TGF-β is associated with poor prognosis and promotes osteosarcoma progression via PI3K/Akt pathway activation

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ABSTRACT
Transforming growth factor beta (TGF-β) is a multifunctional cytokine with important functions in cell proliferation and differentiation. TGF-β is highly expressed in several types of cancers and promotes tumor invasion and metastasis. However, the role of TGF-β in osteosarcoma progression is poorly understood. In the present study, we found that TGF-β is highly expressed in osteosarcoma cells and tissues, and is associated with high Ennecking stage (P = 0.033), metastasis, and recurrence. TGF-β-knockdown osteosarcoma cell lines were established using siRNA (si-TGF-β). Cells transfected with si-TGF-β exhibited significantly reduced proliferation, migration/invasion, and colony formation abilities, and increased levels of cell apoptosis. In addition, si-TGF-β treatment reduced spheroid size, the ratio of CD133-positive cells, and expression of SOX-2, Nanog, and Oct-3/4 in osteosarcoma cells. Mechanistically, PI3K/mTOR phosphorylation is inhibited by TGF-β knockdown. Pretreatment with 25 µM LY294002, a PI3K-specific inhibitor, further enhanced the si-TGF-β-induced suppression of osteosarcoma progression. Taken together, these results reveal a novel role for TGF-β in osteosarcoma progression and modulation of stemness-related traits and indicate that TGF-β may be of value as a therapeutic target for the treatment of osteosarcoma.

Introduction
Osteosarcoma is the most frequently occurring and malignant bone tumor in children and adolescents [1]. Osteosarcoma is a neoplasm derived from mesenchymal tissue, and is characterized by the production of new bone tissue by spindle-shaped stromal cells [2]. However, 50–60% of patients with osteosarcoma have advanced or metastatic disease at the time of diagnosis. Although new adjuvant chemotherapies have been developed within the past decade, many patients with osteosarcoma have developed within the past decade, many patients with osteosarcoma develop chemoresistance and lung metastasis, even when treated with chemotherapy combined with surgical resection [3,4]. To date, the etiology and pathogenesis of osteosarcoma remain poorly understood.

The transforming growth factor-beta (TGF-β) family of cytokines belongs to a superfamily of structurally similar, but functionally diverse, growth factors that include activin, bone morphogenetic protein (BMP), and growth differentiation factor (GDF) [5]. TGF-β can inhibit cell proliferation, initiate cell differentiation, and induce apoptosis, and promote tumor invasion and metastasis [6–8]. The TGF-β signaling pathway, and related pathways, contribute to enhanced stemness in triple-negative breast cancer, colon cancer, and ovarian clear cell carcinoma [9–11]. Furthermore, TGF-β levels in serum are significantly higher in patients with osteosarcoma than in controls [12], and the serum TGF-β levels are higher in patients with metastasis than in those without metastasis. Therefore, we hypothesized that TGF-β could be a biological marker for, and play a role in the regulation of stemness traits in, osteosarcoma.

TGF-β is involved in regulating the biological function of malignant tumors through several pathways, including the Jagged1/Notch, Wnt, JAK2/STAT3, and PI3K/AKT/mTOR signaling pathways [13–16]. Moreover, regulation of Akt/mTOR pathway activity can inhibit osteosarcoma cell proliferation, arrest the cell cycle, induce apoptosis, and suppress invasion and metastasis, suggesting the involvement of this pathway in the
development of osteosarcoma [17–20]. We hypothesized that TGF-β may regulate the stemness and metastatic potential of osteosarcoma stem cells by suppressing Akt/mTOR pathway activity. In this study, we investigated the correlation between TGF-β expression and the clinicopathological characteristics and prognosis of patients with osteosarcoma. We explored the effect of TGF-β knockdown on proliferation, invasion/migration, and regulation of stemness in osteosarcoma cells and examined the underlying molecular mechanisms. This study was designed to determine whether TGF-β is a promising novel molecular target for preventing osteosarcoma metastasis and an effective predictive biomarker for patients with osteosarcoma.

