Interleukin-22 Promotes Osteosarcoma Cell Proliferation and Invasion via STAT3 Activation

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCEF 1 Panpan Li*
BCDE 2 Xin Shi*
BD 1 Yonghui Xu
BC 3 Binggang Zhong
BC 4 Yu Lu
ABCDEFG 1 Yong Sun

1 Department of Radiology, The Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, P.R. China
2 Department of Traumatology, The Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, P.R. China
3 Department of Orthopedics, The First People's Hospital of Zhao Tong, Zhaotong, Yunnan, P.R. China
4 Department of Orthopedics, The Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, P.R. China

Corresponding Author:
Source of support:

* Contributed equally
Yong Sun, e-mail: sunyong0504@163.com
Departmental sources

Background: Interleukin-22 (IL-22) is one of the cytokines secreted by T-helper 17 (Th17) cells. It belongs to the IL-10 cytokine family and influences a variety of immune reactions. Studies have indicated that IL-22 can promote cancer progression and metastases. However, the function of IL-22 in osteosarcoma (OS) remains unclear.

Material/Methods: In this study, the expression of IL-22 in the OS cell line was detected by qRT-PCR. The role of IL-22 in proliferation and invasion in OS cells was tested by MTT and Transwell assays. The protein expression of STAT3, phospho-STAT3, AKT, and phospho-AKT was detected by Western blot analysis.

Results: The results showed that IL-22 was upregulated in OS cells. IL-22 dose-independently promoted OS cells proliferation and invasion, which could be reversed by IL-22 antibody or STAT3 siRNA. Furthermore, IL-22 exposure of OS cells resulted in dose-independently increased levels of phosphorylated STAT3 protein kinases. Interestingly, IL-22 did not influence the expression of phosphorylated AKT.

Conclusions: These results suggest that IL-22 promotes OS cells proliferation and invasion and its effect is mediated by activation of the STAT3 pathway. These findings demonstrate that IL-22 may serve as a promising molecular biomarker for diagnosis and therapy for OS patients.

MeSH Keywords: Cell Proliferation • Neoplasm Invasiveness • Osteosarcoma • STAT3 Transcription Factor
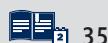
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Background

Osteosarcoma (OS) is the most prevalent primary malignancy of the bones and joints worldwide, accounting for ~2.4% of all malignancies in children and adolescents [1,2]. The annual incidence in children under 15 years old is 5.6 cases per million [3]. OS cells are characteristically aggressive, with rapid growth and early metastasis. The current therapy is a combination of surgical excision, multi-agent neoadjuvant chemotherapy, and adjuvant chemotherapy [4,5]. Although treatment has progressed, the prognosis is still poor. One of the most important causes of ineffective chemotherapy treatment of OSA is multidrug resistance (MDR) [6]. In addition, the presence of synchronous metastases is a factor that significantly reduces the probability of survival [7]. Up to 20% of osteosarcoma patients present with imagological evidence of metastases at the time of diagnosis, with the lungs as the most prevalent metastatic location [8]. The 5-year overall survival rate for non-metastatic patients is 60–70% [9,10]. Many molecular alterations are involved in the critical signal transduction pathways that contribute to OS pathogenesis via promoting key molecular targets [11]. Therefore, it is important to understand the potential molecular mechanisms of rapid growth and early metastasis of OS and to identify new treatment options to prevent early-stage metastasis.

Interleukin-22 (IL-22) is a member of IL-10 cytokine family and is preferentially produced by activated immune cells Th17 and Th22 cells [12]. Increasing evidence shows that IL-22 is involved in host defense, tissue regeneration, and protection against injury [13–15]. Recent studies have reported that IL-22 acts as a tumor promoting cytokine and is closely related to different cancers, including breast, lung, and gastric cancers, and promotes progression and metastasis in many cancer [16,17]. IL-22 induces phosphorylation of kinase Jak1 and Tyk2 and STAT3 transcription factors through the Jak-STAT signaling pathway [18,19], which plays a key role in tumor progression [20]. A previous study has reported that IL-22 overexpression protects lung cancer cell lines from apoptosis by activating STAT3 and its downstream anti-apoptotic proteins Bcl-2 and Bcl-xL and inhibiting MAP kinases [21]. However, the role of IL-22 in osteosarcoma is not clear.

Therefore, in the present study, we investigated the role of IL-22 in OS cell proliferation and invasion. In addition, we further clarified potential mechanisms by which IL-22 stimulates OS cell migration and invasion.

