteosarcoma tissues compared with adjacent tissues. The expression of STMN1 was higher in osteosarcoma tissues than in the non-tumor specimens (Fig. 2B). We also demonstrated that the expression of STMN1 was upregulated in osteosarcoma cell lines (U2OS, SOSP-9607, MG-63 and SAOS-2) and one normal osteoblast cell line (hFOB) (Fig. 2C). Moreover, STMN1 expression was negatively correlated with miR-423-5p expression in osteosarcoma tissues (Fig. 2D).

STMN1 was a direct target gene of miR-423-5p in osteosarcoma cell

We transfected MG-63 cells with miR-423-5p mimic and found that the expression of miR-423-5p was significantly upregulated (Fig. 3A and B). TargetScan was used to search the candidate target genes of miR-423-5p. A conserved domain within the 3'-UTR of STMN1 contained a potential miR-423-5p binding site (Fig. 3C). The MG-63 cell was cotransfected

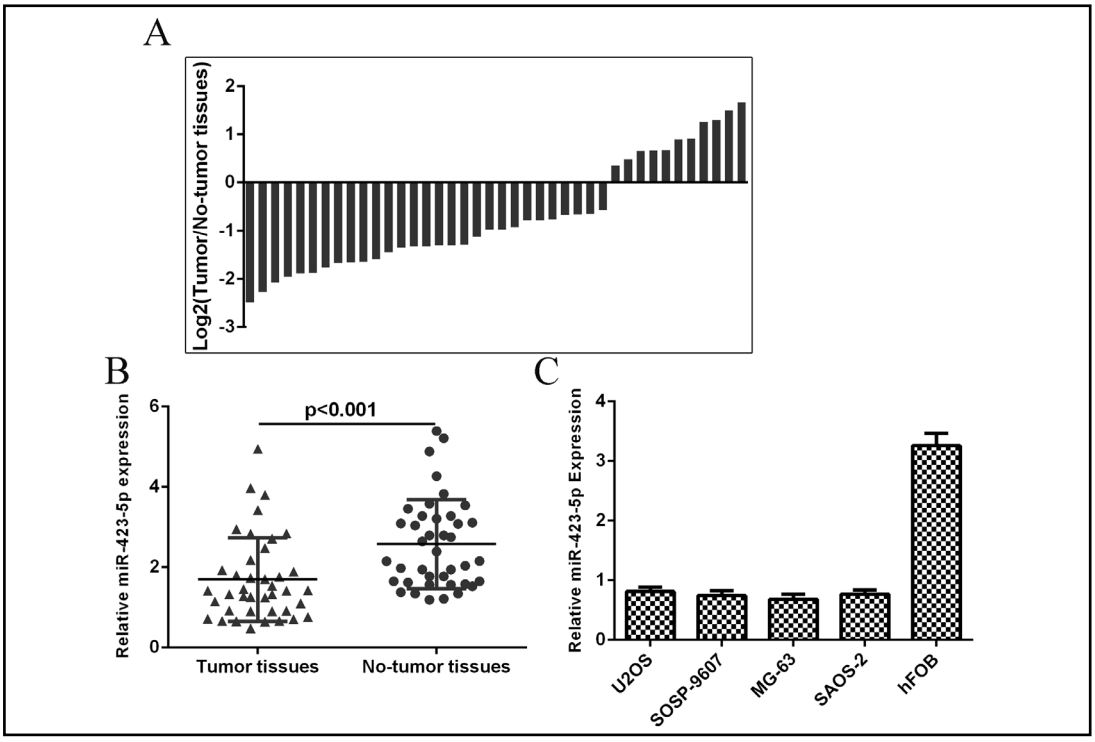

Fig. 1. miR-423-5p expression was downregulated in the osteosarcoma tissues and cell lines. (A) The miR-423-5p expression was downregulated in 29 cases (29/40; 72.5 %) compared with adjacent tissues. Data are shown as log 2 of fold change of osteosarcoma tissues relative to non-tumor tissues. (B) The expression of miR-423-5p was lower in the osteosarcoma tissues than in the no-tumor specimens. (C) The expression of miR-423-5p in the osteosarcoma cell lines (U2OS, SOSP-9607, MG-63 and SAOS-2) and one normal osteoblast cell line (hFOB) was measured by qRT-PCR.

with the mutated (MUT) and wild type (WT) STMN1 luciferase reporter together with miR-423-5p mimic or scramble. Overexpression of miR-423-5p suppressed the luciferase activity of WT reporter, but not the Mut reporter (Fig. 3D). Ectopic expression of miR-423-5p decreased STMN1 expression in the MG-63 cell (Fig. 3E).