Materials and methods

**Tissues**

Data were analyzed in accordance with guidelines approved by the Ethics Committee of Luoyang Orthopedic Hospital of Henan Province and the Orthopedic Hospital of Henan Province. Written informed consent was obtained from all participants prior to specimen collection. All patients were diagnosed with osteosarcoma and underwent surgery in our hospital from 2012 to 2018. Patients did not undergo other treatments, including chemotherapy or radiotherapy, before surgery. Osteosarcoma specimens were obtained from 48 males and 23 females (71 patients). The average patient age was 27.5 years (range, 12–38 years). Hematoxylin and eosin (H&E)-stained sections were obtained for diagnosis. Each section was independently diagnosed by two pathologists following the World Health Organization (WHO) classification of osteosarcoma. The following data were recorded: patient gender, age, tumor size, clinical stage, tumor location, and lymph node metastasis status. Pathological diagnosis was performed following the criteria set by the American Joint Committee on Cancer.

**Cell culture and reagents**

U2OS and MG-63 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) and cultured in DMEM containing 10% fetal bovine serum (FBS), 100 μg/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in an incubator with 5% CO2. The human normal osteoblastic cell line, hFOB 1.19, was maintained in DMEM/F-12 (Gibco) supplemented with 10% FBS (Gibco) and 0.3 mg/ml of genetin (G418; Gibco) at 37°C in a humidified atmosphere containing 5% CO2.

**Gene silencing by small interfering RNA (siRNA)**

Loss-of-function analysis was conducted using siRNA to knock down TGF-β expression. Three siRNAs targeting TGF-β (si-TGF-β) and one control siRNA (si-control) were obtained from Shanghai GenePharma Co., Ltd (Shanghai, China). siRNA sequences were: si-TGF-β#1: 5′-CCATCTTCACATGGAGATT-3′; si-TGF-β#2: 5′-GGAGATTGT GGTAC CCAA-3′; si-TGF -β#3: 5′-TCAAGAGACCAAGGTACAT-3′; and si-control: 5′-TTCTCCGAACGTGTCACGT-3′. Cells were pre-treated with basic culture medium DMEM/F12 with or without 25 μM LY294002 (a specific PI3K inhibitor, that effectively inhibits Akt phosphorylation) for 6 h prior to transfection [21,22]. Each siRNA (100 nM) was mixed with Lipofectamine 3000 (Invitrogen) as a carrier and transfected into U2OS/MG-63 cells (1 × 10⁶) for 10 h at 37°C, following the manufacturer’s protocol. The efficacy of TGF-knockdown was validated by RT-qPCR and western blot analyses and cells were used for experiments 48 h post-transfection.

**MTT assay**

After transfection for 24, 48, and 72 h, U2OS/MG-63 cells were transfected with si-control or si-TGF-β and then cultured in 96-well flat-bottom microtiter plates overnight at a density of 1 × 10⁶ cells/well. Subsequently, 20 μl of MTT solution was added to the cells, followed by incubation for 4 h at 37°C. An automatic multiwell spectrophotometer was then used to calculate the absorbance value for each well at 570 nm.
**Sphere formation assay**

Sphere formation assays were performed on third and fourth passage cells. Osteosarcoma spheroids were cultured in a specialized growth medium (Celprogen Inc, Torrance, CA, USA) containing 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 20 ng/ml human platelet growth factor (Sigma-Aldrich), 100 ng/ml epidermal growth factor (Invitrogen), and 1% antimycotic (Invitrogen). Osteosarcoma spheroids and hFOB 1.19 cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2. Spheres were counted using an inverted microscope and cell colonies with a diameter > 50 μm were measured.

**FACS analysis**

Osteosarcoma cells were harvested with fresh 0.25% trypsin solution (Sigma-Aldrich) and suspended in phosphate-buffered saline (PBS). Cells were blocked on ice for 15 min and labeled with PE-conjugated anti-human CD133 antibody (BioLegend) for 60 min. Cells were then washed twice with PBS and maintained on ice until analysis. Expression levels were determined by flow cytometry (FACS Calibur, BD Bioscience, USA) and data were analyzed using WinMDI software (Scripps Research Institute, La Jolla, CA, USA).

