REG γ knockdown suppresses proliferation by inducing apoptosis and cell cycle arrest in osteosarcoma

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Background: Osteosarcoma (OS) is the most common malignant bone tumor with high mortality in children and adolescents. REG γ is overexpressed and plays important roles in various types of human cancers. However, the expression and potential roles of REG γ in osteosarcoma are elusive. This study aims at exploring possible biological functions of REG γ in the pathogenesis of osteosarcoma and its underlying mechanism.

Methods: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR), western blotting and immunohistochemistry (IHC) 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. The protein levels of apoptosis and cell-cycle related proteins were evaluated using western blotting.

Results: In present study, we found for the first time that REG γ is overexpressed in osteosarcoma tissues and cell lines and knockdown of REG γ significantly inhibits cell proliferation and induces apoptosis and cell cycle arrest in osteosarcoma cells. Furthermore, we observed that p21 and caspase-3 are increased while the expression of cycinD1 and bcl-2 are decreased after REG γ depletion in osteosarcoma cells. In conclusion, REG γ may involve in proliferation of osteosarcoma and serve as a novel therapeutic target in patients with osteosarcoma.

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Abstract:

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Key words: Osteosarcoma; Proliferation; REG γ

Introduction:

Osteosarcoma (OS) is the most common primary malignant bone cancer affecting children and adolescents(Botter et al. 2014). Although OS only accounts for less than 0.2% of all cancers, mortality rate of OS is up to 50% in children(Siegel et al. 2016). Despite rapid development in treatment strategies, prognosis of patients with OS has shown no significant improvement in nearly 20 years(Kempf-Bielack et al. 2005; Marko et al. 2016). As a result, there is an unmet need to identify novel molecules involved in the tumorigenesis of OS, which will be beneficial for treatment of patients with OS.

REG γ, also known as PSME3, PA28g or Ki antigen, is a member of the 11S family of proteasome activator and plays crucial roles in an ubiquitin- and ATP-independent non-lysosomal intracellular protein degradation. Previous researches showed that REG γ-knockout mice and cells display growth retardation, reduced cell proliferation and increased apoptosis(Li et al. 2013). Moreover, REG γ can promote the degradation of multiple cancer-related proteins (Chen et al. 2007; Li et al. 2015b; Li et al. 2006; Wang et al. 2015). Additionally, growing evidence have confirm that REG γ is overexpressed in multiple types of cancers, such as skin carcinogenesis(Li et al. 2015a), breast cancer(Yi et al. 2017), renal cell cancer(Chen et al. 2018) and thyroid carcinoma(Zhang et al. 2012). However, the expression and biological functions of REG γ in OS have never been elucidated.

This study aimed to elucidate the expression and biological functions of REG γ in OS. We found that REG γ is overexpressed in OS tissues and cell lines compared with the adjacent

normal tissues and the normal osteoblast hFOB1.19 cell, respectively. In addition, we found that knockdown of REG γ significantly inhibited proliferation and induced apoptosis and cell cycle arrest in MG-63 and SaoS-2 cell lines. Moreover, we also observed that multiple apoptosis and cell cycle related proteins expression were altered in REG γ-silenced osteosarcoma cells. Our results demonstrated that REG γ plays an oncogenic role in osteosarcoma and may be a molecular target in the treatment of patients with OS.

Materials and methods:

Clinical tissue samples

A total of eight OS tissues and eight adjacent normal tissues were obtained from primary osteosarcoma 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

Human OS cell lines MG-63 and SaoS-2 were cultured in RPMI-1640 medium. The normal osteoblast hFOB1.19 cell was cultured in F-12 medium. All media were supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured in a humidified incubator at 37 °C with 5% CO2. The two OS cell lines and hFOB1.19 were obtained

from Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Immunohistochemistry

Tissue samples were fixed with 4% paraformaldehyde, dehydrated by a gradient series of ethanol, and then 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, be 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-HRP peroxidase. Diaminobenzidine (DAB)-H2O2 was used as a substrate for the peroxidase enzyme. Then, the sections were stained with hematoxylin and dehydration. The primary antibody (REG γ) used for IHC analysis was purchased from Proteintech.

