Original Paper

MiR-129-5p Inhibits Proliferation and Invasion of Chondrosarcoma Cells by Regulating SOX4/Wnt/ β -Catenin Signaling Pathway

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Key Words

Chondrosarcoma • miR-129-5p • SOX4 • Wnt/ β -catenin signaling pathway

Abstract

Background/Aims: Recently, microRNAs (miRNAs) have been identified as novel regulators in Chondrosarcoma (CHS). This study was aimed to identify the roles of miR-129-5p in regulation of SOX4 and Wnt/ β -catenin signaling pathway, as well as cell proliferation and apoptosis in chondrosarcomas. **Materials and Methods:** Tissue samples were obtained from chondrosarcoma patients. Immunohistochemistry, real-time quantitative RT-PCR (RT-qPCR) and western blot analysis were performed to detect the expressions of miR-129-5p and SOX4. Luciferase assay was conducted to confirm that miR-129-5p directly targeted SOX4 mRNA. Manipulations of miR-129-5p and SOX4 expression were achieved through cell transfection. Cell proliferation, migration and apoptosis were evaluated by CCK-8 assay, colony forming assay, wound healing assay and flow cytometry *in vitro*. For *in vivo* experiment, the tumor xenograft model was established to evaluate the effects of miR-129-5p and SOX4 on chondrosarcoma. **Results:** The expression of miR-129-5p was significantly down-regulated in chondrosarcoma tissues as well as cells in comparison with normal ones, while SOX4 was over-activated. Further studies suggested that miR-129-5p suppressed cell proliferation, migration and promoted apoptosis by inhibiting SOX4 and Wnt/ β -catenin pathway. **Conclusion:** MiR-129-5p inhibits the Wnt/ β -catenin signaling pathway by targeting SOX4 and further suppresses cell proliferation, migration and promotes apoptosis in chondrosarcomas.

Introduction

Chondrosarcoma (CHS), a malignant tumor with pure hyaline cartilage differentiation, is the second most frequent primary bone malignancy after osteosarcoma [1, 2]. It is a rare disease that accounts for approximately 3 new cases per 1000,000 population annually and

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mainly occurs in adults [2]. Chondrosarcomas are notoriously resistant to conventional chemotherapy and radiotherapy for lack of vascularity in cartilage which definitely hinders drug delivery and thus, chondrosarcomas are usually settled by surgery [3]. Unfortunately, a majority of patients recur with higher-grade chondrosarcomas which are often lethal to them (10-year survival rate of 29%) [3, 4]. Therefore, alternative strategies are urgently needed in chondrosarcoma diagnosis and therapeutics.

MicroRNAs (miRNAs) are a series of short (approximately consisting of 19-25 nucleotides) non-coding RNA molecules that have vital regulatory functions in modulating target gene expression through translational repression or mRNA cleavage [5, 6]. MiRNAs are often aberrantly expressed and serve as tumor suppressors or oncogenes in carcinomas including chondrosarcomas [1, 7]. Previous studies have revealed that miR-129 plays a tumor suppressor in many cancers, such as breast cancer, lung cancer, prostate cancer, hepatocellular cancer and osteosarcoma [8-13], but few have focused on its corresponding functions and mechanisms in chondrosarcomas.

SOX4 is a member of the SOX (SRY-related HMG-box) family of transcription factors which involves in various physiological processes such as embryonic development [14, 15]. Recently, SOX4 has attracted more attention since it is related with various malignant cancers. Previous studies showed that SOX4 expression was up-regulated in many types of human cancers and over-expression of SOX4 contributed to an increased risk of death in some cancer patients [15]. On the other hand, genome-wide protein expression analysis indicated that SOX4 was associated with a number of transcription factors, including E2F1, E2F4 and c-MYC [1]. Moreover, it was demonstrated that SOX4 over-expression was a malignant prognostic factor for patients with low-grade chondrosarcomas, and that SOX4 functioned as an oncogene in chondrosarcoma cells [1], which provided evidences in potential correlation between SOX4 and chondrosarcoma development.

The Wnt/ β -catenin pathway requires Wnt ligand binding to Frizzled receptors as well as LRP5/6 co-receptors to initiate intracellular signaling via β -catenin nuclear translocation. Wnt/ β -catenin axis affects biological events by targeting series of genes such as C-myc and Cyclin D1 which are known related to cell cycle regulation [16, 17]. Deregulation of the canonical Wnt/ β -catenin pathway is commonly observed in human cancers, and lots of evidences implied that the aberrant activation of the Wnt/ β -catenin signaling pathway may be associated with cancer development including chondrosarcoma [16, 18-21]. In lung cancer, the Wnt signaling components were aberrantly expressed [22] and the over-expression of Wnt1 increased the aggressiveness of non-small cell lung cancers [23]. Jiang *et al.* [24] demonstrated that activated Wnt/ β -catenin signaling pathway could stimulate prostate cancer growth in bone and soft tissues. Chen *et al.* [25] also found that similar aberrant activation of the Wnt/ β -catenin signaling pathway occurred in chondrosarcoma cells and promoted cell proliferation.

