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*Original Article*

# The oncogenic role of REG γ is exerted by activating the Wnt/β-catenin signaling pathway in osteosarcoma

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**Abstract:** Background: Proteasome activator γ (REG γ) expression was found to be upregulated and to play critical roles in several cancers. However, the effect of REG γ on osteosarcoma (OS) remains unclear. The objective of the present study was to explore the clinical significance of REG γ and its function in regulating the progression of OS. Methods: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR), western blotting (WB) and immunohistochemistry (IHC) analyses were performed to detect the expression levels of REG γ in OS tissues and cell lines. Then, the effects of REG γ expression on OS cell behavior *in vitro* were analyzed by Cell Counting Kit-8 (CCK-8), ethylene deoxyuridine (EdU), colony formation, flow cytometry, wound healing and transwell assays. The protein and mRNA levels of components involved in the Wnt/β-catenin pathway were evaluated using WB and qRT-PCR, respectively. Results: We found that REG γ expression was significantly upregulated in both OS tissues and cell lines. Our *in vitro* assay results confirmed that knockdown of REG γ inhibited cell proliferation, migration, and invasion and induced apoptosis and cell cycle arrest in OS. Additionally, through WB and qRT-PCR analyses, we found that REG γ depletion markedly decreased the β-catenin, cyclin D1 and c-myc expression levels and increased the GSK-3β expression levels in OS cell lines. Conclusions: Our results revealed that REG γ plays an oncogenic role in OS by activating the Wnt/β-catenin pathway, indicating that REG γ may be a promising therapeutic target for OS patients.

**Keywords:** REG γ, osteosarcoma, proliferation, metastasis, invasion, Wnt/β-catenin pathway

## Introduction

Osteosarcoma (OS) is the most common primary bone cancer affecting children and adolescents [1]. OS accounts for less than 0.2% of all cancers; however, the OS mortality rate in children can be up to 50% [2, 3]. Metastasis is the most significant contributor to OS patient death, and the lung is the most common site [4, 5]. The 5-year survival rate is approximately 60% in localized OS and less than 25% in patients with metastasis [5]. Despite the rapid development of treatment strategies, the prognosis of patients with OS has shown no significant improvement in nearly 20 years [6, 7]. Thus, there is an unmet need to identify novel molecules involved in the tumorigenesis and development of OS, and identifying these molecules will be beneficial for the treatment of OS patients.

REG γ, also known as PSME3, PA28g or Ki antigen, is a member of the 11S family of proteasome activators and plays crucial roles in ubiquitin- and ATP-independent nonlysosomal intracellular protein degradation [8]. A previous study showed that REG γ-knockout mice and cells display growth retardation, reduced cell proliferation and increased apoptosis [9, 10]. Moreover, REG γ promotes the degradation of multiple important cancer-related proteins, including steroid receptor coactivator 3 (SRC-3), cyclin-dependent kinase inhibitor p21, tumor suppressor p53 and c-myc [11-14]. Thus, REG γ may be involved in tumorigenesis. In recent years, growing evidence has confirmed that REG γ is overexpressed and promotes proliferation, metastasis and invasion in multiple types of cancer, such as skin carcinogenesis [15], breast cancer [16], renal cell cancer [17] and thyroid carcinoma [18]. However, the expres-

sion patterns, significance and biological roles of REG γ in OS have never been clearly elucidated.

This study aimed to elucidate the expression pattern and biological functions of REG γ in OS. We found that REG γ is overexpressed in OS tissues and cell lines compared with that in the adjacent normal tissues and normal osteoblast hFOB1.19 cells. In addition, we found that knockdown of REG γ in MG-63 and SaoS-2 OS cell lines significantly inhibited proliferation, metastasis and invasion by suppressing the Wnt/β-catenin signaling pathway. Our results demonstrate that REG γ is a crucial oncogene in OS and is a potential new treatment target.

## Materials and methods

### *Clinical tissue samples*

A total of ten OS tissues and eight adjacent normal tissues were obtained from primary OS patients who underwent operative treatment at the Third Affiliated Hospital of Harbin Medical University from June 2018 to December 2018. Seven of the patients received preoperative chemotherapy. Partial tissue specimens were snap-frozen immediately in liquid nitrogen and stored at -80°C until use. The other samples were fixed with 4% paraformaldehyde and then immunohistochemically stained. This study was approved by the Ethics Committees of the Third Affiliated Hospital of Harbin Medical University, and written informed consent was obtained from each patient.

### *Cell culture*

The human OS cell lines MG-63 and SaoS-2 were cultured in RPMI-1640 medium. The normal hFOB1.19 osteoblast cell line was cultured in F-12 medium. All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% $CO_2$. The two OS cell lines and hFOB1.19 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

### *Immunohistochemistry (IHC)*

Tissue samples were fixed with 4% paraformaldehyde, dehydrated through a graded series of ethanol, and embedded in paraffin. The 4-μm sections were deparaffinized, rehydrated, and then stained with hematoxylin and eosin (H&E). Next, the tissue sections were subjected to antigen retrieval, blocked with goat serum and incubated with a primary antibody at 4°C overnight. Subsequently, the sections were incubated with a goat anti-rabbit secondary antibody for 20 min at room temperature and then for 30 min with streptavidin-horseradish peroxidase (HRP). Diaminobenzidine (DAB)-$H_2O_2$ was used as a substrate for the peroxidase enzyme. Then, the sections were stained with hematoxylin and dehydrated. The primary antibody (REG γ) used for IHC analysis was purchased from Proteintech.