Material and Methods

Antibodies and reagents

Recombinant human IL-22 and anti-human IL-22 neutralizing antibody was obtained from R&D Systems (Minneapolis, MN, USA). The primary antibodies of total STAT3 and phospho-STAT3, total AKT and phospho-AKT, and GAPDH were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture

Human osteosarcoma cell lines SOSP-9607, MG-63, U2OS, and SAOS-2 and the normal osteoblast cell line hFOB1.09 were obtained from the American Type Culture Collection (Manassas, VA). These cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 0.1g/mL streptomycin (Hyclone) in a humidified 37°C incubator with 5% CO_2.

Cell proliferation assay

Cell proliferation of MG63 and U2OS cells was measured by MTT assay. We cultured 5×10^3 cells per well into 96-well plates with 5% CO_2 at 37°C and 100 µl of MTT solution was added into each well and cultured for 4 h at 37°C. Then, 150 µl DMSO was added and we detected the absorbance at 570 nm using a microplate reader (Thermo Scientific).

Cell invasion assay

For the invasion assay, the invasion ability of MG-63 and U2OS cells was measured using Transwell assay as the reference [22]. Briefly, 3×10^5 MG-63 and U2OS cells were seeded onto the upper chamber of a 24-well Matrigel Transwell invasion insert (BD bioscience, America) containing 0.1% FBS, and the lower chamber was filled with DMEM containing 20% FBS. After 24 h of incubation, a cotton swab was used to remove the non-invading cells and the invaded cells were stained with crystal violet. The number of invaded cells was counted using a light microscope, and the average value was determined through examination of 5 different randomly selected fields.

Real-time PCR

Total RNA was extracted from MG-63 and U2OS cell lines using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We reverse transcribed 2 µg of total RNA using cDNA Reverse Transcription Kits (Applied Biosystems). Quantitative RT-PCR reaction was performed using SYBR Green Master Mix (Roche). The primer sequences were as follows: Human IL-22 forward: 5'-GCTAAGGAGGCTAGCTTG-3'

and reverse: 5'-CAGCAAATCCAGTTCTCC-3'; GAPDH forward: 5'-GGCTGCTTTTAACTCTGGTA-3' and reverse: 5'-ATGCCAGTGAGCTTCCCGT-3'. GAPDH was used as internal control and the expression of IL-22 was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

MG-63 and U2OS cells were lysed using lysis buffer containing protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). The BCA Protein Assay kit (Pierce, USA) was used to detect the total concentration of protein. Protein extract (50 mg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto polyvinylidene difluoride membranes (PVDF, EMD Millipore). The membranes were incubated with the appropriate primary antibody against STAT3 (1: 1000), phospho-STAT3 (1: 1000), AKT (1: 1000), phospho-AKT (1: 1000), or GAPDH (1: 1000) overnight at 4°C. Further, the membranes were incubated with the anti-mouse or rabbit peroxidase-conjugated secondary antibodies (HRP; 1: 5000; Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK) and quantified using Image Lab Software version 4.1 (BIO RAD).

Statistical analysis

Statistical analysis was conducted using SPSS 17.0 (IBM, Armonk, NY, USA). The difference between 2 groups was assessed using one-way ANOVA. Data are presented as the means ± standard error of mean (SEM). P<0.05 was considered significant.

Results

IL-22 was upregulated in osteosarcoma cell lines

To confirm the potential role of IL-22 in osteosarcoma progression, we first detected the expression of IL-22 in the 4 osteosarcoma cells lines MG63, SOSP-9607, U2OS, SAOS2 and normal osteoblast cell line hFOB1.09 using qRT-PCR. The results revealed that IL-22 mRNA expression in the osteosarcoma cell lines was higher than in the normal osteoblast cell line hFOB1.09 (Figure 1).

IL-22 promotes the proliferation and invasion of osteosarcoma cells

To detect the effects of IL-22 on osteosarcoma cell proliferation and invasion, MG63 and U2OS cells were pretreated with different doses of IL-22 (0, 10, 50, and 100 ng/ml). MTT results showed that IL-22 significantly increased the proliferation abilities of MG63 (Figure 2A) and U2OS cells (Figure 2B) in a
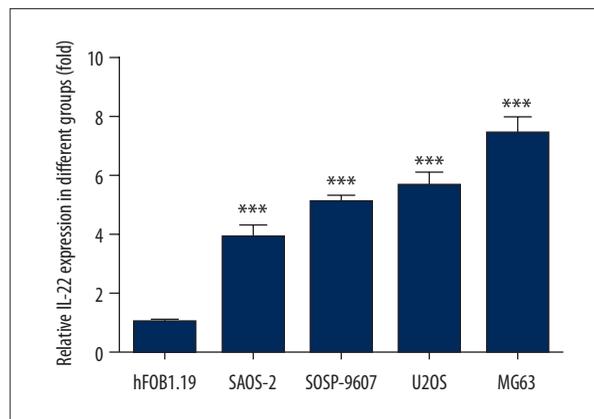