There have been some reports about the interaction between two of miR-129, SOX4 and Wnt/ β -catenin signaling pathway in cancers. Kang *et al.* suggested that the deregulation of miR-129 resulted in aberrant SOX4 expression in esophageal cancer [26]. In addition, Wang *et al.* dug out evidences indicating that up-regulation of SOX4 was inversely correlated with the epigenetic silencing function of miR-129-2 in gastric cancer, and the restoration of miR-129-2 down-regulated SOX4 expression [27]. Furthermore, it has been reported that aberrant activation of the Wnt signaling pathway drove the proliferation of chondrosarcoma cells [15], and SOX4 amplified the Wnt/ β -catenin signaling pathway's effect on proliferation of melanoma cells [28, 29]. However, none has investigated the pathogenesis by taking miR-129-5p, SOX4 and Wnt/ β -catenin signaling into consideration simultaneously.

In the current study, we have systematically validated the relationship among the Wnt/ β -catenin signaling pathway, SOX4 and miR-129-5p in chondrosarcomas and verified the molecular mechanism of miR-129-5p on suppressing proliferation, migration and promoting apoptosis of chondrosarcoma cells.

Materials and Methods

Clinical samples

The chondrosarcoma biopsy specimens were obtained from 92 patients (47 males and 45 females) in Huaihe Hospital of Henan University between Mar 2014 and Oct 2015. Samples were pathologically confirmed and collected during surgical operation before conducting radiotherapy or chemotherapy. All tissue samples were under consent from patients and approved for study by the Ethics Committee of Huaihe Hospital of Henan University.

Immunohistochemistry

Immunohistochemical analysis of SOX4 was performed on tissue samples with the EnVision two-step method as previously described [30]. Conventional dewaxing and antigen microwave thermal retrieval was performed on four-micron-thick serial sections of formalin-fixed and paraffin-embedded tissues. The sections were incubated in a 3% hydrogen peroxide solution to block endogenous peroxidase activity. Primary antibodies (Rabbit anti-human SOX4 monoclonal antibody, 1:400; ab8026, Abcam, Cambridge, MA, USA) were applied to the sections at a refrigerated temperature of 4°C overnight. The sections were incubated for 20 minutes at room temperature. Polymerase secondary antibody was then added and incubated for another 30 minutes at room temperature. Subsequently, secondary antibodies (Bioss, Beijing, China) were applied. The reaction product was visualized by incubation with the substrate/chromogen 3, 3'-diaminobenzidine (DAB, D8001, Sigma-Aldrich, St. Louis, MO, USA). Finally, the sections were counterstained with 0.02% Hematoxylin (H3136, Sigma-Aldrich, St. Louis, MO, USA).

Cells lines

The human chondrosarcoma cell line JJ012 was kindly provided by Dr. Sean P. Scully (University of Miami School of Medicine, Miami, FL), while human chondrosarcoma cell line, SW1353, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). In addition, the normal chondrocyte cell line CHON-002, coming from bone tissues, was purchased from Shanghai Jimian Industrial Co., Ltd. JJ012, HS18.90, SW1353 and CHON-002 cells were authenticated with the PowerPlex® 18D kit (Promega, Madison WI) for short tandem repeat (STR) DNA analysis at their arrival in the laboratory and periodically thereafter. Then cell lines were screened for the contaminations of mycoplasma routinely as previously described [31]. After confirmed mycoplasma-free, cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) under a humidified atmosphere of 5% CO₂ at 37°C.

Transfection

MiR-129-5p mimic, SOX4 siRNA1, SOX4 siRNA2 and SOX4 siRNA3 were obtained from RiboBio Co., Ltd. (Guangzhou, China). pCDNA3.1 vector (Thermo Fisher Scientific, Waltham, MA) was employed to generate miR-129-5p mimic, SOX4 siRNA1 or SOX4 siRNA2 expression plasmid, and the empty pCDNA3.1 vector was as a negative control. Transfection to SW1353 and JJ012 cells was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol.

CCK-8 assay

Cell viability was evaluated with the Cell Counting Kit-8 (CCK-8; Dojindo, Japan). Cells were seeded in 96-well-plates at a density of 3×10^3 per well and incubated over different time (24 hrs, 48 hrs, and 72 hrs). The absorbance value of each well at 450 nm was measured using a microplate reader (BioTek, Biotek Winooski, Vermont, USA). All experiments were repeated in triplicate.

Colony formation assay

Cells were seeded in 6-well plates at a density of 5×10^2 cells/well and incubated for two weeks. After crystal violet staining, colonies were counted under a microscope (Olympus Corporation, Tokyo, Japan).

Wound healing assay

Cells were seeded at a density of 2×10^5 cells/well in 6-well plates. When the cells reached 80% confluence, a wound gap was scratched vertically in the middle of each well with a pipette tip. After being

washed with PBS three times, cells were incubated in growth medium. The wound widths were photographed and measured under a microscope after 0 hr and 24 hrs.