### *Transient transfection*

Three small interfering RNAs (siRNAs) specifically targeting human REG γ (siRNA-REG γ) and a nonspecific negative control oligo (siRNA-NC) were purchased from GenePharma (Shanghai, China). The siRNA-REG γ and siRNA-NC sequences are listed in **Table 1**. Cell transfections were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instructions. The transfection efficiency of siRNA-REG γ-1 and siRNA-REG γ-2 was greater than 50%, so they were used in subsequent experiments. Total RNA and total protein were extracted after 48 h and 72 h of transfection, respectively.

### *Western blot analysis*

Total protein was extracted from tissue samples or cultured cells by using cold radioimmunoprecipitation (RIPA) buffer (Beyotime Biotechnology, Shanghai, China) with a protease inhibitor cocktail (Sigma-Aldrich, USA) on ice. The protein concentration was quantified by a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, MA, USA). Membranes were blocked with 5% fat-free milk in PBS for 2 h at room temperature and incubated with a primary antibody at 4°C overnight. After washing with TBS-T, membranes were incubated with an HRP-conjugated secondary antibody for 1 h at 4°C. Finally, the membranes were washed three times, and the specific signals were visualized by a Tanon Chemilumine-

**Table 1.** siRNA and primer sequences

| Name | Sequence |
|---|---|
| Si-NC | Sense: 5'-UUCUCCGAACGUGUCACGUTT-3' |
| | Antisense: 5'-ACGUGACACGUUCGGAGAATT-3' |
| Si-REG γ-1 | Sense: 5'-GCAGAAGACUUGGUGGCAATT-3' |
| | Antisense: 5'-UUGCCACCAAGUCUUCUGCTT |
| Si-REG γ-2 | Sense: 5'-CCAAGGAACCAAGGUGUUUTT-3' |
| | Antisense: 5'-AAACACCUUGGUUCCUUGGTT-3' |
| Si-REG γ-3 | Sense: 5'-GGAUAGAAGAUGGAAACAATT-3' |
| | Antisense: 5'-UUGUUUCCAUCUUCUAUCCTT-3' |
| GAPDH | Sense: 5'-CCACTCCTCCACCTTTGAC-3' |
| | Antisense: 5'-ACCCTGTTGCTGTAGCCA-3' |
| REG γ | Sense: 5'-CTCCTGATACTGTAGCCTCTTGG-3' |
| | Antisense: 5'-AGCATCTGGACCTCACACTTG-3' |

scence Imaging System (Shanghai, China). The signal intensity was determined using ImageJ software. The primary antibodies used for western blot (WB) analysis were purchased from Proteintech (REG γ) and Cell Signaling Technology (p21, Bcl-2, caspase-3, and cyclin D, c-myc).

*RNA isolation and qRT-PCR analysis*

Total RNA was extracted from the OS tissue and cultured cells using TRIzol Reagent (Invitrogen, CA, USA). Then, the RNA concentrations were measured spectrophotometrically, and equal amounts of total RNA (1 μg) were used to generate the corresponding cDNA with a cDNA synthesis kit (Toyobo, Kyoto, Japan) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Green PCR kit (Toyobo, Kyoto, Japan) with a CFX96 Touch Real-Time machine (Bio-Rad, USA) according to the manufacturer's instructions. The relative target gene mRNA expression level was normalized to β-actin by the $2^{-\Delta\Delta Ct}$ method. All the primers used in this study are listed in **Table 1**.

*CCK-8 assay*

Cells transfected with siRNA for 24 h were seeded in a 96-well plate (100 μl, 1500 cells per well). The viability of transfected MG-63 and SaoS-2 cells was determined by a Cell Counting Kit (CCK-8, Dojindo, Tokyo, Japan) at 24, 48 and 72 h; 10 μl CCK-8 solution was added to each well before measurement. After 2 h of incubation, the optical density at 450 nm was detected by an ultraviolet spectrophotometer (Bio-Rad, Hercules, CA, USA).

*Colony formation assay*

After transfection for 24 h, the cells were plated in 6-well plates at a density of $1.5\times10^3$ cells/well and cultured for approximately five days until visible colonies formed. Cells were washed twice with cold PBS, fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime Biotechnology, Shanghai, China). The number of colonies formed was counted and photographed with a digital camera.