Transient transfection

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

Western blot

105 Total protein was extracted from tissue samples or cultured cells by using a cold RIPA buffer
106 (Beyotime Biotechnology, Shanghai, China) with protease inhibitor cocktail (Sigma-Aldrich,
107 USA) on ice. Protein concentration was quantified by bicinchoninic acid (BCA) protein assay kit
108 (Beyotime Biotechnology, Shanghai, China). Equal amount of protein was separated by 10%
109 SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF, Millipore, MA,
110 USA). Membranes were blocked with 5% fat-free milk in PBS for 2h at room temperature and
111 then incubated with a primary antibody at 4 °C overnight. After washed with TBST, membranes
112 were incubated with an HRP-labeled secondary antibody for 1 h at 4 °C. Finally, membranes
113 were washed three times and the specific signals were visualized by a Tanon
114 Chemiluminescence Imaging System (Shanghai, China). The intensity was determined using
115 ImageJ software. The primary antibodies used for WB were purchased from Proteintech (REG γ,
116 β- actin) and Cell Signaling Technology (p21, bcl-2, caspase-3, cyclin D1).

117 **RNA isolation and qRT-PCR analysis**

118 Total RNA was extracted from the OS tissue and cultured cells using TRIzol reagent (Invitrogen,
119 CA, USA) and cDNA synthesis kit (Toyobo, Kyoto, Japan) was used in generating cDNA
120 according to the manufacturer's protocol. Quantitative reverse transcription-polymerase chain
121 reaction (qRT-PCR) was performed using SYBR Green PCR kit (Toyobo, Kyoto, Japan) with an
122 CFX96 Touch real time machine (Bio-Rad, USA) according to manufacturers' instructions. All
123 the primers used in this study were showed in Table1.

124 **CCK-8 assay**

125 Cells transfected with siRNA for 24 h were seeded in a 96-well plate (100μl, 2000 cells per

126 well). The viability of transfected MG-63 and SaoS-2 cells were determined by a Cell Counting
127 Kit (CCK-8, Dojindo, Tokyo, Japan) at 24, 48 and 72 h and 10 μl CCK-8 solution was added to
128 each well before measurement. After 2 h of incubation, the optical density at 450 nm was
129 detected by an ultraviolet spectrophotometer (Bio-Rad, Hercules, CA, USA).

130 **Colony formation assay**

131 After being transfected for 24 h, the cells were plated into 6-well plates at a density of 1.5×
132 10^3/well and cultured for approximately five days until visible colonies formed. Cells were
133 washed twice with cold PBS, fixed with 4% paraformaldehyde and then stained with 0.1%
134 crystal violet (Beyotime Biotechnology, Shanghai, China). The number of colony formation was
135 counted and photographed with a digital camera.

136 **EdU assay**

137 Cell proliferation was measured using the Cell-Light™ EdU Apollo®488 *In Vitro*
138 Imaging kit (Ribobio, Guangzhou, China) according to the manufacturer's instructions. Briefly,
139 MG-63 and SaoS-2 cells transfected with siRNA for 24 h were seeded into 96-well plates at a
140 density of 6 ×10^3/well. After 24 hours, cells were incubated with 50μM EdU for 2 h. Then, the
141 cells were fixed with 4% paraformaldehyde and the cell nuclei were stained with Hoechst 33342.
142 Subsequently, the EdU-positive cells were captured and counted with a fluorescent microscope.

143 **Flow cytometry**

144 To analyze cells apoptosis rate, FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, San
145 Jose, CA) was used according to the manufacturer's instructions. After being transfected for 48
146 h, cultured cells were collected, washed twice with cold PBS and resuspended in 1 ×binding

147 buffer. Then, cells were stained with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) in the
148 dark for 15 min at room temperature. For the cell cycle analysis, cells transfected with siRNA for
149 48 h were harvested, washed twice with precooled PBS, and fixed in 70% precooled ethanol at 4
150 °C overnight. Then, the cells were washed with precooled PBS and resuspended in 500 μl
151 solution containing PI and 50 μg/ ml RNase A (Sigma-Aldrich) in the dark at room temperature
152 for 20 min. All the flow experiments were performed using BD FACS Calibur (Beckman
153 Coulter, CA, USA).

154 **Statistical analysis**

155 Data were presented as the mean ± SD and analyzed with Student's *t*-test or one-way ANOVA
156 by GraphPad Prism7.0. All tests were two-sided, and a *P*-value of <0.05 was considered
157 statistically significant. All experiments were independently performed three times.

158 **Results:**

159 1. REG γ is upregulated in OS tissue and cell lines at both protein and mRNA levels

160 The expression of REG γ was examined in osteosarcoma tissues and adjacent normal tissues
161 by using IHC, WB and qRT-PCR analysis. Results demonstrated that REG γ expression was
162 significantly overexpressed in OS tissues compared with adjacent normal tissues (Fig. 1A, B, C).
163 To further confirm the upregulated expression of REG γ in OS, western blot and RT-PCR
164 analysis were performed in OS cell lines (MG-63 and SaoS-2) and human normal osteoblast
165 (hFOB1.19) (Fig. 1D, E). Meanwhile, bioinformatic results from the Oncomine open cancer
166 microarray database (https://www.oncomine.org/) also shown that REG γ mRNA levels were
167 higher in sarcoma than in normal tissues (Fig. 1F).