Transwell assay

Cells in five different groups were seeded into the upper chambers (8 μ m pore size; Corning Incorporated, Corning, NY, USA) at the density of 1×10^4 cells/well with serum-free medium, and medium with 20% FBS was added into the lower chamber. Before the inoculation of the cancer cells, the upper chambers were additionally coated with Matrigel basement membrane matrix (354234, BD Bioscience, San Jose, CA, USA) in the invasion assay. After incubation for 24 hours at 37 °C in 5% CO₂, the cells were fixed by 4.0% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min. The swabs were used to remove the cells on the upper side of filters, followed with washes with PBS for three times. Then the number of cells was counted in five randomly selected fields per well under a microscope.

Flow cytometry

For apoptosis assays, staining with FITC-Annexin V and propidium iodide (PI) was performed using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). The apoptosis rates were analyzed by flow cytometry. The Annexin V- and PI+ represented necrotic cell, the Annexin V+ and PI+ represented late apoptotic cell, the Annexin V+ and PI- represented early apoptotic cell, and the Annexin V- and PI- represented normal cells.

Luciferase reporter assay

The SOX4 3' UTR segments containing the miR-129-5p binding sites were amplified by RT-PCR and the mutant segments were also designed. The couple of oligonucleotides were then inserted into pmirGLO, Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI), and subsequently sequenced to prevent any mutant. HEK293 cells purchased from ATCC were seeded in 24-well plates and co-transfected with miR-129-5p mimics or empty pcDNA3.1 vector and recombinant pmirGLO. The relative Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) 48 h after co-transfection.

Tumor formation in nude mice

Sixty SCID nude mice (Laboratory Animal Center of Henan University) at an average age of 4 weeks were obtained to build tumor growth models. The mice were randomly separated into 10 groups. Mice of three groups were each subcutaneously injected with 1×10^6 SW1353 cells or JJ012 cells transfected with miR-129-5p, SOX4 siRNA or SOX4 siRNA+miR-129-5p or miR-129-5p+SOX4 (co-transfected with miR-129-5p and SOX4) respectively and other seven groups were injected with an equivalent number of cells expressing empty vector. The tumor was measured every three days with a caliper, and the volume size was calculated based on the formula: $V (\text{mm}^3) = (L \times W^2)/2$ (L and W represent tumor length and width, respectively). Mice were sacrificed at the end of the observation period (24 days after the injection) and tumors were taken out for further studies.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA extraction from tissues and cells was conducted with the Trizol reagent kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The corresponding cDNA was obtained using the reverse transcription kit (Thermo Fisher Scientific, Waltham, MA). Real-time quantitative PCR assay was performed to detect the relative expression levels of miR-129-5p and SOX4 with U6 as an endogenous control for miRNA and GAPDH for mRNA (Primers were listed in Table 1). The relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method and experiments were repeated in triplicate.

Table 1. Primer sequences involved in RT-PCR.
GAPDH: phosphoglycerate dehydrogenase,
RT-PCR: real time-polymerase chain reaction

Gene		Primer sequence
MiR-129-5p	Sense	5'-GGGGGCTTTTTCGGTCTGG-3'
	Antisense	5'-AGTGCCTGTCGTGGAGTC-3'
SOX4	Sense	5'-GACCTGCTCGACCTGAACC-3'
	Antisense	5'-CCGGGCTCGAAGTTAAATCC-3'
U6	Sense	5'-CTCGCTTCGGCAGCAC-3'
	Antisense	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	Sense	5'-GTCAGCCGATCTTCTTTTG-3'
	Antisense	5'-GCGCCAATACGACCAATC-3'

Western blot

Cells were lysed with M-PER Protein Extraction Reagent (Pierce, Rockford, IL) and supplemented with protease inhibitors. Nucleoproteins were extracted using the CellLytic™ NuCLEAR™ extraction Kit (NXTRACT, Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined using a BCA protein assay kit (QPBCA, Sigma-Aldrich, St. Louis, MO, USA). Equal amount of protein was separated using SDS-polyacrylamide gel electrophoresis, transferred to equilibrated polyvinylidene difluoride membranes and blocked in 5% non-fat milk Tris-buffered saline (pH 7.4) containing 0.1% Tris buffered saline with Tween (TBST) for 2 hrs. Then the membranes were incubated over night at 4 °C with primary antibodies purchased from Sigma-Aldrich (St. Louis, MO, USA) against SOX4 (SAB2108306), β -catenin (C2206), Cyclin D1 (SAB4503499), C-myc (ROAMYC), nuclear β -catenin (C8249), histone H3 (H9289), β -actin (Mab1987), Mcl-1 (M8434) and Bcl-2 (S3335), while anti-cleaved caspase-3 from Abcam (ab32351, Cambridge, MA, USA). Next, membranes were incubated with secondary antibody (Pierce, Rockford, IL) for 1 h at room temperature. Proteins were visualized with the ECL Plus Western Blotting Detection Reagent (Amersham Biosciences, USA) and detected with enhanced chemiluminescence.