Figure 1. IL-22 was upregulated in osteosarcoma cell lines. The levels of IL-22 in the 4 osteosarcoma cells lines MG63, SOSP-9607, U2OS, and SAOS2 and normal osteoblast cell line hFOB1.09 were detected by qRT-PCR. Data are expressed as mean ± standard error. *** P<0.01 *vs.* hFOB1.09 cells.

concentration-dependent manner. In addition, when osteosarcoma cells were pretreated with IL-22, the invasive ability of MG63 and U2OS cells increased significantly in a concentration-dependent manner (Figure 2C–2E). Then, MG63 and U2OS cells were pretreated simultaneously with anti-IL-22 antibody (10 ng/ml) to inhibit the effect of IL-22. IL-22 antibody treatment reduced IL-22-induced proliferation and invasion of MG63 and U2OS cells (Figure 2F–2H).

IL-22 stimulation activates the phosphorylation of STAT3 in osteosarcoma cells

We further studied the molecular mechanism pathways potentially involved in the effect of IL-22 on the proliferation and invasion of osteosarcoma. The expression of p-STAT3 was markedly and dose-dependent increased in MG63 and U2OS cells stimulated with IL-22. Interestingly, IL-22 did not influence the expression of phosphorylated AKT (Figure 3A, 3B). Furthermore, IL-22 (10 ng/ml) stimulation for 30 min markedly induced the phosphorylation of STAT3 without affecting phosphorylation of AKT (Figure 3C, 3D).

IL-22 stimulation promotes osteosarcoma cell proliferation and invasion via STAT3 signaling