2. SiRNAs targeting REG γ reduce the expression of REG γ at mRNA and protein level in OS cells

To reduce the expression of REG γ and avoid off-target phenomenon, the cells were transfected with three different siRNAs targeting REG γ and with Si-NC as control. The qRT-PCR analysis showed significantly decreased levels of REG γ mRNA in Si-REG γ-1 and Si-REG γ-2 groups compared to Si-NC group (Fig. 2A, B). Consistently, Si-REG γ-1 and Si- REG γ-2 also markedly inhibited the REG γ expression at protein levels as shown as in western blot analysis (Fig. 2C, D). Conclusively, Si-REG γ-1 and Si-REG γ-2 efficiently downregulated REG γ expression.

3. REG γ knockdown inhibits proliferation in MG-63 and SaoS-2 cells.

To confirm REG γ biological functions in osteosarcoma, we performed a series of functional assays in cells after transfection. Compared to Si-NC, siRNA-REGγ-1 and siRNA-REGγ-2 were able to effectively suppressed OS cells growth determined by CCK-8 (Fig. 3A). Similarly, results of colony formation assay also demonstrated that the colon formation rates were obviously lower in REG γ silenced group than that in control group and gradually decreased in REG γ expression-dependent manner (Fig. 3B). In addition, data from EdU assay also revealed that REG γ depletion significantly decreased the number of cells in proliferative period (Fig. 3C). Taken together, we found that REG γ knockdown inhibits OS cells proliferation.

4. REG γ knockdown induces apoptosis and cell cycle arrest in OS cell lines

To elucidate the underlying mechanism of silencing REG γ inhibiting OS proliferation, we performed flow cytometry experiments and western blot analysis. We observed that the

189 percentage of OS cells was increased in G0/G1 phase while decreased in S and G2/M phase
190 following REG γ downregulation in flow cytometry (Fig. 4A). Furthermore, we also measured
191 the effect of REG γ knockdown on OS cells apoptosis and found that the reduction of REG γ
192 dramatically increased OS cells apoptotic rate determined by flow cytometry (Fig. 4B). In
193 addition, we quantified the apoptosis and cell cycle related genes in OS cells after REG γ
194 knockdown at protein levels. We found that the expression of bcl-2 was downregulated after
195 transfected with siRNA-REG γ, while caspase-3 was upregulated (Fig. 4C, D). Furthermore, cell
196 cycle related genes were also significantly altered by siRNA- REG γ. Compared with Si-NC
197 group, higher expression of p21 and lower expression of cyclinD1 were observed in siRNA-REG
198 γ groups (Fig. 4C, D).

199 **Discussion:**

200 In this study, we investigated the potential role of REG γ in human osteosarcoma. We revealed
201 that REG γ was significantly overexpressed in human osteosarcoma tissues and cell lines for the
202 first time. Knockdown of REG γ inhibited osteosarcoma cells proliferation, prompted cells
203 apoptosis and cell cycle arrest, indicating that targeting REG γ could be an alternative for human
204 osteosarcoma therapy in the future.

205 Murata et al found that REG γ-deficient mice display growth retardation compared with wild
206 type mice. Meanwhile, previous studies reported that REG γ was strongly implicated in many
207 kinds of cancer cells proliferation(Chen et al. 2018; Li et al. 2015a; Zhang et al. 2012). Our
208 results are consistent with these data, indicating that REG γ plays a critical role in osteosarcoma
209 proliferation. To seek further the underlying mechanism that REG γ prompting human

osteosarcoma proliferation, we used RNA interference to reduce REG γ expression in MG-63 and SaoS-2 human osteosarcoma cell lines, then performed cell-cycle profile and apoptosis analysis by flow cytometry and measured the cell cycle and apoptosis related proteins level.

Knockdown of REG γ lead more cells to be arrested at the G0/G1 phase in our experiments, that was parallel with other independent studies on prostate cancer cells and renal carcinoma cells(Chen et al. 2018). However, another study also demonstrated that REG γ downregulation resulted in cell cycle arrest at the G2/M phase in HeLa cells(Chen et al. 2007). Therefore, we speculated that REG γ depletion lead to cell cycle arrest by different mechanisms in different cell types. Moreover, apoptotic percent of osteosarcoma cells transfected with Si-REG γ was higher than that in Si-NC group. Meanwhile, we found that proapoptotic protein (caspase-3) level was significantly increased, while antiapoptotic protein (bcl-2) was decreased following REG γ knockdown. Present results were supported by previous researches(Chen et al. 2018; Moncsek et al. 2015).