Statistical Method

SPSS 19.0 (Chicago, Illinois, USA) and GraphPad Prism (Version 6.0, San Diego, CA, USA) were used for data analysis. The measurement data was expressed as mean $\pm$ standard deviation (SD). The data were analyzed with Student's *t* test when only two groups were compared. A one-way analysis of variance (ANOVA) together with Fisher's least significant difference (LSD) test was conducted to evaluate the differences among the multiple groups. $P < 0.05$ was considered statistically significant.

Results

The up-regulation of SOX4 and down-regulation of miR-129-5p in chondrosarcoma tissues and cells

The immunohistochemistry results indicated that tumor tissues with higher grades showed higher SOX4 expression (Fig. 1A). miR-129-5p exhibited a significant lower expression level in both tumor tissues ($P < 0.05$, Fig. 1B) and human chondrosarcoma cell lines (JJ012 and SW1353) ($P < 0.05$, Fig. 1D) according to qRT-PCR analysis. On the contrary, qRT-PCR confirmed higher expression of SOX4 at mRNA level in tumor tissues ($P < 0.05$, Fig. 1C) as well as two different human chondrosarcoma cell lines ($P < 0.05$, Fig. 1E). In addition, the expression of miR-129-5p was negative correlated with SOX4 at tissue level and cell level (for all online suppl. material, see www.karger.com/doi/10.1159/000477323, Fig. S1). The protein expression of SOX4 is significant higher in tumor tissues, SW1353 cells and JJ012 cells compared to normal samples ($P < 0.05$, Fig. 1F-G). Moreover, the expression of SOX4 was measured at both mRNA and protein level in normal chondrocytes CHON-002, in which miR-129-5p was introduced in the pilot experiment. However, no remarkable differences were observed, and the corresponding data were not shown.

miR-129-5p directly targeted SOX4

TargetScan and miRBase suggested that 3'UTR of SOX4 contained the miR-129-5p seed sequence (Fig. 2A). To determine whether miR-129-5p could actually target SOX4, dual-luciferase analysis was conducted after HEK293 cells were co-transfected with the SOX4-wt (wild-type 3'UTR) or SOX4-mut (with mutant sites) reporter plasmids and miR-129-5p or miR-NC respectively. The results showed that miR-129-5p down-regulated the luciferase activity of SOX4-wt plasmid ($P < 0.05$), but had no effects on that of SOX4-mut plasmid ($P > 0.05$, Fig. 2B). The results manifested that SOX4 was one of miR-129-5p targets. Furthermore, the expression level of SOX4 in chondrosarcoma cells (SW1353 cells and JJ012 cells) with miR-129-5p over-expressed was evaluated using Western blot. As shown in Fig. 2C-D, miR-129-5p down-regulated the protein level of SOX4 in both SW1353 cells and JJ012 cells.

MiR-129-5p inhibited the Wnt/ β -catenin signaling pathway by targeting SOX4

The Wnt/ β -catenin-related proteins in SW1353 cells and JJ012 cells were investigated with miR-129-5p over-expressed or SOX4 suppressed. As shown in Fig. 3A and see

Fig. 1. The up-regulation of SOX4 and down-regulation of miR-129-5p in chondrosarcoma tissues and cells. (A) Immunohistochemical staining with SOX4 in tumor and adjacent normal tissues. (B) RT-PCR analysis of miR-129-5p in tissue samples. (C) RT-PCR analysis of SOX4 mRNA in tissue samples. (D) RT-PCR analysis of miR-129-5p in normal chondrocyte cell line CHON-002 (CHON), and two different human chondrosarcoma cell lines (JJ012 and SW1353). (E) RT-PCR analysis of SOX4 mRNA in CHON, JJ012 and SW1353 cell lines. (F) Western blot and the relative intensity of SOX4 protein in tissue samples. (G) Western blot and the relative intensity of SOX4 protein in cell samples. Each tissue sample was measured once, while cell samples with three parallels were tested for three times. The Student's *t*-test was used to analyze the difference between Normal and Tumor groups, while One-way analysis of variance (ANOVA) followed with Fisher's least significant difference (LSD) test was applied for comparison among CHON, SW1353 and JJ012 cells. * $P < 0.05$.

Fig. 2. MiR-129-5p directly targets SOX4. (A) Fragments containing the predicted miR-129-5p binding site in the 3'UTR of SOX4. (B) Luciferase reporter of SOX4 was constructed. Then SW1353 cells were co-transfected with either miR-129-5p mimics or negative control and the recombinant pmirGLO Vector containing SOX4-wt (wild-type 3'UTR) or SOX4-mut (with mutant site). The relative luciferase activity was measured 48 h after. (C-D) Western Blot was performed to detect SOX4 protein levels in cells with miR-129-5p over-expressed.

in SW1353 and JJ012 cells. Each group has three parallels, and each sample was measured thrice. The student's *t*-test was used to analyze the difference between miR-129-5p and NC groups, * $P < 0.05$.

supplementary material, Fig. S2, cells in the miR-129-5p group displayed significant lower expression of β -catenin in the nucleus than that in the control group ($P < 0.05$). In addition, SOX4, β -catenin, Cyclin D1 and C-myc, extracted from both cell cytoplasm and nucleus, also represented decreased expression levels ($P < 0.05$). Similar results were obtained from the