*EdU assay*

Cell proliferation was measured using a Cell-Light™ EdU Apollo® 488 *In Vitro* Imaging kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. Briefly, MG-63 and SaoS-2 cells transfected with siRNA for 24 h were seeded in 96-well plates at a density of $6\times10^3$ cells/well. After 24 h, the cells were incubated with 50 μM EdU for 2 h. Then, the cells were fixed with 4% paraformaldehyde, and the cell nuclei were stained with Hoechst 33342. Subsequently, the EdU-positive cells were imaged and counted under a fluorescence microscope.

*Flow cytometry*

To analyze the apoptosis rate, an FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA) was used according to the manufacturer's instructions. After being transfected for 48 h, cultured cells were collected, washed twice with cold PBS and resuspended in 1× binding buffer. Then, the cells were stained with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) in the dark for 15 min at room temperature. The apoptosis rate was measured by flow cytometry using a BD FACSCalibur instrument (Beckman Coulter, CA, USA).

For the cell cycle analysis, cells transfected with siRNA for 48 h were harvested, washed twice with precooled PBS, and fixed in 70% precooled ethanol at 4°C overnight. Then, the cells were washed with precooled PBS and resuspended in 500 μl of solution containing PI and 50 μg/ml RNase A (Sigma-Aldrich) in the dark at room temperature for 20 min. Subse-

quently, the cell cycle analysis was performed by flow cytometry using a BD FACSCalibur instrument.

*Wound healing assay*

Wound healing assays were used to evaluate the migration ability of cells after transfection. Briefly, the cells transfected with siRNAs (Si-NC, Si-REG γ-1 and Si-REG γ-2) were plated in 6-well plates and cultured until the cell confluence reached 90-100%. Confluent cells were scraped with a 200 μl pipette tip to generate an artificial wound, washed with PBS three times to remove the cell debris and then maintained in serum-free medium for 48 h. Wound closure was observed, photographed, and then analyzed by ImageJ software at 0 h, 24 h and 48 h.

*Transwell invasion assay*

The invasion ability of OS cells was evaluated by a transwell invasion assay. First, the transwell chambers (Corning, MA, USA) were precoated with 50 μl of a 1:8 mixture of Matrigel (BD Bioscience, CA, USA): serum-free medium according to the manufacturer's instructions. After transfection with siRNAs for 48 h, $8\times10^4$ cells suspended in 200 serum-free medium were added to the upper chamber, and 600 μl of culture medium with 10% FBS was added to the lower chamber. After incubating for 24 h, cells on the lower side of the membrane were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet. After being washed twice with PBS, the invasive cells were counted and imaged using a microscope.

*Statistical analysis*

Data were expressed as the mean ± SD and analyzed with Student's *t*-test or one-way ANOVA by GraphPad Prism 7.0. All tests were two-tailed, and a *P*-value of <0.05 was considered statistically significant. All experiments were independently performed three times.

## Results

*REG γ is upregulated in OS tissue and cell lines at both the protein and mRNA levels*

The expression of REG γ was examined in OS tissues and adjacent normal tissues by using IHC, WB and qPCR analyses. The results demonstrated that REG γ expression in OS tissues was significantly upregulated compared with that in adjacent normal tissues (**Figure 1A-C**). Consistently, the expression of REG γ was also obviously higher in OS cell lines (MG-63 and SaoS-2) than in normal human osteoblasts (hFOB1.19) (**Figure 1D**, **1E**).

*SiRNAs targeting REG γ reduce the expression of REG γ at the mRNA and protein levels in OS cells*

To reduce the expression of REG γ and avoid off-target phenomena, the cells were transfected with three different siRNAs targeting REG γ and with Si-NC as a control. The qRT-PCR analysis showed significantly decreased levels of REG γ mRNA in the Si-REG γ-1 and Si-REG γ-2 groups compared to that in the Si-NC group (**Figure 2A**, **2B**). WB analysis was performed to determine the expression of the REG γ protein, and there was more than a 50% decrease in the expression of the REG γ protein in the Si-REG γ-1 and Si-REG γ-2 groups compared to that in the Si-NC group (**Figure 2C**, **2D**). Conclusively, these results demonstrated that Si-REG γ-1 and Si-REG γ-2 efficiently downregulate REG γ expression.

*REG γ knockdown inhibits proliferation and induces apoptosis and cell cycle arrest in OS cells*

REG γ expression was markedly upregulated in OS tissues and cell lines, so we hypothesized that REG γ may play an oncogenic role in OS. To confirm the biological functions of REG γ in OS, three siRNAs targeting REG γ were transfected to suppress the expression of REG γ, and several functional assays were performed. Compared to the Si-NC control, siRNA-REG γ-1 and siRNA-REG γ-2 reduced the expression of REG γ by more than 50% at the protein and mRNA levels in the OS cell lines (MG-63 and SaoS-2); the inhibition effect of siRNA-REG γ-2 was higher than that of siRNA-REG γ-1 (**Figure 2A-D**). In addition, a CCK-8 assay showed that knockdown of REG γ effectively suppressed OS cell growth (**Figure 3A**). Similarly, the results of the colony formation assay also demonstrated that the colony formation rate was obviously lower in the REG γ-silenced group than in the control group. The colony formation rate gradually decreased in an REG γ expression-dependent manner (**Figure 3B**). In addition, data from

