Knockdown of YAP1 inhibits the proliferation of osteosarcoma cells in vitro and in vivo

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Abstract. Yes-associated protein 1 (YAP1) is a candidate oncogene that is involved in tumorigenesis and progression of many malignant tumors. Recently, many studies have revealed that YAP1 is highly expressed in human osteosarcoma. To investigate the role of YAP1 in osteosarcoma tumorigenesis, the expression of YAP1 in the osteosarcoma cell lines (MG-63 and HOS) was knocked down by small hairpin RNA (shRNA), and the cell proliferation and colony formation assay showed that knockdown of YAP1 significantly suppressed the cell proliferation and colony formation of osteosarcoma cells. Subsequently, cell cycle distribution was analyzed by flow cytometry, and the results showed an accumulation of YAP1-knockdown cells in the G0/G1 phase, suggesting that YAP1 knockdown results in the arrest of cell cycle progression. Additionally, the knockdown of YAP1 also inhibited the tumorsphere formation in vitro and the growth of xenograft tumors in vivo. Therefore, these data suggest that YAP1 knockdown inhibits the proliferation of osteosarcoma cells. However, the mechanism of action was unclear. Further investigation showed that in the YAP1-knockdown MG-63 and HOS cells, the level of cyclinD1 and c-myc expression, target genes of the Wnt signaling pathway and TOP-Flash reporter activity were all significantly decreased, which indicated that the inhibitory effect of YAP1 knockdown on osteosarcoma might be associated with the Wnt signaling pathway. Taken together, our results demonstrated that YAP1 is an important regulator of osteosarcoma tumorigenesis and knockdown of YAP1 would be a novel therapeutic strategy for osteosarcoma.

Introduction

Osteosarcoma is the most common malignant primary bone tumor that occurs in children and adolescents, and accounts for 20% of all bone tumors and approximately 5% of pediatric tumors overall (1). Osteosarcoma is characterized by a highly malignant and metastatic potential, and the leading cause of death of osteosarcoma patients is distant metastases (2). In recent years, although improvements in patient survival rates have been achieved through multimodal therapeutic approaches (3), the overall relapse-free survival rate over 5 years still remains at 65%-75% (4). Therefore, to the development of new effective therapeutic strategies for osteosarcoma is required.

In recent years, much research concerning osteosarcomagenesis has been carried out; however, the pathogenesis of osteosarcoma is not fully understood. There is increasing evidence that genetic alterations are responsible for the tumorigenesis, including anomalous activation of oncogenes and/or inactivation of tumor suppressors. Recently, yes-associated protein 1 (YAP1) has been suggested to be a candidate oncogene in multiple tumors, and it was found to be highly expressed in a variety of human cancers, such as liver, colon, prostate, ovarian, lung and breast cancers (5-7), suggesting that YAP1 plays an important role in tumorigenesis (8,9). YAP1, a 65-kDa proline-rich phosphoprotein, is the main downstream effector of the Hippo pathway and functions as a transcriptional coactivator which can bind transcription factor Sd to enhance the expression of several proliferation and anti-apoptosis-related genes (10), including cycE, diap1 and cyclinD1 and therefore regulates cell proliferation and apoptosis depending on the cellular context (11,12). YAP1 was originally identified by virtue of its binding to the Src family member non-receptor tyrosine kinase YES (13). A number of studies have shown that the upregulation of YAP1 promotes tumorigenesis in most but not all tumor types evaluated (14), and the overexpression of YAP1 is an independent poor prognostic factor in hepatocellular and urothelial carcinoma of the bladder (15,16). In addition, transgenic mice with liver-specific YAP1 overexpression were found to exhibit a marked increase in liver size and eventually developed tumors (17). However, to date, our understanding of the molecular mechanism of the tumor promotion by YAP1 is still quite limited.