Fig. 3. MiR-129-5p inhibited the Wnt/ β -catenin signaling pathway related proteins and the ability of proliferation and mobility of chondrosarcoma cells by targeting SOX4. **A:** Western blot analysis of SOX4, β -catenin, Cyclin D1 and C-myc in cell samples with β -actin as internal control, and nuclear β -catenin with histone H3 as internal control in both SW1353 and JJ012 cells. **(B)** Effects of miR-129-5p on SW1353 and JJ012 cell viability were estimated using CCK-8 assay. **(C)** Clonogenicity was evaluated by colony formation assay. **(D)** Wound closure of cells was evaluated using wound healing assay. **(E)** Migration of cells was evaluated by Transwell assay. **(F)** Invasion of cells was detected through Transwell assay. Each group has three parallels, and each sample was measured for three times. ANOVA was performed for the comparison among control, miR-129-5p, SOX4 siRNA1, SOX4 siRNA2 and miR-129-5p+SOX4 groups, followed with LSD test for pairwise comparisons. The p-value of ANOVA test was less than 0.05. * $P < 0.05$ versus control group. # $P < 0.05$ versus miR-129-5p+SOX4 group in LSD test.

groups with SOX4 inhibited cells (SOX4 siRNA1 group and SOX4 siRNA2 group) ($P < 0.05$). In addition, SOX4 overexpression antagonized the effects of miR-129-5p on the expressions of SOX4, β -catenin, Cyclin D1 and C-myc in whole cells and β -catenin in cell nucleus ($P < 0.05$) in both SW1353 cells and JJ012 cells.

MiR-129-5p suppressed proliferation, migration and promoted apoptosis of chondrosarcoma cells

CCK-8 proliferation assay and colony formation assay were carried out in SW1353 cells and JJ012 cells to investigate cell viability and proliferation. As suggested by Fig. 3 B-C and see

Fig. 4. MiR-129-5p promotes apoptosis of chondrosarcoma cells. (A) Cell apoptosis rate was evaluated using flow cytometry. (B) Western blot was performed to detect the expressions of apoptosis-related proteins. Each group has three parallels, and each well was measured thrice.

supplementary material, Fig. S3A). SW1353 and JJ012 cells transfected with exogenous miR-129-5p or SOX4 siRNA (SOX4 siRNA1 and SOX4 siRNA2) had lower proliferation rate compared to the control group ($P < 0.05$), while over-expression of SOX4 antagonized the inhibitory effect of miR-129-5p on cell proliferation. As Fig. 3D-E and (see supplementary material) Fig. S3B-C show, the ability of cell migration was suppressed in cells transfected with miR-129-5p or SOX4 siRNA (SOX4 siRNA1 and SOX4 siRNA2) ($P < 0.05$). Moreover, the results of invasion assay indicated that the invasion ability of SW1353 cells and JJ012 cells was inhibited by miR-129-5p and SOX4 siRNA ($P < 0.05$, Fig. 4F, and see supplementary material, Fig. S3D).

Apoptosis was subsequently detected via AnnexinV-PI double staining flow analysis and the results were shown in Fig. 4A and see supplementary material, Fig. S4A. We found that the transfected of miR-129-5p or SOX4 siRNA (SOX4 siRNA1 and SOX4 siRNA2) increased the proportion of apoptotic cells, whereas up-regulation of SOX4 antagonized the effect of miR-129-5p ($P < 0.05$). In addition, we detected the expressions of apoptosis-related proteins including Mcl-1, Bcl-2 and caspase-3. Compared with control group and miR-129-5p+SOX4 group, miR-129-5p group and SOX4 siRNA (SOX4 siRNA1 and SOX4 siRNA2) group had significantly lower expressions of Mcl-1, Bcl-2, and higher expression of cleaved caspase-3 ($P < 0.05$, Fig. 4B, see supplementary material, Fig. S4B-D). Thus, all these evidences manifested that miR-129-5p suppressed proliferation, migration and promoted apoptosis of SW1353 cells and JJ012 cells by targeting SOX4.

MiR-129-5p suppressed formation of chondrosarcoma xenografts by targeting SOX4

Given that miR-129-5p acted as a chondrosarcoma suppressor *in vitro*, we further assessed its effect *in vivo*. As shown in Fig. 5A, tumors induced with miR-129-5p over-expressed cells or SOX4 blocked cells grew at a much slower rate. Significant reductions in tumor size were observed at the termination of experiment ($P < 0.05$). Moreover, in tumor tissues, miR-129-5p was significantly over-expressed in both miR-129-5p group and miR-

Fig. 5. MiR-129-5p suppressed formations of chondrosarcoma xenografts by targeting SOX4 *in vivo*. (A) The tumor was measured in six nude mice in each group, and each measurement was repeated for three times every three days. (B) The expression of miR-129-5p in tumor tissues of SCID nude mice was determined 24 days after chondrosarcoma cells (SW1353 and JJ012 cells) injection. (C) The expression of SOX4 in tumor tissues was detected 24 days after chondrosarcoma cells injection. ANOVA was performed for the comparison among control, miR-129-5p, SOX4 siRNA1, SOX4 siRNA2 and miR-129-5p+SOX4 groups, followed with LSD test for pairwise comparisons. The p-value of ANOVA test was less than 0.05. * $P < 0.05$ verse control group, # $P < 0.05$ verse miR-129-5p+SOX4 group in LSD test.