To determine whether IL-22 regulates osteosarcoma cell proliferation and invasion via STAT3 signaling, MG63 and U2OS cells were treated with IL-22 or STAT3 siRNA. The results showed that inhibition of STAT3 signaling by STAT3 siRNA significantly inhibited the proliferation and invasion of MG63 and U2OS cells promoted by IL-22 treatment (Figure 4A–4E). These findings suggest that IL-22 promotes MG63 and U2OS cell proliferation and invasion via STAT3 signaling.
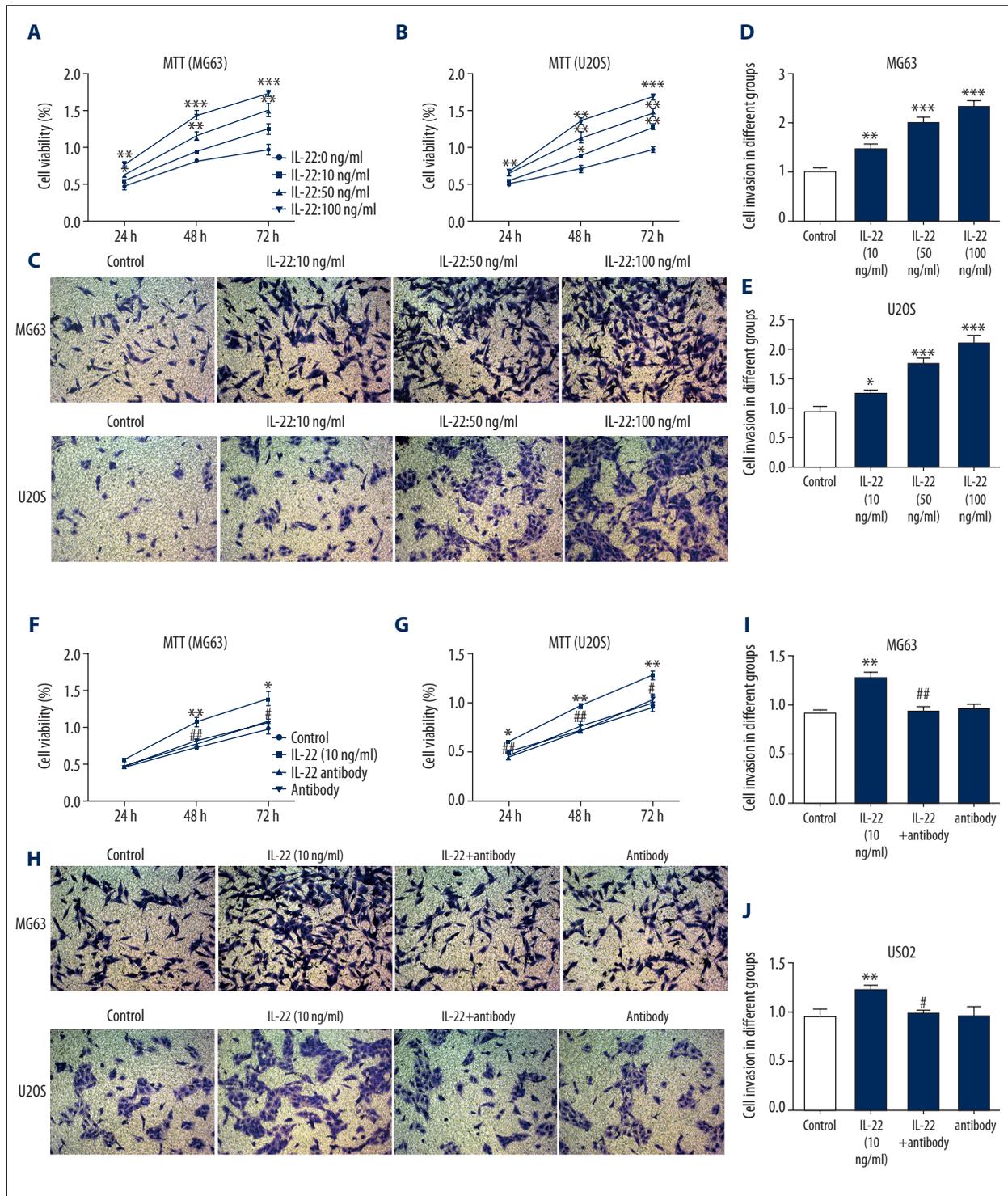
Figure 2. IL-22 promotes the proliferation and invasion of osteosarcoma cells. MG63 and U2OS cells were pretreated with different dosages of IL-22 (0, 10, 50, 100 ng/ml). Then, in order to inhibit the effect of IL-22, MG63 and U2OS cells were treated concomitantly with anti-IL-22 antibody (10ng/ml). MTT assay was used to examine cell proliferation of MG63 (**A, F**) and U2OS cells (**B, G**). Transwell assay was used to examine cell invasion of MG63 and U2OS cells (**C–E, H–J**). Data are expressed as mean ± standard error. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* control. # $P<0.05$, ## $P<0.01$ *vs.* IL-22 (10 ng/ml) group.