**Western blotting**

Cells were extracted and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (P0013B, Beyotime Biotechnology) supplemented with phenylmethanesulfonyl fluoride (PMSF; 1 μM). Extracted proteins were treated with Enhanced BCA Protein Assay Reagent (P0009, Beyotime Biotechnology), following the manufacturer’s instructions, and quantified using a microplate reader. Next, equal amounts of total protein (40 μg) were separated by 10% SDS-PAGE (P0012A; Beyotime Biotechnology) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk at room temperature for 2 h, and incubated overnight at 4°C with the following primary antibodies (all raised in a rabbit host): anti-CD133 (1:1000, ab19898), anti-SOX-2 (1:1000, ab97959), anti-Oct-4 (1:1000, ab18976), anti-NANOG (1:1000, ab80892), anti-MMP2 (D8N9Y, 1:1000, ab13132), anti-E-cadherin (1:1000, ab15148), and anti-vimentin (1:1000, ab92547) (Abcam, Cambridge, UK); anti-p-PI3K (1:500; 4228s), anti-PI3K (1:1000, 4257s), anti-p-AKT (1:1000, 4060s), anti-AKT (1:500, 9272s) (Cell Signaling Technology, Inc); and anti-GAPDH (1:2000, sc-47,724; Santa Cruz Biotechnology, Inc). PVDF membranes were then incubated with the following secondary antibodies: goat anti-mouse IgG (cat. no. 2305) and goat antirabbit IgG (cat.no. 2301) (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) at room temperature for 1 h. Protein bands were visualized by enhanced chemiluminescence detection using a Bio-Rad gel imaging system (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Densitometric analysis was performed using Quantity One software (Bio-Rad Laboratories, Inc.) and GAPDH was used as a loading control.

**Transwell assays**

Cells were seeded in 10 mm diameter transwell plates with polycarbonate filters (8 μm pore size). The upper and lower compartments of the plates were separated by a filter coated with 25 mg of Matrigel, which formed a reconstituted basement membrane at 37°C. After treatment with si-control or si-TGF-β, cells were seeded into the upper well, and the lower well was filled with DMEM containing 10% fetal calf serum. After incubation in the presence of 5% CO2, cells were fixed for 30 min in 4% formaldehyde and stained for 15 min. Nonmigrating cells were removed from the upper surface of the transwell chamber using a wet cotton swab, and the number of cells that had migrated or invaded the bottom surface of the filter was determined. For each well, six evenly spaced fields of cells were counted using an inverted phase-contrast microscope.

**Wound healing assay**

Cells were seeded in six-well plates at a density of 5 x 10^5 cells/well in DMEM supplemented with
10% FBS. Twelve hours after seeding, cells were treated with si-control or si-TGF-β for 48 h. A scratch was made in the cell monolayer, and the rate of wound closure was observed at 24 h and 48 h. The resultant data were analyzed using ImageJ software.

**RT-qPCR analysis**

Total RNA was extracted using Trizol reagent (Invitrogen). qPCR to detect TGF-β expression included 1 l of cDNA, 1 l each of forward and reverse primer, and 12 l of Master Mix (Promega, USA) and was performed on a Thermo ABI 7500 Real-Time PCR system (USA). All experiments were performed following the manufacturer’s instructions. GAPDH was used as the internal control for gene expression. The primer sequences were: TGF-β forward: 5′-AGGGCTACCATGCCAACTTC-3′ and reverse: 5′-GCGGCACGCAGCACGGTGAT-3′; and GAPDH forward: 5′-TGCCCAG AACAT CATCCCT-3′ and reverse: 5′-GTCCCTCAGTGLAGCCCAAG-3′.

**Tumor xenografts**

Animal care protocols and experiments were approved by the animal ethical committee of Luoyang Orthopedic Hospital of Henan Province and the Orthopedic Hospital of Henan Province and performed in accordance with the guidelines specified in the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). Twenty female BALB/c mice (4–6 weeks old) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Cells (1 × 10⁶) were subcutaneously injected into each mouse. Nude mice were anesthetized with a 3–5% (v/v) mixture of isoflurane (Aerrane; isoflurane, Baxter) in synthetic air (200 ml/min), killed by cervical dislocation, and the tumors were obtained. The body weight and tumor length and width were measured in each mouse every 3 days. Additionally, tumor volume was calculated using the formula: volume = 1/2 (width² × length). After 4 weeks, the mice were euthanized, and the tumors excised and weighed.