129-5p+SOX4 group ($P < 0.05$), while the expression of SOX4 was obviously inhibited in miR-129-5p group, SOX4 siRNA1 group and SOX4 siRNA2 group ($P < 0.05$, Fig. 5B-C). Therefore, miR-129-5p suppressed formation of chondrosarcoma xenografts by targeting SOX4 and these results supported our *in vitro* findings.

Discussion

Chondrosarcoma is a lethal disease with poor survival rates particularly when it progresses to higher grade chondrosarcoma. However, it is a pity that the conventional chemotherapy is ineffective for it due to the avascular nature of cartilages [2, 3, 32]. Therefore, it is essential to focus on the tumorigenesis mechanisms of chondrosarcomas in order to discover the way to manage this disease effectively. Recently, an increasing number of studies have indicated that miRNAs were often aberrantly expressed in cancer cells and served as tumor suppressors or oncogenes in carcinogenesis [1, 7]. Hence, we have every reason to believe that identification of chondrosarcoma related miRNAs as well as their downstream targets, their biological functions and the molecular mechanism would contribute to chondrosarcoma treatments.

In this study, we sought to investigate the role of miR-129-5p on chondrosarcomas and the possible molecular mechanism. We had performed *in-vitro* and *in-vivo* experiments to evaluate the effects of miR-129-5p on chondrosarcoma cell proliferation and migration by altering its expression level via cell transfection. In addition, SOX4 is predicted as one of the targets of miR-129-5p by both miRBase (<http://www.mirbase.org/>) and Targetscan 7.1

(http://www.targetscan.org/vert_71/). And our results of luciferase reporter assay were consistent with the prediction. Furthermore, we have identified that miR-129-5p regulates Wnt/ β -catenin signaling pathway by targeting SOX4.

On the basis of our study, miR-129-5p showed decreased expression levels in chondrosarcoma tumor, which was in accordance with previous studies performed in other cancers [33-35]. Thus, it was surmisable that miR-129-5p might serve as a tumor suppressor. Na et al. reported that the invasion and migration of laryngeal cancer cells were inhibited when the expression of miR-129 was up-regulated [36]. Likewise, Xiao et al. suggested that miR-129 exerted suppressive effects on cell proliferation, migration and invasion in lung cancer [10]. Similarly, our results also indicated that miR-129-5p repressed the development of chondrosarcoma. Up-regulation of miR-129-5p attenuated cell proliferation and migration while stimulated apoptosis in chondrosarcomas.

On the other hand, we also studied the biological functions of SOX4 in chondrosarcoma cells. SOX4 was previously considered to be an oncogene according to many other researches and was confirmed with our findings in chondrosarcomas. We determined that SOX4 was up-regulated in chondrosarcomas cells, which was in agreement with the results of Li et al. [1]. Next, we blocked the expression of SOX4 with SOX4 siRNA1 and SOX4 siRNA2 and observed similar inhibitory effects on cell viability and mobility as overexpression of miR-129-5p. For this reason, we subsequently focused on the specific relationship between miR-129-5p and SOX4 as well as the potential downstream signaling pathways.

Our further studies indicated that SOX4 and Wnt/ β -catenin were involved in the molecular mechanism of miR-129-5p's tumor suppressive effect. There have been some studies investigating the regulatory relationship among miR-129-5p, SOX4 and Wnt/ β -catenin pathway. For example, Li et al. reported that overexpressing miR-129 results in a decreased expression of the tumor suppressor APC, thereby activating the Wnt signaling pathway in laryngeal squamous cell carcinoma (LSCC) [37]. Saegusa et al. demonstrated that SOX4 positively regulated β -catenin through TCF and resulted in promotion of cell proliferation of endometrial carcinomas [38]. In our study, we have indicated that SOX4 could form transcriptional complexes with β -catenin and promoted β -catenin mediated transcription of the *Slug* gene in uterine carcinosarcoma [39]. Different with previous studies, we designed and conducted experiments by considering miR-129-5p, SOX4 and the Wnt/ β -catenin pathway altogether. We verified the regulator relationships between miR-129-5p and SOX4 at first and our results suggested that miR-129-5p directly targeted SOX4. Subsequently, we manipulated miR-129-5p or SOX4 expression through cell transfection and observed abnormal expression of β -catenin as well as the downstream targets of the Wnt/ β -catenin pathway (CyclinD1 and C-myc) in chondrosarcoma cells. With all these evidences, we determined that miR-129-5p regulated Wnt/ β -catenin pathway by targeting SOX4.

In conclusion, our study demonstrates that miR-129-5p can inhibit the over-activation of the Wnt/ β -catenin signaling pathway via targeting SOX4 and further regulate proliferation, migration of chondrosarcoma cells.