Additionally, we discovered that proteins regulating cell cycle also were altered by siRNA interference of REG γ. CyclinD1, known as a regulator of cyclin-dependent kinases, is indispensable in transition from G0/G1 phase to S phase and has been reported to serve as an oncogene in several types of cancers, including colorectal cancer(Wei et al. 2019), breast cancer(Hosseini et al. 2019) and osteosarcoma(Li et al. 2017). Therefore, we investigated the cyclinD1 expression and disclosed that level of cyclinD1 protein was gradually downregulated in Si-REG γ groups. Consistent with the phenomenon that more cells transfected with Si-REG γ were arrested at the G0/G1 phase than cells transfected with Si-NC. A previous study also has

231 shown that the depletion of REG γ leads to a striking decrease in cyclinD1 levels in prostate
232 cancer cells. Taking all these results into account, we demonstrated that cyclinD1 may play an
233 important part in the REG γ-related control of cell cycle progression in osteosarcoma. In
234 addition, p21 serves as a broad-spectrum cyclin-dependent kinases inhibitor and takes part in
235 regulating the cell cycle in many types of cells(Weinberg & Denning 2016). However, there has
236 been significant debate as to whether the REG γ depletion increases p21 protein expression level.
237 Previous studies revealed that the reduction of REG γ lead to a markedly increase in p21 in TPC
238 cells, but only had a relatively slight effect on p21 levels in MCF-7 cells and even had no effect
239 on p21 expression in HepG2 and 3T3-L1 cells(Chen et al. 2007), indicating a cell type-specific
240 effect of REG γ depletion on p21 protein expression. Given distinct effect of REG γ depletion on
241 different kinds of cells, we measured the p21 level in cells after transfection. Interestingly, we
242 conformed for the first time that REG γ knockdown significantly increases the level of the p21
243 protein in osteosarcoma cell lines in the present study.

244 **Conclusions:**

245 We revealed for the first that REG γ was significantly overexpressed in human osteosarcoma
246 tissues and cell lines for the first time. Knockdown of REG γ inhibited osteosarcoma cells
247 proliferation, prompted cells apoptosis and cell cycle arrest, we speculate that the REG γ-
248 controlled proliferation of osteosarcoma cells is probably due to the role of REG γ in regulating
249 the cell-cycle and apoptosis relevant proteins, but the specific mechanism is still unclear. Thus,
250 further study is needed to comprehensively understand the importance of REG γ in the
251 development and progression of osteosarcoma.

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None.

Disclosure of conflict of interest:

The authors declare no conflict of interest.

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Figure legends:

Fig. 1 REG γ expression is upregulated in OS. A, B,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, 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 and qRT-PCR. F REG γ expression (median expression

322 intensity) in sarcoma tissues and adjacent normal tissues derived from the Oncomine database
323 (https://www.oncomine.org/).

324 **Fig. 2** Si-REG γ reduce the expression of REG γ at mRNA (A,B) and protein level (C,D) in OS
325 cells. Compared to Si-NC, Si-REG γ-1 and Si-REG γ-2 inhibit more than 50 percent of REG γ
326 expression and Si- REG γ-3 inhibit less than 50 percent of REG γ expression at mRNA level
327 (A,B) and protein level (C,D).