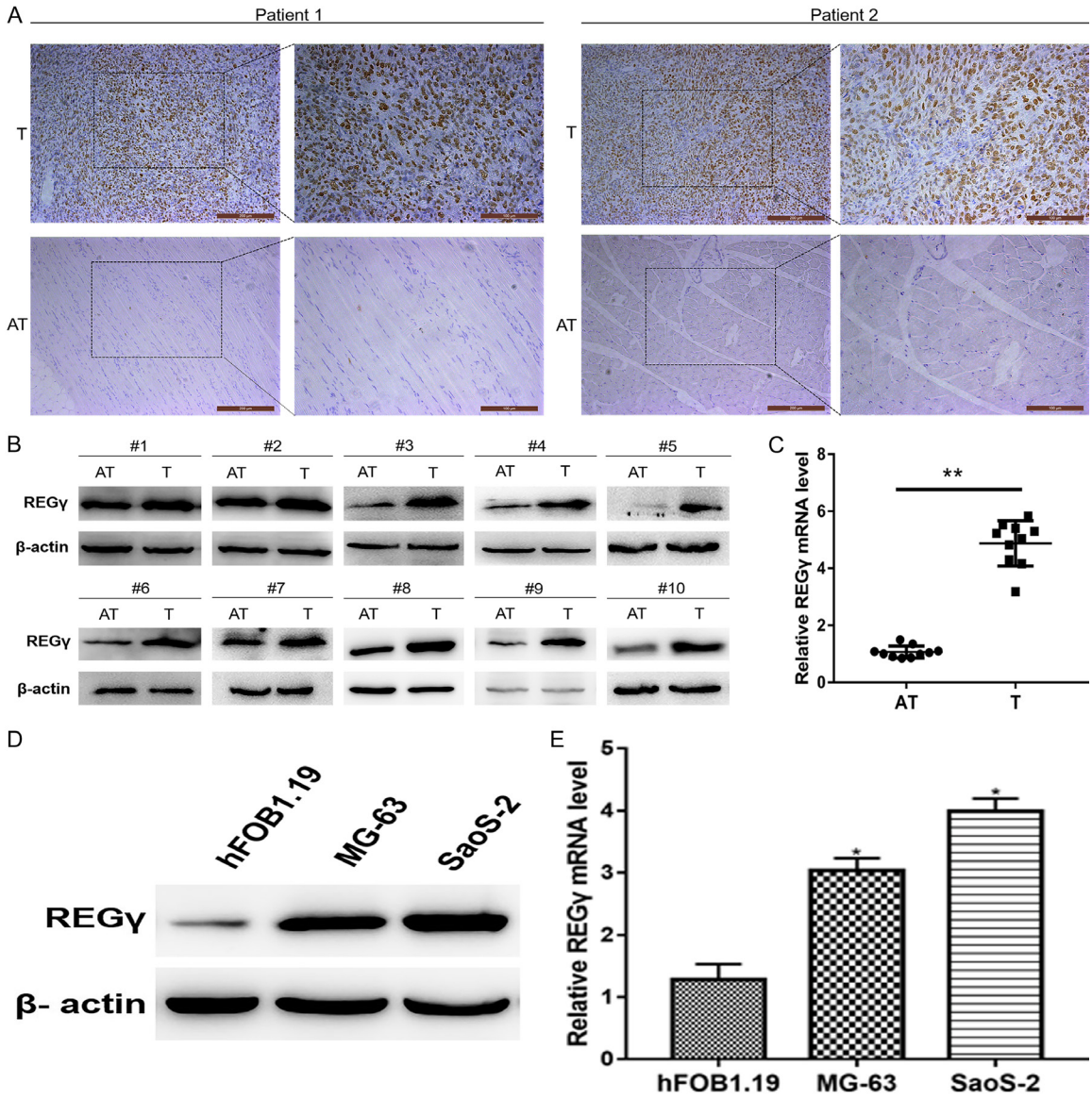



**Figure 1.** REG γ expression is upregulated in OS. (A-C) Expression of REG γ in OS tissues (T) and adjacent normal tissues (AT) as detected by IHC (A), WB (B) and qRT-PCR (C). (D and E) Expression of REG γ in two OS cell lines (MG-63 and SaoS-2) and a normal osteoblast cell line (hFOB1.19), as detected by WB (D) and qRT-PCR (E). Data are shown as the mean ± SD. *P<0.05.

the EdU assay also revealed that REG γ depletion significantly decreased the number of cells in the proliferative period (**Figure 3C**). Consistent with the EdU results, we observed in the flow cytometry analysis that the percentage of OS cells in G0/G1 phase was increased, but the percentage in S and G2/M phase was decreased following REG γ downregulation (**Figure 3E**). Furthermore, we measured the effect of REG γ knockdown on OS cell apoptosis and found that the REG-γ-knockdown group had a dramatically increased apoptosis rate compared to that of the NC group, as determined by flow cytometry (**Figure 3D**). Given these results, we found that REG γ knockdown inhibits OS cell proliferation and induces apoptosis and cell cycle arrest.