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Apart from the Hippo signaling pathway, it was reported that YAP1 is also implicated in the Wnt signaling pathway (18), which plays a key role in the pathogenesis of colon and other cancers (19). Binding of Wnts to frizzled (Fz) receptor and low-density lipoprotein 5 or 6 (LRP5/6) co-receptors inactivates destruction complex composed of AXIN1, GSK3β and adenomatous polyposis coli (APC), leading to β-catenin accumulation and subsequent translocation into the nucleus. In the nucleus, β-catenin forms a complex with TCF/LEF transcription factors to drive the transcription of genes that contribute to cell proliferation (19,20). Increasing evidence indicates that Wnt signaling increases osteosarcoma cell proliferation and their development (21,22). Furthermore, it was reported that YAP1 and the transcription factor TBX5 form a complex with β-catenin and then phosphorylation of YAP1 leads to localization of this complex to the promoters of anti-apoptotic genes including BCL2L1 and BIRC5 (9). However, the role of YAP1 in osteosarcoma initiation and development still remains unclear. Therefore, we hypothesized that YAP1 is likely to play a key role in the progression of osteosarcoma.

In this study, for the first time, we investigated the contribution of YAP1 in osteosarcoma cell proliferation and tumor formation through shRNA-based technique. We found that knockdown of endogenous YAP1 inhibited the cell proliferation and tumor formation of osteosarcoma, and the underlying mechanism was associated with the inhibition of the Wnt signaling pathway. Our findings provide a potential target for osteosarcoma therapeutics.

Materials and methods

Cell lines and cell culture. Human osteosarcoma cell lines, MG-63 and HOS, were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, USA) at 37˚C in a humidified incubator with 95% air and 5% CO2.

Western blot analysis. The lysates from the cell lines were separated by SDS-PAGE and transferred onto PVDF membranes by wet transfer. After being blocked with 5% fat-free milk, the membranes were incubated with the primary antibody for YAP1 (1:500; Abcam, Cambridge, UK), β-actin (1:1000; Santa Cruz, CA, USA), cyclinD1 (1:500; Santa Cruz) and c-myc (1:500; Santa Cruz) overnight at 4˚C, and then rinsed thrice with Tris-buffered saline containing 0.05% Tween-20 (TBST), followed by secondary incubation with anti-rabbit or anti-mouse IgG (1:5000; Cell Signaling) linked with horse-radish peroxidase (HRP) for 1 h at room temperature. Blots were developed with enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) on X-ray film.

RNA interference. RNA interference was performed using short hairpin RNA (shRNA), and the shRNA expression vector containing the YAP1-specific sequence was generated by GeneChem Co., Ltd. (Shanghai, China). The targeting sequence of the shRNA was 5'-UUUAUAAGUAUUUCUCCC-3' and 5'-UUAGAAGUACUGACC-3'. A scrambled shRNA was used as a negative control. The shRNA expression vectors were transfected into MG-63 and HOS cells, respectively, using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocols. Transfected cells were treated with G418 (Invitrogen) for 2 weeks, and drug-resistant colonies were collected by trypsinization and used without cloning.

Cell proliferation assay. The anti-proliferative effect of YAP1 silencing on osteosarcoma cells was assessed through cell count and MTT assay, respectively. Briefly, MG-63 and HOS cells with YAP1 silencing were seeded in 6-well plates at 5x10^4 cells/well and incubated as described above. The cells were harvested and counted continuously for 7 day using a hemocytometer.

For the MTT assay, the cells were seeded in 96-well plates at 2x10^3 cells/well and incubated for 1, 3, 5 and 7 days. At each time point, MTT (Sigma-Aldrich) was added into each well. After 4 h of incubation, the resulting formazan was then dissolved in 100 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich), and the absorbance was recorded at 490 nm using a Bio-Rad 3350 microplate reader.

Colony formation assay. Cells were plated in 60-mm culture dishes at 200 cells/well and cultured in DMEM supplemented with 10% FBS for 3 weeks. Then the colonies were rinsed with PBS, fixed with absolute methanol for 15 min, and stained with Giemsa solution for 30 min. The colonies containing ≥50 cells were counted as positive for growth.

Tumorsphere formation assay. To obtain tumorspheres, cells were seeded in 24-well ultra-low attachment plates at a concentration of 10^3 cells/well and cultured in serum-free DMEM/F12 medium supplemented with 1X B-27 (Invitrogen), 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich) and 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech, USA) at 37˚C under 5% CO2 for 2 weeks. The formed tumorspheres were quantified using an inverted microscope.

Cell cycle analysis. Cells were cultured in 6-well plates until they reached 60-70% confluency. Cells (1x10^6) were harvested and washed twice with ice-cold PBS and then fixed overnight with 70% ethanol at 4˚C. Following incubation with 50 mg/ml RNase A and 20 mg/ml propidium iodide (PI; Sigma) for 30 min at room temperature, the cell cycle was analyzed by FACSCalibur flow cytometry (Becton-Dickinson, Franklin Lakes, USA) using CellQuest software.