Disclosure Statement

The authors declare no conflict of interest.

References

- 1 Lu N, Lin T, Wang L, Qi M, Liu Z, Dong H, Zhang X, Zhai C, Wang Y, Liu L, Xiang L, Qi L, Han B, Li J: Association of SOX4 regulated by tumor suppressor miR-30a with poor prognosis in low-grade chondrosarcoma. *Tumour Biol* 2015;36:3843-3852.
- 2 van Oosterwijk JG, Anninga JK, Gelderblom H, Cleton-Jansen AM, Bovee JV: Update on targets and novel treatment options for high-grade osteosarcoma and chondrosarcoma. *Hematol Oncol Clin North Am* 2013;27:1021-1048.

- 3 Gelderblom H, Hogendoorn PC, Dijkstra SD, van Rijswijk CS, Krol AD, Taminiau AH, Bovee JV: The clinical approach towards chondrosarcoma. *Oncologist* 2008;13:320-329.
- 4 Bovee JV, Hogendoorn PC, Wunder JS, Alman BA: Cartilage tumours and bone development: molecular pathology and possible therapeutic targets. *Nat Rev Cancer* 2010;10:481-488.
- 5 Tan TW, Chou YE, Yang WH, Hsu CJ, Fong YC, Tang CH: Naringin suppress chondrosarcoma migration through inhibition vascular adhesion molecule-1 expression by modulating miR-126. *Int Immunopharmacol* 2014;22:107-114.
- 6 Hammond SM: An overview of microRNAs. *Adv Drug Deliv Rev* 2015;87:3-14.
- 7 Dyrskjot L, Ostenfeld MS, Bramsen JB, Silahatoglu AN, Lamy P, Ramanathan R, Fristrup N, Jensen JL, Andersen CL, Zieger K, Kauppinen S, Ulhoi BP, Kjems J, Borre M, Orntoft TF: Genomic profiling of microRNAs in bladder cancer: miR-129 is associated with poor outcome and promotes cell death *in vitro*. *Cancer Res* 2009;69:4851-4860.
- 8 Fesler A, Zhai H, Ju J: miR-129 as a novel therapeutic target and biomarker in gastrointestinal cancer. *Targets Ther* 2014;7:1481-1485.
- 9 Tang X, Tang J, Liu X, Zeng L, Cheng C: Downregulation of miR-129-2 by promoter hypomethylation regulates breast cancer cell proliferation and apoptosis. *Oncol Rep* 2016;33:2969-2976.
- 10 Xiao Y, Li X, Wang H, Wen R, He J, Tang J: Epigenetic regulation of miR-129-2 and its effects on the proliferation and invasion in lung cancer cells. *J Cell Mol Med* 2015;19:1000-1009.
- 11 Bijnsdorp IV, Hodzic J, Lagerweij T, Westerman B, Krijgsman O, Broeke J, van der Wal AC, Nilsson RJA, Rozendaal L, Beusechem VVW: miR-129-3p controls centrosome number in metastatic prostate cancer cells by repressing CP110. *Oncotarget* 2016;7:16676-16687.
- 12 Zhai J, Qu S, Li X, Zhong J, Chen X, Qu Z, Wu D: miR-129 suppresses tumor cell growth and invasion by targeting PAK5 in hepatocellular carcinoma. *Biochem Biophys Res Commun* 2015;464:161-167.
- 13 Long XH, Zhou YF, Peng AF, Zhang ZH, Chen XY, Chen WZ, Li JM, Liang SH, Liu ZL: Demethylation-mediated miR-129-5p up-regulation inhibits malignant phenotype of osteogenic osteosarcoma by targeting Homo sapiens valosin-containing protein (VCL). *Tumour Biol* 2015;36:3799-3806.
- 14 Bao ZQ, Zhang CC, Xiao YZ, Zhou JS, Tao YS, Chen YC: Overexpression of Sox4 and beta-catenin is associated with a less favorable prognosis of osteosarcoma. *J Huazhong Univ Sci Technolog Med Sci* 2016;36:193-199.
- 15 Chen YF, Yang CC, Kao SY, Liu CJ, Lin SC, Chen YW: MicroRNA-211 Enhances the Oncogenicity of Carcinogen-Induced Oral Carcinoma by Repressing TCF12 and Increasing Antioxidant Activity. *Cancer Res* 2016;76:4872-4886.
- 16 Xue G, Romano E, Massi D, Cerrito M, D'Alagni M: Wnt/beta-catenin signaling in melanoma: Preclinical rationale and novel therapeutic implications. *Cancer Treat Rev* 2016;49:1-12.
- 17 Logan CY, Nusse R: The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004;20:781-810.
- 18 Le PN, McKeown-Metzger A: Targeting the Wnt pathway in human cancers: therapeutic targeting with a focus on Wnt2-54F28. *Pharmacol Ther* 2015;146:1-11.
- 19 Lewis P: Wnt signaling in cancer. *Cold Spring Harb Perspect Biol* 2012;4.
- 20 Martre Y, Lin YM, Kahn M: The Wnt signaling pathway in cancer. *Crit Rev Oncol Hematol* 2016;99:141-144.
- 21 Gao J, Li H, Lei G: Wnt/beta-catenin pathway in bone cancers. *Tumour Biol* 2014;35:9439-9445.
- 22 Yang J, Chen J, He J, Li J, Shi J, Cho WC, Liu X: Wnt signaling as potential therapeutic target in lung cancer. *Expert Opin Ther Targets* 2016;20:999-1015.
- 23 Nakashima N, Huang CL, Liu D, Ueno M, Yokomise H: Intratumoral Wnt1 expression affects survivin gene expression in non-small cell lung cancer. *Int J Oncol* 2010;37:687-694.
- 24 Jiang Y, Dai J, Zhang H, Sottnik JL, Keller JM, Escott KJ, Sanganee HJ, Yao Z, McCauley LK, Keller ET: Activation of the Wnt pathway through AR79, a GSK3beta inhibitor, promotes prostate cancer growth in soft tissue and bone. *Mol Cancer Res* 2013;11:1597-1610.
- 25 Chen C, Zhao M, Tian A, Zhang X, Yao Z, Ma X: Aberrant activation of Wnt/beta-catenin signaling drives proliferation of bone sarcoma cells. *Oncotarget* 2015;6:17570-17583.
- 26 Kang M, Li Y, Liu W, Wang R, Tang A, Hao H, Liu Z, Ou H: miR-129-2 suppresses proliferation and migration of esophageal carcinoma cells through downregulation of SOX4 expression. *Int J Mol Med* 2013;32:51-58.