*REG γ knockdown impairs the migration and invasion abilities of OS cell lines*

After confirming that REG γ plays an essential role in the regulation of OS cell proliferation, we next sought to investigate the effect of REG γ on the mobility of OS cells. Wound healing assays illustrated that suppressing the expres-

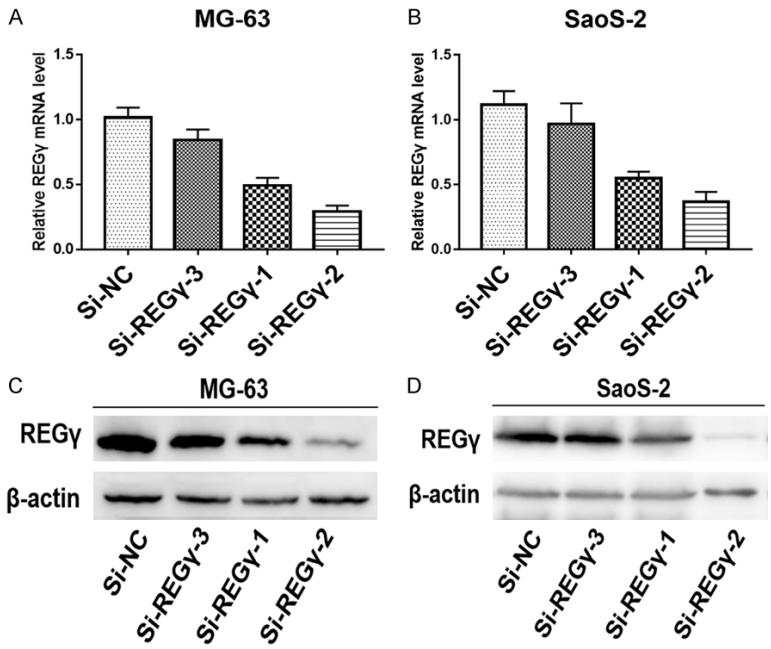



**Figure 2.** Knockdown of REG γ in OS cell lines (MG-63 and SaoS-2) confirmed by qRT-PCR (A and B) and WB (C and D). Si-REG γ reduces the expression of REG γ at the mRNA (A and B) and protein levels (C and D) in OS cells. Compared to Si-NC, Si-REG γ-1 and Si-REG γ-2 inhibit more than 50% of REG γ expression and Si-REG γ-3 inhibits less than 50% of REG γ expression.

sion of REG γ notably attenuated the metastatic abilities of MG-63 and SaoS-2 cells (**Figure 4A**, **4B**). We then performed transwell invasion assays to assess the impact of REG γ deficiency on cell invasion. We found that REG γ depletion markedly decreased the number of invaded MG-63 and SaoS-2 cells (**Figure 4C**, **4D**). Therefore, these data demonstrated that REG γ is involved in OS cell metastasis and invasion.