**Immunohistochemistry**

Immunohistochemical (IHC) staining was performed to measure Ki-67, PI3K, and Akt protein levels. Tumor tissue was embedded in paraffin, cut into 4-μm sections, and heated overnight at 60°C. The sections were dewaxed in xylene and rehydrated in a 100, 95, 80, and 75% alcohol series. Slides were incubated overnight with primary antibodies against Ki-67, PI3K, and Akt at 4°C. The next day, sections were incubated with the appropriate secondary antibodies for 1 h at 37°C. Data were then analyzed using a light microscope (Olympus Corporation).

**Evaluation of IHC staining**

The H-score system was used to evaluate the immunointensity of tumor cells. The score for staining intensity was: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The formula used to evaluate the immunointensity of tumor cells was: H-score = (percentage of cells with weak intensity × 1) + (percentage of cells with moderate intensity × 2) + (percentage of cells with strong intensity × 3). The scoring was performed using the Densito Quant module of Quant Center software. Scores of duplicate specimens were averaged. At a total H-score of 276, the threshold for TGF-β expression was set at 165.38. TGF-β staining results were classified as TGF-β-low (≤ 165.38) or TGF-β-high (> 165.38).

**Statistical analysis**

Data are presented as means ± standard deviation (SD). Fisher’s exact test was applied for multiple comparisons. Statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc.). P < 0.05 was considered to indicate significance.

**Results**

**TGF-β is upregulated in osteosarcoma tissues and positively correlates with poor survival**

IHC was performed on 71 osteosarcoma samples and corresponding normal adjacent tissues to
assess TGF-β expression and localization. TGF-β was expressed in low levels in the cytoplasm of normal tissue (Figure 1(a), left) and in high levels in the cytoplasm of osteosarcoma specimens (Figure 1(a), right). The immunointensity of TGF-β staining in tissue samples was evaluated using H-score. The proportion of TGF-β-high osteosarcoma samples was substantially greater than that of normal adjacent tissue samples (74.65% vs. 18%, for osteosarcoma and normal samples, respectively; P = 0.003) (Table 1).

There was no correlation between TGF-β expression and gender, age, or histological grade (P > 0.05). TGF-β expression levels were markedly positively correlated with high Enneking stage (P = 0.033), metastasis, and recurrence (P = 0.008) (Table 2).

Western blot and RT-qPCR analyses (Figure 1(b, d)) showed that TGF-β levels were higher in U2OS, MG-63, and HOS cells than in normal human osteogenic hFOB.1.19 cells (P 0.05). Kaplan-Meier analysis was used to assess the prognostic value of TGF-β expression in patients with osteosarcoma. Overall survival was shorter in patients with osteosarcoma and higher levels of TGF-β expression, indicating that TGF-β is a potential prognostic factor for osteosarcoma (Figure 1(c)).

**TGF-β knockdown inhibits osteosarcoma cell proliferation and promotes their apoptosis**

To clarify the biological functions of TGF-β in U2OS and MG-63 cells, TGF-β expression was knocked down using short interfering (si) RNA. si-control and si-TGF-β#1, #2, and #3 were transfected into cells and TGF-β knockdown was confirmed by RT-qPCR and western blotting (Figure 2(a,b)). si-TGF-β#1 produced the best knockdown efficiency (P < 0.01) and used for subsequent studies.

Compared with si-control transfection, si-TGF-β transfection significantly reduced the viability of U2OS and MG-63 cells (P < 0.05; Figure 2(c)). Colony formation assays, to assess cell proliferation, revealed that TGF-β knockdown suppressed clonogenicity in U2OS and MG-63 cells (P < 0.05; Figure 2(d,e)). Additionally, pretreatment with si-TGF-β increased S-phase cell cycle arrest in both cell lines (figure 2(f,g)). Flow cytometry assessment of apoptosis showed that si-TGF-β treatment increased the apoptotic ratio in both U2OS and MG-63 cells (Figure 2(h,i)). Moreover, TGF-β knockdown led to a substantial increase in cleaved PARP, caspase-3, and BAX levels and a decrease in Bcl-2 levels (Figure 2(j,k)).

**TGF-β knockdown of suppresses invasion, migration, and the epithelial-mesenchymal transition (EMT) in osteosarcoma cells**

Transwell assays were performed to examine the role of TGF-β in cell migration and invasion in U2OS and MG-63 osteosarcoma cells. TGF-β knockdown reduced the invasion potential of
Moreover, si-TGF-β treatment increased wound widths in U2OS and MG-63 cells (Figure 3(c,d)). Taken together, these results indicate that TGF-β knockdown inhibits the migratory and invasive capacities of U2OS and MG-63 osteosarcoma cells.