STMN1 inhibited osteosarcoma cell proliferation, colony formation and invasion

Ectopic expression of miR-423-5p inhibited MG-63 cell proliferation (Fig. 4A). Elevated miR-423-5p expression decreased the expression of ki-67 in the MG-63 cell (Fig. 4B). Overexpression of miR-423-5p inhibited the expression of Cyclin D1 in MG-63 cell (Fig. 4C). Moreover, ectopic expression of miR-423-5p suppressed the MG-63 cell colony formation (Fig. 4D and E). Elevated miR-423-5p expression inhibited the MG-63 cell invasion (Fig. 4F and G).

STMN1 was involved in miR-423-5p-mediated cell behavior

qRT-PCR and Western blot assay disclosed that transfection with pCDNA3.1-STMN1 vector lead to an increase in STMN1 expression in MG-63 cells, compared with the control vector (Fig. 5A and B). STMN1 overexpression partially rescued the cell proliferation effect induced by miR-423-5p (Fig. 5C). Overexpression of STMN1 enhanced the expression of ki-67 in the miR-423-5p-induced MG-63 cell (Fig. 5D). Ectopic expression of STMN1 enhanced the expression of Cyclin D1 in the miR-423-5p-induced MG-63 cell (Fig. 5E). Re-introduction of STMN1 significantly reversed the inhibition of cell colony formation in the miR-423-5p-expressing cells (Fig. 5F and G). Overexpression of STMN1 significantly enhanced the inhibition of cell invasion in the miR-423-5p-expressing cells (Fig. 5H and I).