β-catenin reporter assay. β-catenin transcriptional activity was determined by TOP/FOP-Flash assays. Briefly, TOP/FOP-Flash plasmids were transfected into cells with Lipofectamine 2000 in 24-well plates. Firefly and Renilla luciferase assays were performed using Dual-Glo Luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's recommendations.

Tumor xenograft assay. Six week old BALB/c nude mice were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Cells (1x10^6) were subcutaneously injected into the dorsum of the mice, and tumor size was measured every week. The tumor volume (V) was determined by the...
length \((a)\) and width \((b)\) as: \(V = ab^{2}/2.\) At the end of the experiment, the mice were sacrificed, and the formed tumors were dissected out and their wet weights were determined. Animals were treated humanely in accordance with institutional policies, and all studies performed were approved by the Animal Care and Use Committee of Xi'an Jiaotong University.

Figure 1. Knockdown of YAP1 inhibits the proliferation of osteosarcoma cells. Western blot assays were used to characterize the expression of YAP1 in MG-63, Hs-188T, HOS, U-2OS, IHNC cells (A) and YAP1 knockdown MG-63 and HOS cells (B). The cell proliferation of YAP1-knockdown MG-63 cells was evaluated by cell count (C) and MTT assay (D). The cell proliferation of YAP1-knockdown HOS cells was evaluated by cell count (E) and MTT assay (F). (G) The knockdown of YAP1 significantly inhibited the colony formation of MG-63 cells. (H) The knockdown of YAP1 significantly inhibited the colony formation of HOS cells. Data are shown as mean ± SD of three experiments in duplicate. *\(P<0.05, **P<0.01\) compared to the control. YAP1, yes-associated protein 1.
Statistical analysis. Statistical analysis was performed using SPSS 16.0 software. The experiments were performed in triplicate and repeated three times independently. Comparisons among all groups were performed using one-way analysis of variance (ANOVA), and the Student’s t-test was used for comparison of differences between two groups. Differences were considered significant at P<0.05.

Results

Knockdown of YAP1 inhibits the proliferation and colony formation of osteosarcoma cells in vitro. Recently, a number of studies have reported that YAP1 protein is highly expressed in human osteosarcoma tissues (23,24); however, the role of YAP1 in osteosarcoma tumorigenesis is unclear. Western blot analysis showed that YAP1 expression in osteosarcoma cell lines MG-63, Hs 188T, HOS and U-2OS was much higher than that in normal human osteoblasts (IHNC), therefore we established shRNA-mediated YAP1-knockdown MG-63 and HOS cell lines to examine the effect of YAP1 on osteosarcoma cell proliferation (Fig. 1A). The level of YAP1 expression in the YAP1-knockdown cells was confirmed to be markedly downregulated compared with the control cells (Fig. 1B). Subsequently, the cell growth curve showed that the knockdown of YAP1 resulted in a significant inhibition of MG-63 cell growth (Fig. 1C). Moreover, the cell viability assay by MTT revealed that the viability of the YAP1-knockdown MG-63 cells was markedly decreased when compared with that of the control cells (Fig. 1D). Similar results were observed in the YAP1-knockdown HOS cells (Fig. 1E and F). These results suggest that the knockdown of YAP1 inhibits the proliferation of osteosarcoma cells.

Furthermore, colony formation assays were performed to confirm the inhibitory effect of YAP1 knockdown on osteosarcoma cells. Compared with the control cells, the percentage of colony formation in the YAP1-knockdown cells (MG-63 and HOS) was significantly decreased (P<0.05; Fig. 1G and H), suggesting that knockdown of YAP1 also inhibited the colony formation ability of osteosarcoma cells. Collectively, all the results demonstrated that YAP1 knockdown inhibits the proliferation of osteosarcoma cells.