The EMT is a dynamic biological process through which epithelial cells develop mesenchymal cell properties. In cancer cells, the EMT ultimately leads to their metastasis [23]. TGF-β knockdown decreased MMP-2, vimentin, and α-SMA expression and increased E-cadherin expression in U2OS and MG-63 cells (Figure 3(e,f)).

TGF-β knockdown reduces stemness and the ratio of CD133+ subpopulations in osteosarcoma cells

Spheroid formation assays were performed to evaluate the effect of TGF-β on the self-renewal ability of osteosarcoma cells. TGF-β knockdown reduced spheroid volume and numbers in ultralow attachment plates (Figure 4(a,b)). Notably, we observed that TGF-β knockdown reduced the number of CD133+ cells (Figure 4(c,d)) and the expression levels of several stem cell-associated gene products, including SOX2, NANOG, and OCT4 (Figure 4(e,f)).

TGF-β accelerates osteosarcoma progression through PI3K/mTOR signaling pathway activation

The PI3K/mTOR signaling pathway facilitates the proliferation, invasion/migration, vascularization, and tumorigenicity of cancer cells [24–27]. si-TGF-β treatment markedly attenuated p-PI3K and p-Akt expression in U2OS and MG-63 cells (Figure 5(a)), indicating that the PI3K/mTOR signaling pathway may mediate the effects of TGF-β on osteosarcoma progression. To further understand the role of the PI3K/mTOR signaling pathway in osteosarcoma, we exposed U2OS and MG-63 cells to LY294002, a PI3K-specific inhibitor that inhibits Akt phosphorylation. Pretreatment with 25 µM LY294002 significantly enhanced TGF-β knockdown-mediated colony formation inhibition (P< 0.05; Figure 5(b,c)). Following LY294002+ si-TGF-β cotreatment, significantly fewer invading cells and wider wound widths were observed (Figure 5(d–g)), as were smaller spheroid volumes and fewer CD133+ cells (Figure 5(h–k)). Together, these results imply that TGF-β regulates osteosarcoma development and stemness traits via the PI3K/mTOR pathway.

TGF-β knockdown inhibits tumorigenicity in vivo

A xenograft nude mouse model of osteosarcoma was established using U2OS cells to investigate

U2OS and MG-63 cells (Figure 3(a,b)). Moreover, si-TGF-β treatment increased wound widths in U2OS and MG-63 cells (Figure 3(c,d)). Taken together, these results indicate that TGF-β knockdown inhibits the migratory and invasive capacities of U2OS and MG-63 osteosarcoma cells.

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Table 1. TGF-β expression in the samples

| Group       | Samples | TGF-β expression (n (%)) | X2 | P    |
|-------------|---------|-------------------------|----|------|
| Low (H-score ≤165.38) | Control | 50 41(82) 9(18) | 10.689 | 0.003* |
|            | osteosarcoma | 71 18(25.35) 53(74.65) |      |      |

*P>0.05

Table 2. The relationship between TGF-β expression and pathological characteristics.

| Characteristics          | TGF-β+ | TGF-β− | P     |
|--------------------------|--------|--------|-------|
| Gender                   |        |        | 0.312 |
| Male                     | 33     | 15     |       |
| Female                   | 20     | 3      |       |
| Age(year)                |        |        | 0.865 |
| >16                      | 47     | 8      |       |
| <16                      | 14     | 2      |       |
| Tumor size(d/cm)         |        |        | 0.187 |
| >5                       | 37     | 16     |       |
| ≤5                       | 16     | 2      |       |
| Tumor location           |        |        | 0.412 |
| Femur                    | 28     | 10     |       |
| Tibia and fibia          | 17     | 6      |       |
| others                   | 8      | 2      |       |
| Histological type        |        |        | 0.238 |
| Osteoblastic             | 28     | 6      |       |
| Chondroblastic           | 10     | 3      |       |
| Fibroblastic             | 10     | 2      |       |
| Ennecking stage          |        |        | 0.0023|
| IIA                      | 5      | 16     |       |
| IIB/III                  | 48     | 2      |       |
| Metastasis               |        |        | 0.018 |
| Yes                      | 40     | 3      |       |
| No                       | 13     | 15     |       |