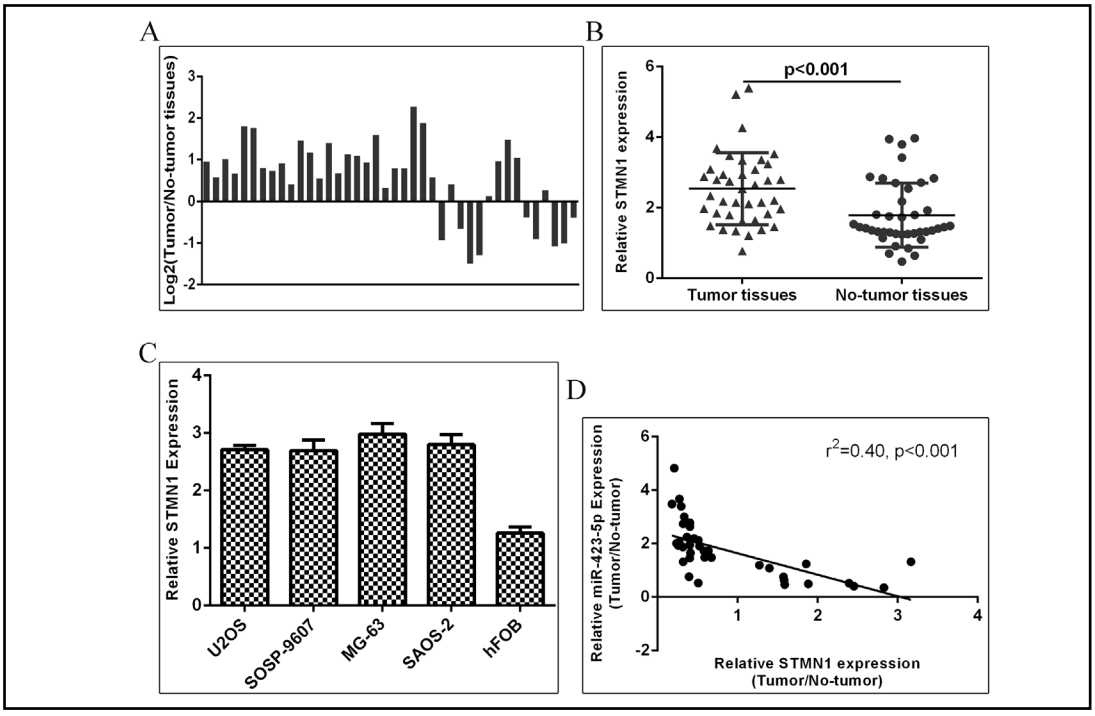

Fig. 2. STMN1 expression was upregulated in the osteosarcoma tissues and cell lines. (A) The STMN1 expression was upregulated in 31 cases (31/40; 77.5 %) compared with adjacent tissues. Data are shown as log 2 of fold change of osteosarcoma tissues relative to non-tumor tissues. (B) The expression of STMN1 was higher in the osteosarcoma tissues than in the no-tumor specimens. (C) The expression of STMN1 in the osteosarcoma cell lines (U2OS, SOSP-9607, MG-63 and SAOS-2) and one normal osteoblast cell line (hFOB) was measured by qRT-PCR. (D) The expression of STMN1 was negatively related with the expression of miR-423-5p in the osteosarcoma tissues.

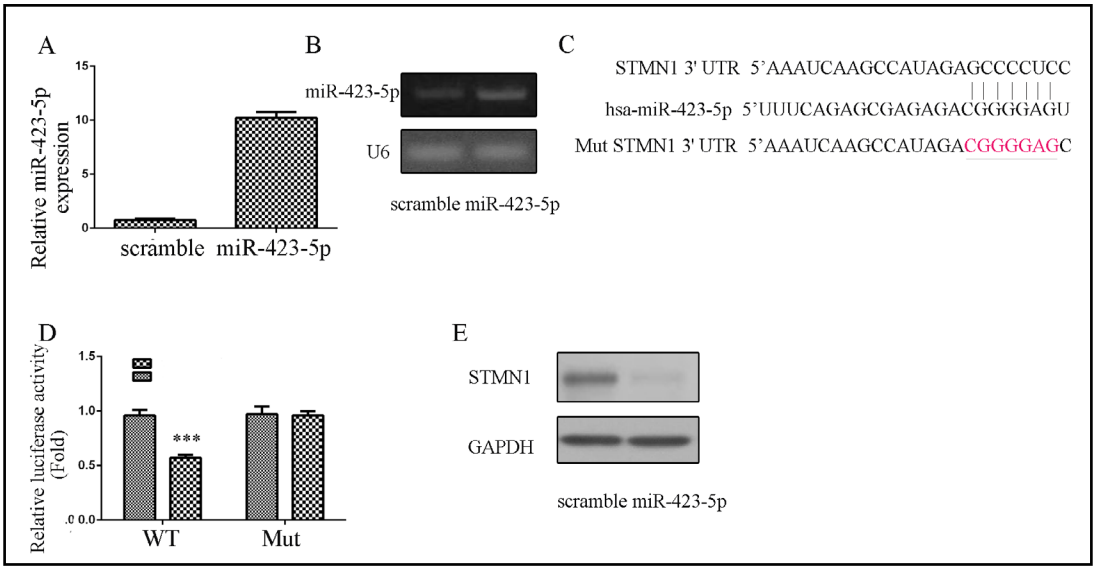

Fig. 3. STMN1 was a direct target gene of miR-423-5p in the osteosarcoma cell. (A) The expression of miR-423-5p was determined by qRT-PCR. (B) The expression of miR-423-5p was determined by PCR. (C) A conserved domain within the 3'-UTR of STMN1 with a potential miR-423-5p binding site was shown. (D) Overexpression of miR-423-5p suppressed the luciferase activity of WT reporter, but not the Mut reporter. (E) Ectopic expression of miR-423-5p inhibited the expression of STMN1 in the MG-63 cell. ***p<0.001.