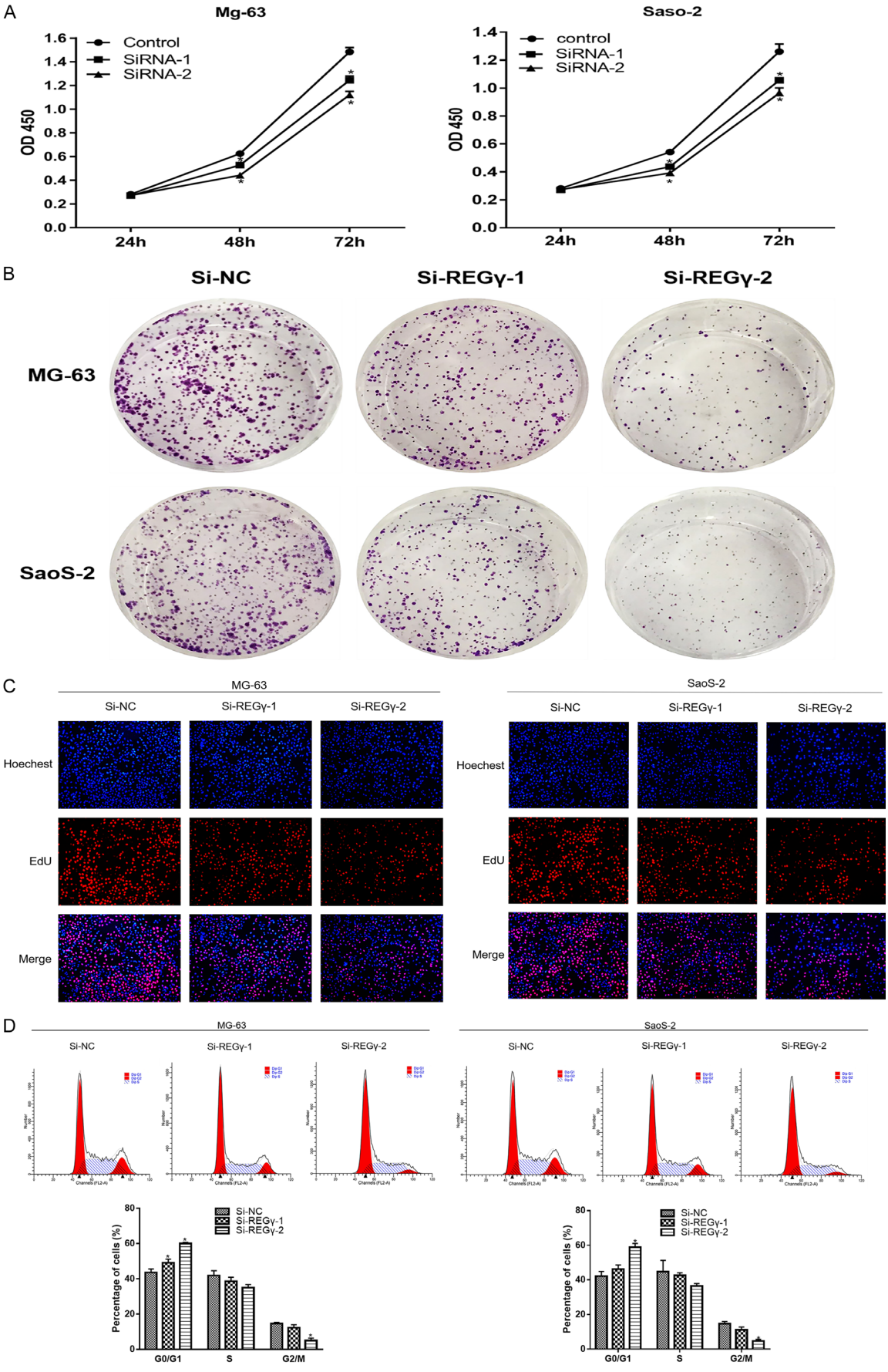
*REG γ depletion suppresses the Wnt/β-catenin signaling pathway in OS cell lines*

To further research the oncogenic molecular mechanisms of REG γ in OS, we investigated the activation of the REG γ-related Wnt/β-catenin signaling pathway. We mainly focused on the four key components of this signaling pathway: GSK-3β, β-catenin, cyclin D1, and c-myc. The WB results showed that the expression of β-catenin, cyclin D1, and c-myc was lower in cells transfected with Si-REG γ than in cells transfected with Si-NC, while the level of GSK-3β was higher (**Figure 5A**, **5B**). Previous studies demonstrated that REG γ can regulate the stabilization of GSK-3β via direct degradation, and we confirmed these results with SaoS-2 cells. After transfection for 24 h with Si-NC or Si-REG γ-2, SaoS-2 cells were cultured with complete media with 100 μg/ml cycloheximide (Chx) for different time periods, and the protein level of GSK-3β was analyzed by WB. We observed that knockdown of REG γ enhanced the stabilization of GSK-3β in SaoS-2 cells (**Figure 5C**). Therefore, these results illustrate that REG γ depletion inhibits the activation of the Wnt/β-catenin signaling pathway in OS.

## Discussion

In the present study, we analyzed the potential role of REG γ in human OS using siRNAs. We demonstrated that REG γ functions as an oncogene for OS. We confirmed for the first time that REG γ is overexpressed in OS tissues and cell lines. Moreover, REG γ silencing inhibited the proliferation, metastasis and invasion of OS by suppressing the activation of the Wnt/β-catenin signaling pathway, indicating that targeting REG γ could be an alternative approach for OS therapy in the future.

The proteasome system is an important regulator of the development and progression of cancers, and specific proteasome inhibitors can act as novel anticancer agents [19]. REG γ, a crucial activator of the proteasome system, has attracted increasing attention in the field of oncology. In 1999, Murata et al. first illustrated the important roles played by REG γ in regulating cell proliferation and apoptosis [9]. Since then, accumulating evidence has revealed that REG γ is extensively involved in the proliferation and progression of multiple cancers. Lei Li et al. reported that REG γ acts in skin tumorigenesis via MAPK/p38-mediated activation of the Wnt/β-catenin pathway [15]. Moreover, REG γ overexpression increases the proliferation, migration and invasion of breast cancer and correlates with shorter overall survival of patients with breast cancer [20, 21]. In addition, previous studies demonstrated that knockdown of REG γ suppresses the growth of cancer cells by inducing apoptosis and cell cycle arrest at the