in osteosarcoma patients
whether TGF-β knockdown can inhibit tumor growth in vivo. The effects of TGF-knockdown were assessed following intratumoral injection of si-TGF-β or si-control. A significant decrease in tumor volume and weight was observed after si-TGF-β injection (P < 0.05 for each; Figure 6(a,b)). Immunohistochemical staining revealed that Ki-67, p-PI3K, and p-Akt expression levels were markedly reduced in xenograft tumors originating from si-TGF-β-transfected cells (Figure 6(c)).
Figure 3. TGF-β promotes cell invasion/migration and the EMT. (a and b) TGF-β knockdown reduces the invasive ability of U2OS and MG-63 cells. TGF-β knockdown decreased the invasive ability of U2OS and MG-63 cells (lower panel) (*P < 0.05). Magnification, ×200. (c and d) The rate of U2OS and MG-63 cell migration. Right panels, quantitative data. *P < 0.05 vs. the si-control group. (e and f) E-cadherin, vimentin, α-SMA, and MMP-2 protein expression levels in MG-63 and U2OS cells with or without si-TGF-β treatment. GAPDH was used as a loading control. Results are expressed as means ± SD of three independent experiments; *P < 0.05 vs. the si-control group.

Figure 4. TGF-β enhances osteosarcoma cell stemness and increases the proportion of CD133+ cells. (a and b) Spheroid formation assay for U2OS and MG-63 cells transfected with si-TGF-β. Representative images (left panel) and statistical measurements (right panel). Representative micrographs of formed spheres were analyzed in cells treated with si-TGF-β or si-control. Scale bar, 100 μm. *P < 0.05 vs. the si-control group. (c and d) The percentage of CD133+ cells in U2OS- and MG-63-derived spheroids was analyzed by flow cytometry. *P < 0.05 vs. the si-control group. (e and f) Stem cell-associated protein expression was measured by western blot. *P < 0.05 vs. the si-control group.
Discussion

Osteosarcoma is the most common type of bone tumor in children and adolescents, and is the second leading cause of cancer-related deaths in pediatric patients. Once diagnosed, osteosarcoma often develops into the final stage and late diagnosis and chemoresistance lead to high mortality rates [28]. Given the current poor prognosis for osteosarcoma patients, specific molecular targets for improving the prognosis of osteosarcoma and the effectiveness of intervention are urgently needed.

TGF-β is a multifunctional cytokine that plays a role in various cancers, including hepatic, gastric, and colon cancers [29–31]. Furthermore, the TGF-β pathway is associated with poor prognosis in hepatocellular carcinoma, clear cell renal carcinoma, and colorectal cancer [32–34]. The serum level of TGF-β is significantly increased in osteosarcoma patients [12], but no study has
investigated the role of TGF-β in the pathogenesis of this cancer to date.

In this study, TGF-β was found to be highly expressed both in human osteosarcoma tissues and osteosarcoma cell lines, indicating that TGF-β may contribute to osteosarcoma procession. Immunohistochemical staining and statistical analysis further indicated that TGF-β was not associated with gender, age, tumor location, or histological grade \( (P < 0.05) \), but was positively correlated with high Ennecking stage and metastasis. Additionally, overall survival was shorter in patients with osteosarcoma and higher TGF-β expression. Taken together, these results support the suggestion that TGF-β may be a prognostic factor for osteosarcoma \[12\].

To determine how TGF-β affects osteosarcoma, we performed in vitro and in vivo experiments. Verrecchia et al. reported that TGF-β exerts its oncogenic function in primary bone tumors by promoting angiogenesis, bone remodeling, and cell migration and inhibiting immunosurveillance \[35\]. We found that si-TGF-β treatment significantly decreased the viability of U2OS/MG-63 cells \( (P < 0.05; \text{Figure~2(a–c)}) \). TGF-β has been shown to play a role in modulating osteosarcoma cell apoptosis \[36\]. Our results show that TGF-β downregulation directly induces an increase in S-phase cell cycle arrest and upregulates the expression of apoptosis-related markers. These results indicate that indicating that TGF-β plays a role in promoting osteosarcoma progression.