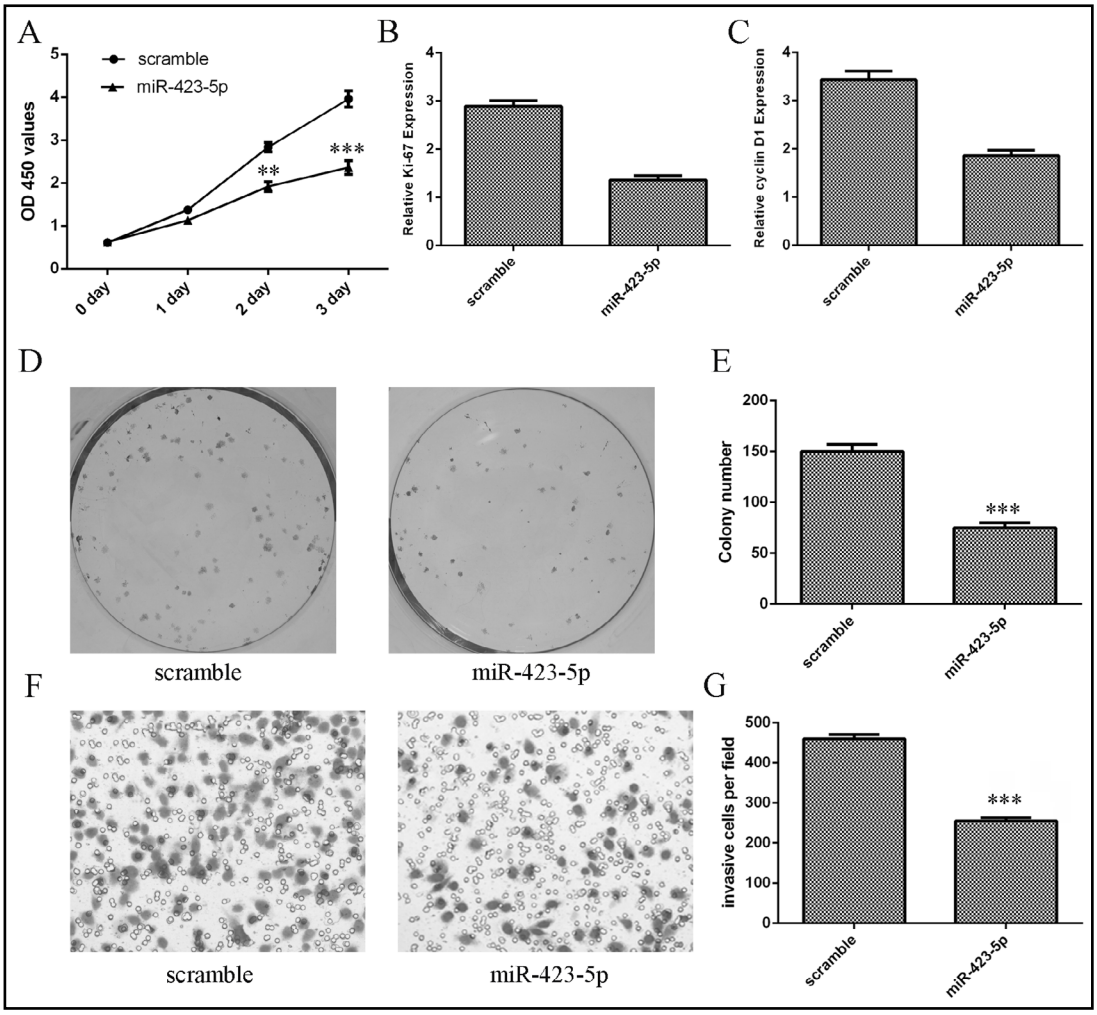

Fig. 4. STMN1 suppressed the osteosarcoma cell proliferation, colony formation and invasion. (A) Ectopic expression of miR-423-5p suppressed the MG-63 cell proliferation. (B) Elevated miR-423-5p expression decreased the ki-67 expression in the MG-63 cell. (C) Overexpression of miR-423-5p inhibited the cyclin D1 expression in the MG-63 cell. (D) Ectopic expression of miR-423-5p suppressed the MG-63 cell colony formation. (E) The relative colony numbers were shown. (F) Elevated miR-423-5p expression inhibited the MG-63 cell invasion. (G) The relative invasive cells were shown. **p<0.01 and ***p<0.001.