A
Mg-63
Control
SiRNA-1
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Si-NC
Si-REGγ-1
Si-REGγ-2
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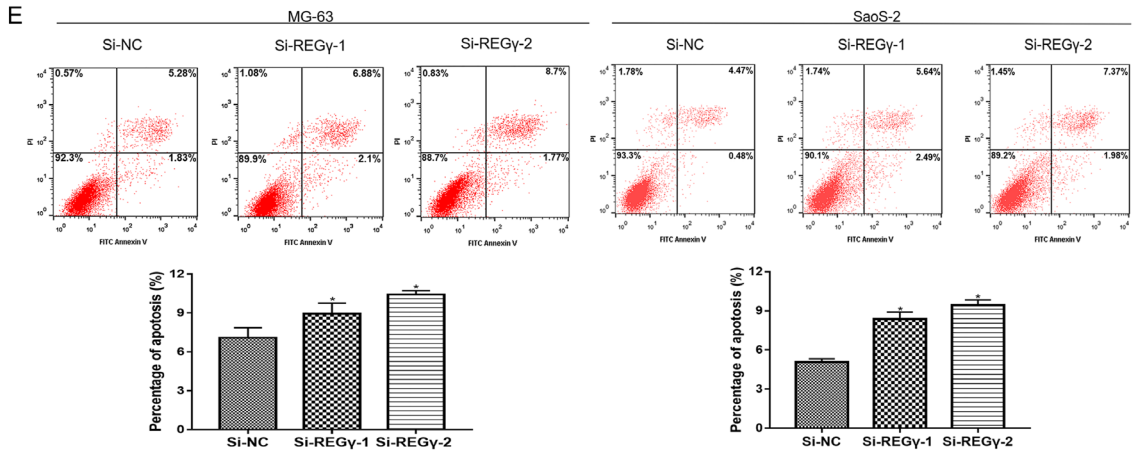


**Figure 3.** REG γ depletion suppresses OS cell progression *in vitro*. A. Effect of Si-REG-γ-1 and Si-REG-γ-2 on OS cell growth as determined by a CCK-8 assay. B. Representative OS cell colony formation images after transfection of Si-REG γ versus Si-NC. C. Representative images of an EdU incorporation assay after transfection of Si-REG γ compared to after transfection of Si-NC. D. Apoptosis rates of MG-63 and SaoS-2 cells after transfection with Si-REG γ and Si-NC, as determined by flow cytometry. E. Representative flow cytometry analysis of the cell cycle distribution of MG-63 and SaoS-2 cells transfected with Si-REG γ and Si-NC. Data are shown as the mean ± SD. *P<0.05.

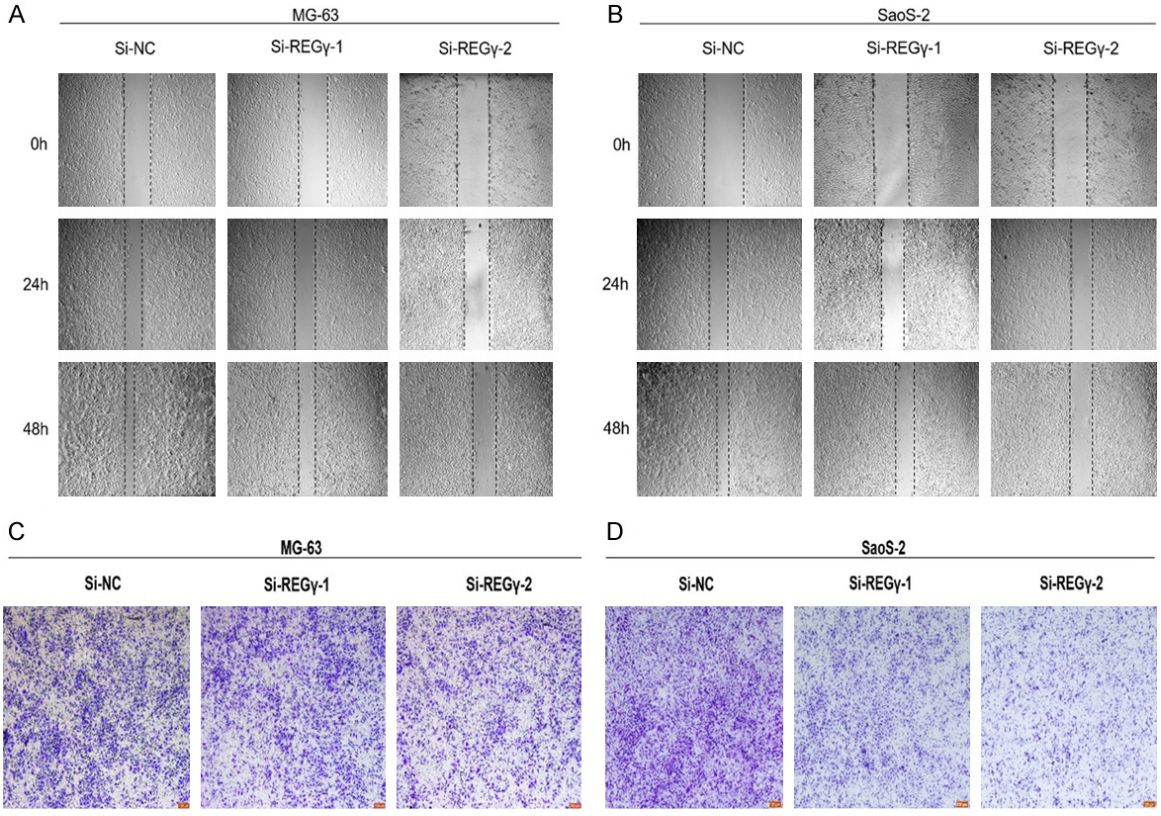


**Figure 4.** REG γ depletion inhibits OS cell migration and invasion. A and B. Representative images of the wound healing assay in OS cells after transfection. C and D. Representative images of the transwell invasion assay in MG-63 and SaoS-2 cells after transfection.