- 27 Huang YW, Liu JC, Deatherage DE, Luo J, Mutch DG, Goodfellow PJ, Miller DS, Huang TH: Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 oncogene in endometrial cancer. *Cancer Res* 2009;69:9038-9046.
- 28 Bhattaram P, Penzo-Mendez A, Kato K, Bandyopadhyay K, Gadi A, Taketo MM, Lefebvre V: SOXC proteins amplify canonical WNT signaling to secure nonchondrocytic fates in skeletogenesis. *J Cell Biol* 2014;207:657-671.
- 29 Cai H, Ni A, Li W, Li J: Inhibition of melanoma cell proliferation by targeting Wnt/ β -catenin pathway through Sox4 RNA interference. *J Huazhong Univ Sci Technolog Med Sci* 2011;31:565-569.
- 30 Ou DL, Chen CL, Lin SB, Hsu CH, Lin LI: Chemokine receptor expression profiles in nasopharyngeal carcinoma and their association with metastasis and radiotherapy. *J Pathol* 2006;210:363-373.
- 31 Radons J, Falk W, Schubert TE: Interleukin-10 does not affect IL-1-induced interleukin-6 and metalloproteinase production in human chondrosarcoma cells, SW1353. *Int J Mol Med* 2006;17:1083-1088.
- 32 Soderstrom M, Ekfors TO, Bohling TO, Teppo LH, Vuorio EI, Aro HT: No improvement in the overall survival of 194 patients with chondrosarcoma in Finland in 1971-1990. *Acta Orthop Scand* 2007;74:144-150.
- 33 Zhao JJ, Yang J, Lin J, Yao N, Zhu Y, Zheng J, Xu J, Cheng JQ, Lin JY, Ma X: Identification of miRNAs associated with tumorigenesis of retinoblastoma by miRNA microarray analysis. *Chin J Cancer Res* 2013;25:13-20.
- 34 Shen R, Pan S, Qi S, Lin X, Cheng S: Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 in gastric cancer. *Biochem Biophys Res Commun* 2010;394:1047-1051.
- 35 Liu Y, Hei Y, Shu Q, Dong J, Gao Y, Fu H, Zheng X, Yang G: VCP/p97, down-regulated by microRNA-129-5p, could regulate the progression of hepatocellular carcinoma. *PLoS One* 2012;7:e33300.
- 36 Shen N, Huang X, Li J: Upregulation of miR-129-5p affects breast cancer cell proliferation, invasiveness, and migration by affecting STAT3 expression. *Tumour Biol* 2014;35:1789-1796.
- 37 Li M, Tian L, Wang L, Yao H, Zhang J, Lu J, Sun Y, Gao X, Xie H, Li J: Down-regulation of miR-129-5p inhibits growth and induces apoptosis in laryngeal squamous cell carcinoma by targeting APC. *Plos One* 2013;8:e77829-e77829.
- 38 Saegusa M, Hashimura M, Kuwata T: Sox4 functions as a positive regulator of β -catenin signaling through upregulation of TCF4 during morulation of endometrial carcinomas. *Lab Invest* 2012;92:511-521.
- 39 Inoue H, Takahashi H, Hashimura M, Kuwata T, Akiya M, Matsumoto T, Saegusa M: Cooperation of Sox4 with β -catenin/p300 complex in transcriptional regulation of the Slug gene during divergent sarcomatous differentiation in murine carcinosarcoma. *BMC Cancer* 2015;16:53.