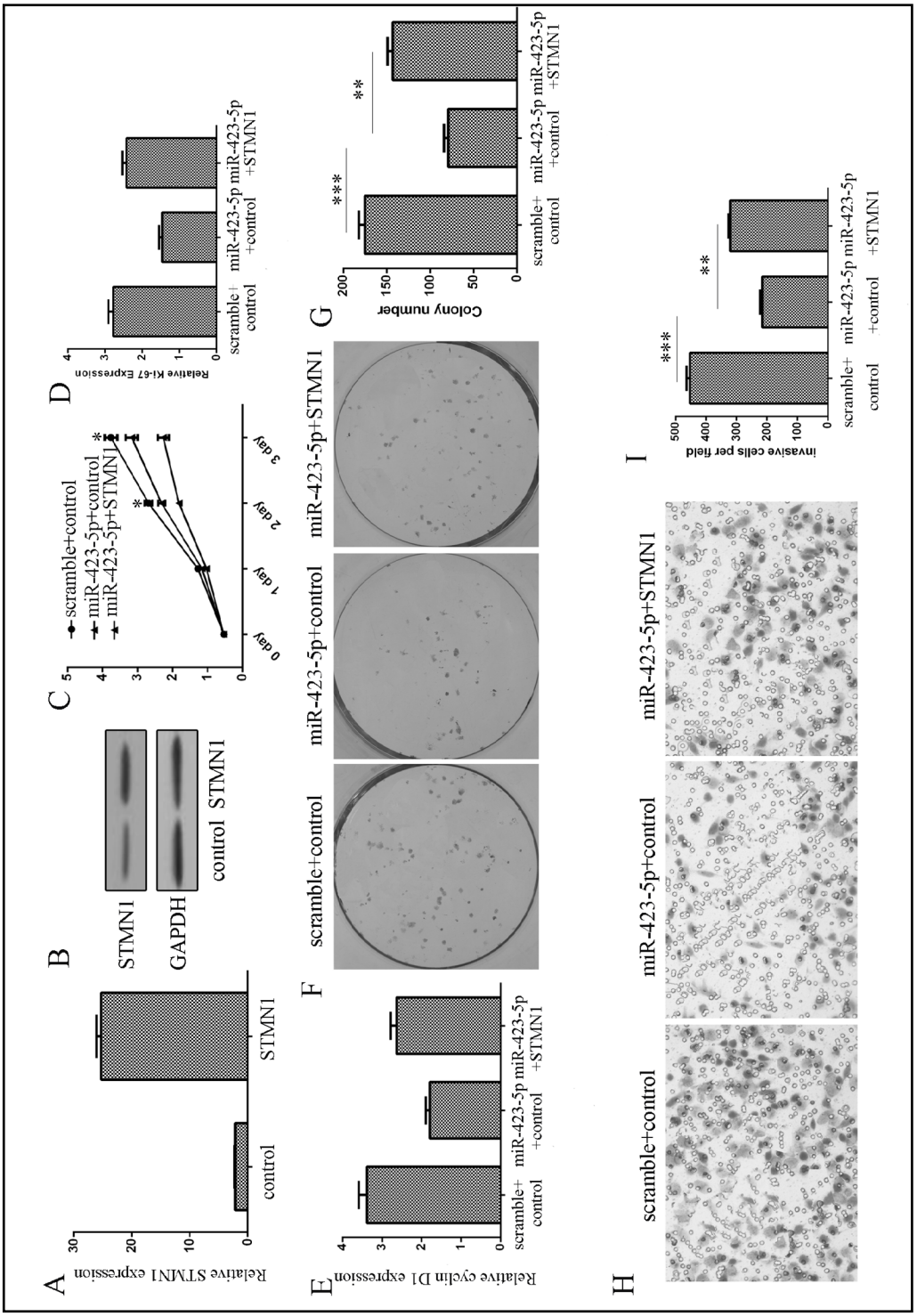

Fig. 5. STMN1 was involved in miR-423-5p-mediated cell behavior. (A) The mRNA expression of STMN1 was determined by qRT-PCR. (B) The protein expression of STMN1 was determined by western blot. (C) The cell proliferation of different groups was determined by CCK-8 analysis. (D) The mRNA expression of ki-67 was measured by qRT-PCR. (E) The mRNA expression of cyclin D1 was measured by qRT-PCR. (F) Re-introduction of STMN1 significantly reversed the inhibition of cell colony formation in the miR-423-5p-expressing cells. (G) The relative colony numbers were shown. (H) Overexpression of STMN1 significantly enhanced the inhibition of cell invasion in the miR-423-5p-expressing cells. (I) The relative invasive cells were shown. *p<0.05, **p<0.01 and ***p<0.001.