G0/G1 phase in prostate cancer [22], renal carcinoma [17] and thyroid carcinoma [18]. In parallel with these results, we first found in our experiments that REG γ depletion leads to an

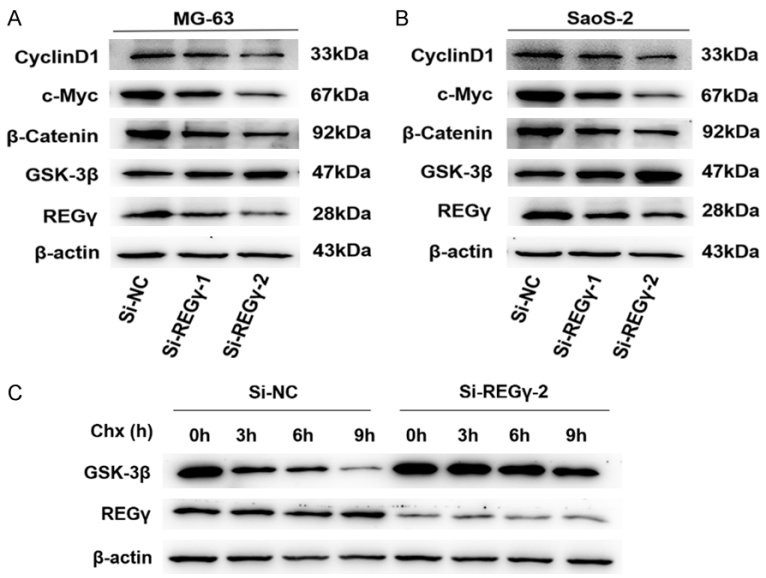


**Figure 5.** REG γ deficiency impairs the activation of the Wnt/β-catenin signaling pathway in OS development. (A and B) Four important components of the Wnt/β-catenin signaling pathway (GSK-3β, β-catenin, c-myc and cyclin D1) as evaluated by WB in MG-63 (A) and SaoS-2 (B) cells after transfection. (C) GSK-3β level in SaoS-2 cells transfected with Si-NC or Si-REG γ-2 was detected by WB after Chx (100 μg/ml) treatment.

increase in the number of OS cells arrested at the G0/G1 phase. Nevertheless, another study demonstrated that REG γ downregulation contributes to cell cycle arrest at the G2/M phase in HeLa cells [12]. This inconsistency between the results may be attributed to the different mechanisms by which REG γ depletion results in cell cycle arrest at different phases in different cell lines. Interestingly, the percentage of apoptotic OS cells transfected with Si-REG γ was higher than that of cells transfected with Si-NC, and this result is also supported by previous research [17, 22, 23].

OS is characterized by early metastasis and invasion, and metastasis lowers the 5-year survival rate by more than 35% [5]. Thus, an optimal therapeutic target should play pivotal roles in the migration and invasion of OS. Next, we investigated the effect of REG γ on migration and invasion in MG-63 and SaoS-2 cells and clarified for the first time that knockdown of REG γ impairs OS cell line metastasis and invasion. Above all, REG γ may be a novel therapeutic target in patients with OS.

Wnt/β-catenin is a highly conserved signaling pathway that regulates gene transcription, differentiation, and proliferation [24]. This pathway has been implicated in the pathogenesis of various cancers, especially OS [25], by modulating its downstream targets, including GSK-3β, β-catenin, cyclin D1 and c-myc [14, 15, 26, 27]. In addition, a previous study demonstrated that REG γ activates the Wnt/β-catenin signaling pathway by degrading GSK-3β in skin carcinoma [15] and renal carcinoma [28]. To determine whether REG γ promotes the proliferation, migration and invasion of OS cells by modulating the Wnt/β-catenin signaling pathway, we performed WB and qRT-PCR analyses to detect the protein and mRNA expression levels of four key components of this signaling pathway (GSK-3β, β-catenin, cyclin D1, and c-myc) in MG-63 and SaoS-2 cells after REG γ knockdown. The results showed that the mRNA and protein levels of β-catenin, cyclin D1, and c-myc gradually decreased in the REG γ-knockdown group, while the expression of GSK-3β gradually increased. Therefore, knockdown of REG γ upregulated GSK-3β expression and inhibited the Wnt/β-catenin signaling pathway, leading to a decrease in cyclin D1 and c-myc expression. In summary, REG γ has an oncogenic effect on human OS cells by degrading GSK-3β to activate the Wnt/β-catenin signaling pathway.

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## Disclosure of conflict of interest

None.

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