Increasing evidence has shown that TGF-β exerts a promigratory effect on osteosarcoma cells and stimulates tumor growth \[37,38\]. Here, we found that TGF-β accelerates the EMT in osteosarcoma cells. When carcinoma cells undergo EMT, they become resistant to chemotherapy and acquire the ability to suppress immune responses, thereby promoting tumor progression. Exogenous TGF-β promotes an EMT-like phenotype in several osteosarcoma cell lines, and the development of lung metastases in osteosarcoma \[39\]. Similarly, we observed that TGF-β knockdown markedly reduced the number of invading cells and increased wound widths, indicative of reduced

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**Figure 6.** TGF-β promotes tumorigenicity in vivo. (a and b) Representative images of the xenograft tumors formed in nude mice injected with siRNA targeting TGF-β (si-TGF-β) or control siRNA (si-control). The volumes and weights of xenograft tumors are summarized. (c) Representative images of immunohistochemical staining for Ki-67, p-Pi3K, and p-Akt in tumor nodules.
cell migration (Figure 3(a–d)). These results, together with the observed downregulation of vimentin, α-SMA, and MMP-2 expression levels, and increased E-cadherin levels, indicate that TGF-β induces invasion/migration and the EMT phenotype in osteosarcoma. This is consistent with previously reported results [40,41].

Metastasis and cancer stem cell (CSC) emergence are major causes of therapy failure. Xu et al. reported that MB-231/Epi cells express high levels of TGF-β and contain a larger population of breast CSCs [42]. However, they did not evaluate the effect of TGF-β on the modulation of stemness in breast cancer. Peng et al. showed that exogenous TGF-β application increases the percentage of CD44+/EpCAM+ cells, and vimentin and N-cadherin levels [12]. Meanwhile, Katsuno et al. showed that prolonged TGF-β exposure leads CD44+CD24− cell population and mammosphere formation increases [43]. However, these reports only assessed the effect of exogenous TGF-β on the regulation of stemness markers. In the current study, we observed that si-TGF-β treatment resulted in smaller spheroids sizes, a reduced ratio of CD133-positive cells, and downregulation of stemness marker expression (Figure 4(a–d)). These results indicate that endogenous TGF-β positively modulates stemness in osteosarcoma and contribute to the understanding of stemness property modulation by TGF-β.

The PI3K/Akt, NF-κB, and Wnt pathways are involved in osteosarcoma progression. Katsuno et al. showed that prolonged TGF-β exposure enhances and stabilizes Akt-mTOR signaling [43]. Furthermore, Tsubaki et al. showed that the Ras/PI3K/Akt pathway positively regulates TGF-β levels in mouse osteosarcoma [44]. Consistent with these results, we observed reduced levels of p-PI3K and p-Akt following si-TGF-β treatment (Figure 5(a)). Moreover, pretreatment with 25 µM LY294002 (a PI3K-specific inhibitor) enhanced the si-TGF-β-mediated inhibition of PI3K and Akt phosphorylation. Cotreatment with si-TGF-β and LY294002 reduced the proliferative and invasive/migratory ability of osteosarcoma cells, spheroid size, and the ratio of CD133-positive cells (Figure 5(b–j)).

Taken together, our in vitro experimental results, and those of other studies, show that the TGF-β/PI3K/Akt pathway drives osteosarcoma progression. In nude mice we observed si-TGF-β-mediated reduction in tumor volume and Ki67, p-PI3K, and p-Akt levels (Figure 6(a–c)), confirming the osteosarcoma-promoting effects of TGF-β.

This is the first study to systematically characterize the role of TGF-β in osteosarcoma progression. The results of our in vitro and in vivo experiments show that TGF-β is positively correlated with high Ennecking stage and metastasis. Our results reveal the mechanism underlying the role of TGF-β in osteosarcoma progression and modulation of stemness.

This study had several limitations. First, the sample size was relatively small and may have led to regional bias. Therefore, multicenter and multi-area samples are required to validate our findings. Second, clinical trials are warranted to assess the application of TGF-β as a biomarker in the management of osteosarcoma. Our findings may help in the development of novel therapeutic strategies to promote the long-term survival of patients with osteosarcoma.

Disclosure statement

No potential conflict of interest was reported by the authors.

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