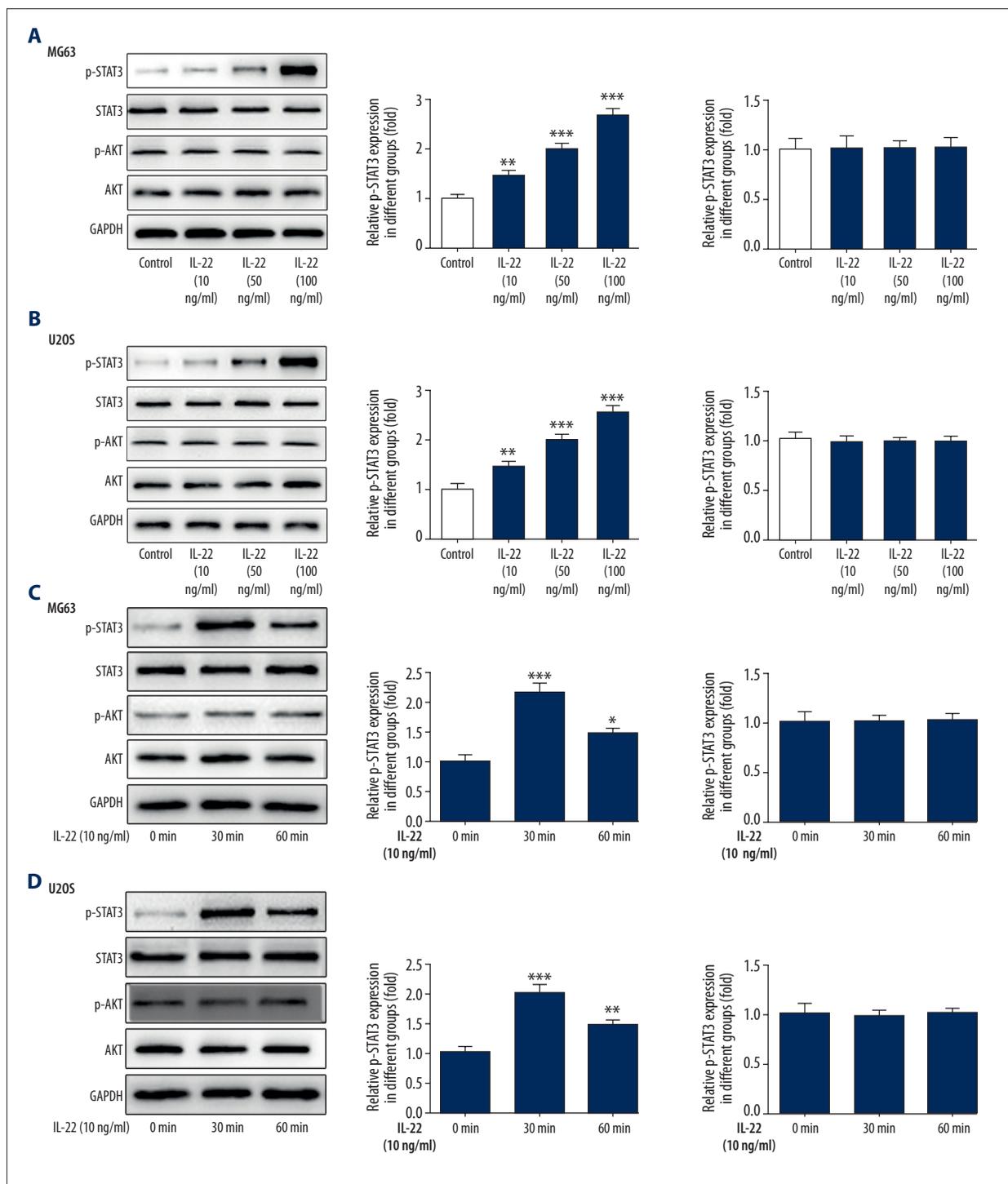
Figure 3. IL-22 stimulation activates the phosphorylation of STAT3 in osteosarcoma cells. Western blot analysis was used to examine the protein expression levels of P-STAT3, STAT3, P-AKT, and AKT in MG63 (**A**) and U2OS cells (**B**) pretreated with different dosages of IL-22 (0, 10, 50, 100 ng/ml). Western blot analysis was used to examine the protein expression levels of P-STAT3, STAT3, P-AKT, and AKT in MG63 (**C**) and U2OS cells (**D**) pretreated with IL-22 (10 ng/ml) for 0 min, 30 min, and 60 min. Data are expressed as mean ± standard error. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* control.
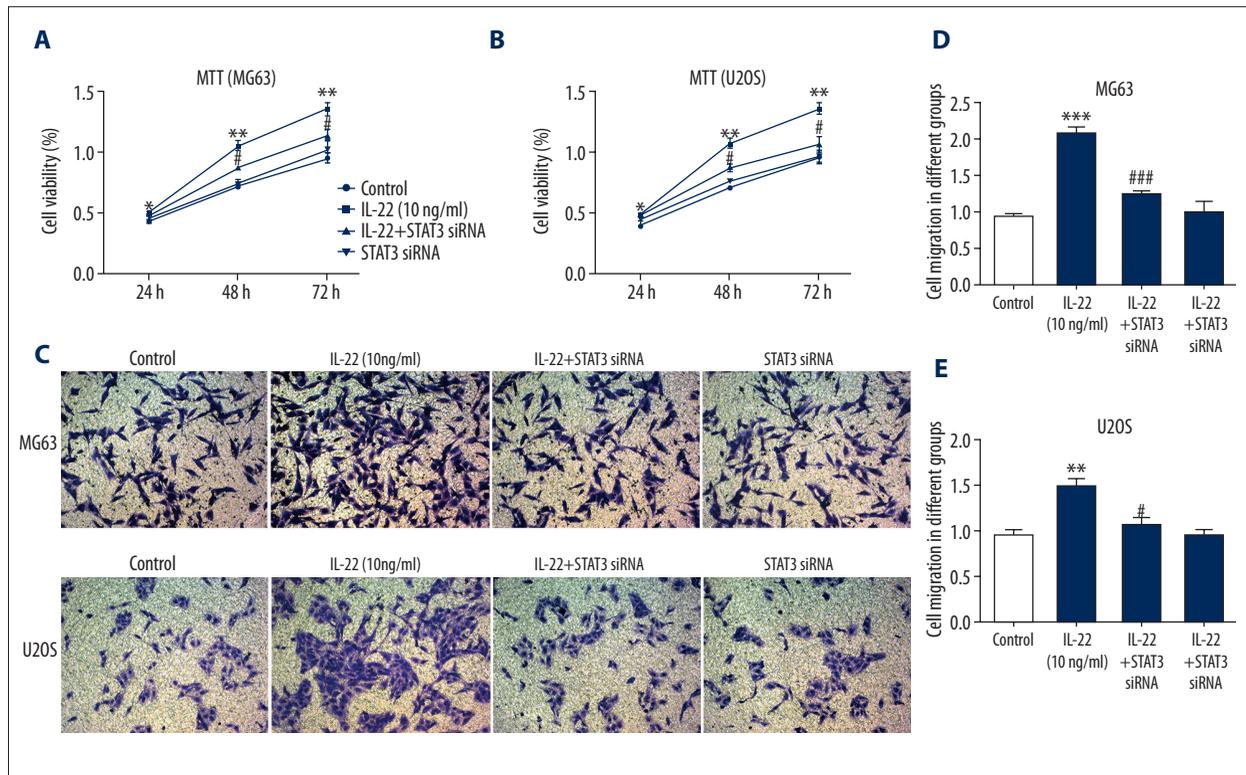
Figure 4. IL-22 stimulation promotes osteosarcoma cell proliferation and invasion via STAT3 signaling. MG63 and U2OS cells were treated with IL-22 or STAT3 siRNA. MTT assay was used to examine cell proliferation of MG63 (**A**) and U2OS cells (**B**). Transwell assay was used to examine cell invasion of MG63 and U2OS cells (**C–E**). Data are expressed as mean ± standard error. ** $P<0.01$, *** $P<0.001$ *vs.* control. # $P<0.05$, ### $P<0.001$ *vs.* IL-22 (10 ng/ml) group.