Knockdown of YAP1 suppresses the tumor formation of osteosarcoma cells in vitro and in vivo. Since the knockdown of YAP1 inhibited the proliferation of osteosarcoma cells in vitro, we hypothesized that YAP1 knockdown may affect the tumorigenic potential of osteosarcoma cells. Therefore, firstly, the tumorsphere formation in vitro was assessed to examine the role of YAP1 in the tumorigenesis of osteosarcoma. As shown in Fig. 2A and C, the YAP1-knockdown MG-63 and HOS cells formed fewer and smaller tumorspheres compared to the control cells. Further quantitative analysis showed that the numbers of tumorspheres formed by the YAP1-knockdown cells were significantly decreased compared to the control cells (P<0.05; Fig. 2B and D). These results indicate that the knockdown of YAP1 inhibits the tumorsphere formation of MG-63 and HOS cells.

Next, YAP1-knockdown MG-63 and HOS cells were injected into nude mice, and the development and growth of tumors were monitored every week in terms of tumor growth.
During the course of the study, the knockdown of YAP1 led to a significant inhibition of xenograft tumor growth (Fig. 3A and B). Moreover, the weights of the tumors generated from the YAP1-knockdown MG-63 and HOS cells were reduced significantly compared with the control cells (P<0.05; Fig. 3C and D). All these data suggest that knockdown of YAP1 also inhibits tumor growth in vivo. Taken together, these results demonstrated that knockdown of YAP1 inhibits the tumor formation ability of osteosarcoma cells.

**YAP1 regulates the cell cycle progression of osteosarcoma cells.** It is well known that the alteration in cell cycle progression is frequently involved in cell proliferation. To explore the mechanism by which YAP1 knockdown inhibits tumor growth, cell cycle distribution was analyzed by flow cytometry. Representative histograms are shown in Fig. 4A and C. The knockdown of YAP1 led to a marginal accumulation of MG-63 cells in the G0/G1 phase and a significant decrease in cells in the S phase of the cell cycle (P<0.05; Fig. 4B). Similar to the MG-63 cell results, the percentage of YAP1-knockdown HOS cells distributed in the S phase was sharply decreased compared to the control cells (Fig. 4D). These results suggest that the knockdown of YAP1 arrests the cell cycle progression of osteosarcoma cells.

**Knockdown of YAP1 inhibits the Wnt signaling pathway in osteosarcoma cells.** It has been reported that YAP1 potentiates the Wnt signaling pathway through forming a complex with β-catenin (9). To test whether YAP1 also affects the Wnt signaling pathway in osteosarcoma cells, we detected the expression of cyclinD1 and c-myc proteins, target genes of the Wnt signaling pathway, in YAP1-knockdown MG-63 and HOS cells through western blot assay. As shown in Fig. 5A and B, the levels of cyclinD1 and c-myc expression in the YAP1-knockdown MG-63 and HOS cells were significantly decreased compared to the control cells (P<0.05), suggesting that the Wnt signaling pathway was inhibited in the YAP1-knockdown cells.

Furthermore, TOP/FOP-Flash reporter assay was performed, a canonical experiment for the detection of the activity of the Wnt signaling pathway. The results showed that TOP-Flash reporter activity in the YAP1-knockdown MG-63 cells was significantly decreased compared to the control cells (P<0.05), and FOP-Flash reporter activity had no obvious difference between the YAP1-knockdown and the control cells (Fig. 5C). Similar results were also observed in the YAP1-knockdown HOS cells (Fig. 5D). These results confirmed the inhibitory effect of YAP1 knockdown on the Wnt signaling pathway. Therefore, all these data demonstrated that knockdown of YAP1 inhibits the Wnt signaling pathway in osteosarcoma cells.

**Discussion**

YAP1, a downstream component of the Hippo pathway, is a key regulator of organ size and tissue expansion and regulates the balance between cell proliferation and apoptosis to maintain the steady state of the cellular environment (25,26). Recently, research has revealed that YAP1 is implicated in tumorigenesis and promotes the progression of various tumors, including...
Figure 4. Knockdown of YAP1 blocks cell cycle at G0/G1 phase in osteosarcoma cells. The cell cycle was analyzed by flow cytometry. (A) The representative cell cycle histograms of MG-63 cells were shown and (B) YAP1 knockdown significantly reduced the number of cells in S phase. (C) The representative cell cycle histograms of HOS cells were shown. (D) YAP1 knockdown significantly reduced the number of cells in S phase. Data were shown as mean ± SD from three independent experiments. *P<0.05 compared to the control. YAP1, yes-associated protein 1.