Discussion

Although several miRNAs have been found to be deregulated in osteosarcoma cells and tissues, the exact mechanism by how miRNA regulated the tumorigenesis is still not fully elucidated [31-34]. Our study showed that miR-423-5p expression was decreased in osteosarcoma tissues and cell lines. The expression of STMN1 was upregulated in osteosarcoma tissues and cell lines. Moreover, STMN1 expression was negatively related with miR-423-5p expression in osteosarcoma tissues. We identified STMN1 as a direct target gene of miR-423-5p in osteosarcoma cell. Overexpression of miR-423-5p suppressed osteosarcoma cell proliferation, colony formation and invasion. Furthermore, we demonstrated that STMN1 was involved in miR-423-5p-mediated cell behavior such as cell proliferation, colony formation and invasion in osteosarcoma cell. Our research indicated that miR-423-5p acted as a tumor suppressor gene in osteosarcoma partly through inhibiting STMN1 expression.

Previous studies revealed that miR-423-5p acted significant roles in the development of tumors [35-37]. For example, Li et al. [38] showed that miR-423-5p expression was increased in gliomas tissues and ecoptic expression of miR-423-5p increased glioma cell angiogenesis, proliferation and invasion through targeting ING-4. Lu et al. [39] demonstrated that serum miR-423-5p was upregulated in the stage I-II colorectal cancer patients compared with the control. Liu et al. demonstrated that miR-423-5p increased cell proliferation and invasion through regulating trefoil factor 1 (TFF1) in gastric cancer cells [40]. However, the expression and effect of miR-423-5p are still unknown in osteosarcoma. Our study showed that miR-423-5p expression was decreased in osteosarcoma tissues. The miR-423-5p expression was downregulated in 29 cases (29/40; 72.5 %) of osteosarcoma tissues compared with the adjacent tissues. Furthermore, we indicated that the expression of miR-423-5p was upregulated in osteosarcoma cell lines (U2OS, SOSP-9607, MG-63 and SAOS-2) and one normal osteoblast cell line (hFOB). Moreover, overexpression of STMN1 suppressed the osteosarcoma cell proliferation, colony formation and invasion. Our results demonstrated that miR-423-5p acted as a tumor suppressor gene in osteosarcoma.

Next, we examined the molecular mechanism about the function of miR-423-5p and demonstrated that STMN1 was a target for miR-423-5p in the osteosarcoma cell. STMN1 is a 17-kDa cytoplasmic protein, which is also named as oncoprotein 18 (OP18) [41-43]. Previous research showed that STMN1 acted as an important regulator in cell cycle, proliferation, invasion and survival through regulating microtubule dynamics [44-46]. STMN1 was upregulated in several human tumors such as lung cancer, gastric cancer, oral squamous-cell carcinoma, colorectal tumor, gallbladder cancer and breast cancer [47-51]. Our data identified STMN1 as a direct target gene of miR-423-5p in osteosarcoma cell. TargetScan system showed that there was a conserved domain within the 3'-UTR of STMN1 with a potential miR-423-5p binding site. Luciferase reporter assay demonstrated that ectopic expression of miR-423-5p suppressed the luciferase activity of WT reporter, but not the Mut reporter. Overexpression of miR-423-5p suppressed the expression of STMN1 in the MG-63 cell. Furthermore, we demonstrated thatSTMN1 expression was increased in osteosarcoma tissues and cell lines. STMN1 expression was negatively corelated with miR-423-5p expression in osteosarcoma tissues. In addition, STMN1 was demonstrated to be involved in miR-423-5p-mediated cell behavior such as cell proliferation, colony formation and invasion in the osteosarcoma cell.

Conclusion

We found that miR-423-5p expression was downregulated in osteosarcoma tissues and cell lines. Overexpression of miR-423-5p inhibited osteosarcoma cell proliferation, colony formation and invasion through suppressing STMN1 expression. Our data suggested that miR-423-5p acted as a tumor suppressor gene in osteosarcoma partly through inhibiting the expression of STMN1.

Disclosure Statement

The authors declare no conflicts of interest.

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