Discussion

Osteosarcoma is a universally prevalent primary malignant bone cancer with heterogeneous pathogenesis [1]. Inflammatory mediators and cellular effectors are important components of the local environment of tumors and are important factors in inducing tumorigenesis [23]. IL-22 plays an important role in various diseases, including autoimmune diseases, malignant tumors, and infectious diseases [24]. Levels of IL-22 are up-regulated in a variety of human tumors, including prostate, ovarian, and hepatocellular cancers [25]. In addition, experimental data revealed that increased levels of IL-22 are related to tumor growth and metastasis of gastric cancer [26]. In the present study, our data first confirmed that IL-22 was upregulated in the osteosarcoma cells. Furthermore, cell proliferation and invasion tests were performed to assess the effects of IL-22 on the proliferation and invasiveness of OS cell lines MG63 and U2OS. The results showed that IL-22 treatment significantly increased OS cell migration and invasion in a dose-independent manner and its effect could be reversed by IL-22 antibody.

Previous studies indicated that the activity of most inflammatory cytokines is focused on STAT3 [27]. STAT molecules are inherently latent transcription factors that in stay the cytoplasm (inactivated) until they are activated by JAK (Janus kinase) [28]. STATs play a crucial role in normal cell signaling. However, recent evidence has suggested another possible role of STATs in the development of cancer. According to these studies, the constitutively activated STATs, especially STAT3, are the main culprits in the development of cancer [29,30]. It has been reported that multiple signal pathways, such as STAT3, MAPK, and NF-κB, can be activated by IL-22 in different types of cells [31–33]. There is increasing evidence that IL-22 treatment increases cell proliferation and suppresses apoptosis through activation of STAT3 and/or ERK signaling [34,35]. A study reported that IL-22 produced by cancer-associated fibroblasts promotes invasion of gastric cancer cells by activating STAT3 and ERK signaling [34]. IL-22 promotes epithelial cell transformation and breast tumorigenesis by activating MAP3K8 [35]. In the present study we ought to determine whether IL-22 promotes proliferation and invasion of osteosarcoma cells as mediated by STAT3, MG63, and U2OS cells pretreated with si-STAT3. Our results showed that si-STAT3 abolished the effects of IL-22 on proliferation and invasion ability of osteosarcoma cells. This result suggests that STAT3 activation plays important role in IL-22-induced tumor growth.

Conclusions

In conclusion, we found that IL-22 increases osteosarcoma cell proliferation and invasion via STAT3 activation. IL-22 may serve as a molecular biomarker for diagnosis and treatment of OS patients.

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Conflict of interest

None.

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