Figure 5. Knockdown of YAP1 suppressed the activity of Wnt signaling pathway. The expression of cyclinD1 and c-myc proteins in YAP1 knockdown MG-63 and HOS cells were measured by western blotting assay, YAP1 significantly inhibited the expression of cyclinD1 and c-myc proteins in MG-63 (A) and HOS (B) cells. TOP/FOP-Flash reporter plasmid were transfected into the YAP1 knockdown MG-63 and HOS cells, the reporter activities were determined by Luciferase assay at 48 h after transfection. YAP1 knockdown significantly decreased the reporter activities in MG-63 (C) and HOS (D) cells. Data were shown as mean ± SD from three independent experiments. *P<0.05 compared to the control. YAP1, yes-associated protein 1.
The knock: Prognostic closely related to the Wnt signaling pathway (36), which sarcoma tumors. Crucial role of YAP1 in the development and growth of osteosarcoma derived from the YAP1-knockdown cells was much lighter NIH3T3 cells resulted in tumor formation when transplanted previous report which showed that YAP1-overexpressing tumorsphere experiments in nude mice were conducted. We observed that YAP1 knockdown significantly retarded tumor cells and then regulate the growth of osteosarcoma Hence, YAP1 may confer some of the properties of stem cells of tumorspheres formed in the culture conditions that allowed the proliferation of only CSCs and progenitor cells (Fig. 2). Therefore, our data indicated that the inhibitory effect of YAP1 knockdown on cell proliferation was probably associated with regulation of the cell cycle. Recently, accumulating evidence supports that a subpopulation of cancer cells with stem-like properties (CSCs) exist in bone sarcomas (31), which have high tumorigenic potential and are resistant to chemotherapy and irradiation. CSCs have self-renewal and differentiation abilities and may be responsible for tumor development and relapse (32,33). Moreover, it was reported that YAP1 is associated with intestinal stem cell proliferation and colonic tumorigenesis (34). Accordingly, the role of YAP1 in osteosarcoma tumorigenesis was assessed through tumorsphere formation assay in vitro. The knockdown of YAP1 significantly decreased the number and size of tumorspheres formed in the culture conditions that allowed the proliferation of only CSCs and progenitor cells (Fig. 2). Hence, YAP1 may confer some of the properties of stem cells to tumor cells and then regulate the growth of osteosarcoma by promoting the proliferation of CSCs. In addition, to further determine the role of YAP1 in tumor formation in vitro, tumor xenograft experiments in nude mice were conducted. We observed that YAP1 knockdown significantly retarded the development and growth of implanted osteosarcoma tumors (Fig. 3). Moreover, the weight of the tumor xenografts derived from the YAP1-knockdown cells was much lighter than that of the control cells. Our result is consistent with a previous report which showed that YAP1-overexpressing NH3T3 cells resulted in tumor formation when transplanted into nude mice (35). Collectively, these data confirmed the crucial role of YAP1 in the development and growth of osteosarcoma tumors. Notably, some researchers have noted that YAP1 is closely related to the Wnt signaling pathway (36), which plays a critical role in the modulation of stem cell proliferation and tumorigenesis (37). Moreover, the Wnt canonical pathway is known to be involved in cell proliferation and fate. McQueen et al reported that the Wnt signaling pathway is dysregulated in osteosarcoma (38). In the present study, we found that YAP1 knockdown in the osteosarcoma cell lines (MG-63 and HOS) resulted in a significant reduction in cyclinD1 and c-myc protein expression, target genes of the Wnt signaling pathway, implying that the Wnt signaling pathway may be inhibited. TOP/FOP-Flash reporter assay further confirmed that the knockdown of YAP1 in MG-63 and HOS cells suppressed the activities of the Wnt signaling pathway (Fig. 5). Therefore, these data suggest that the suppression of the proliferation of osteosarcoma cells following YAP1 knockdown was probably associated with the inhibition of the Wnt signaling pathway. In summary, our study demonstrated that the knockdown of YAP1 resulted in suppression of osteosarcoma cell proliferation and tumor growth in vitro and in vivo, and the mechanism was associated with the Wnt signaling pathway. However, the precise mechanisms that are ultimately involved in the interaction between YAP1 and Wnt signaling molecules remain to be elucidated. Nevertheless, our findings suggest the potential important role of YAP1 in the regulation of osteosarcoma growth and also provide a new target for gene therapy of osteosarcoma.

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YAP1 KNOCKDOWN INHIBITS OSTEOSARCOMA GROWTH