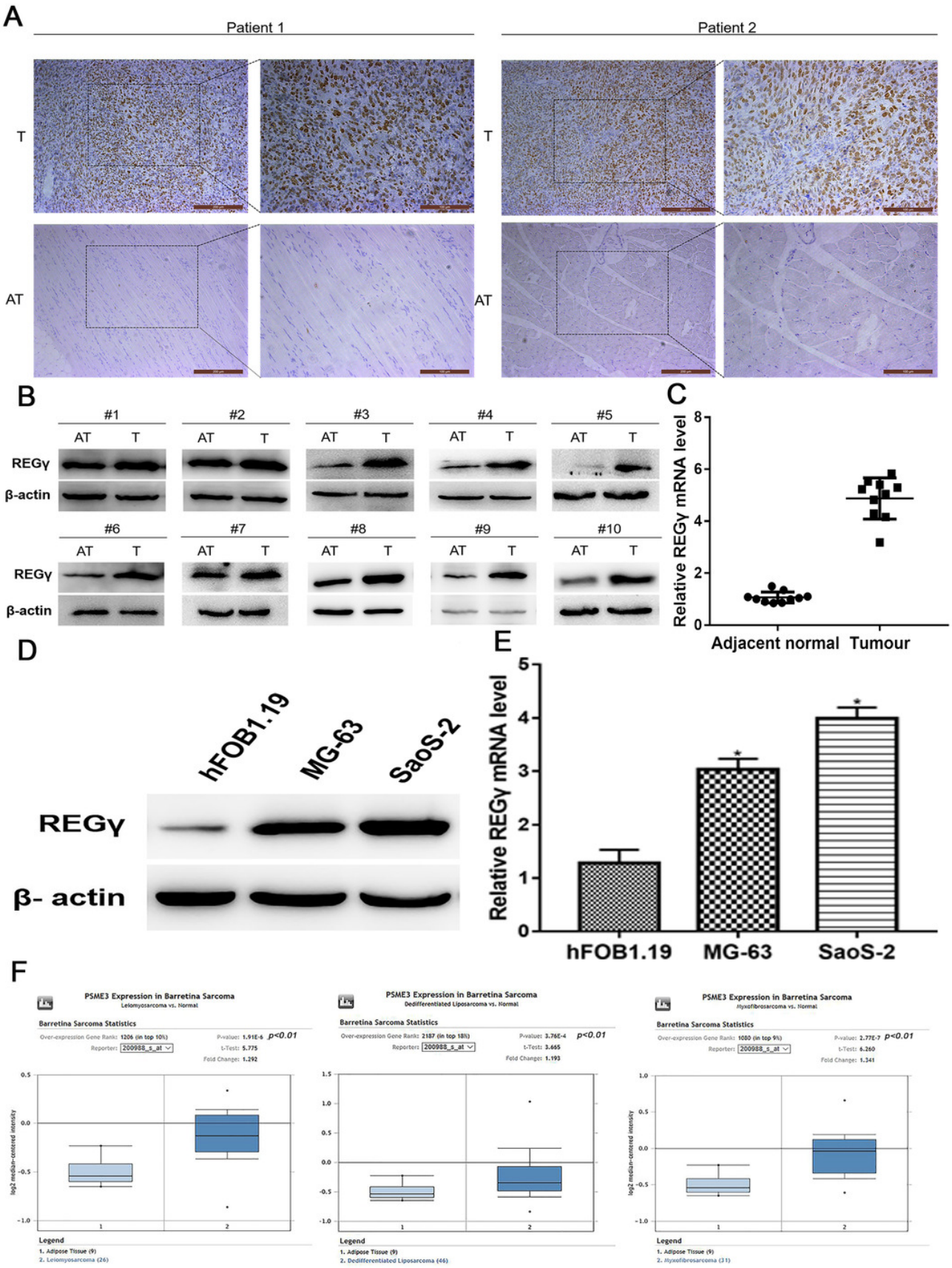
328 **Fig. 3** REG γ depletion suppresses OS cell progression *in vitro*. A Effect of Si-REG-γ-1 and Si-
329 REG-γ-2 on OS cell growth as determined by CCK-8 assay. B Representative OS cell colony
330 formation images after transfection of Si-REG γ versus Si-NC. C, D Representative images of
331 the EdU incorporation assay after transfection of Si-REG γ compared to after transfection of Si-
332 NC in Mg-63 (C) and in SaoS-2（D. Data are shown as the mean ± SD. *P <0.05.

333 **Fig. 4** REG γ deficiency induce apoptosis and cell cycle arrest and alters multiple cell apoptosis
334 and cell cycle related genes in MG-63 and SaoS-2. A, B Representative flow cytometry analysis
335 of the cell cycle distribution of MG-63 and SaoS-2 cells transfected with Si-REG γ and Si-NC.
336 C, D Apoptosis rate of MG-63 and SaoS-2 cells after transfection with Si-REG γ and Si-NC, as
337 determined by flow cytometry. E, F Si- REG γ alters apoptosis and cell cycle related genes at
338 protein levels. Data are shown as the mean ± SD, *P <0.05.

Figure 1

REG γ expression is upregulated in OS.

A, B,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, 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 and qRT-PCR. F REG γ expression (median expression intensity) in sarcoma tissues and adjacent normal tissues derived from the Oncomine database (https://www.oncomine.org/).


A
Patient 1
Patient 2
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AT
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#1
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#3
#4
#5
#6
#7
#8
#9
#10
REGγ
β-actin
C
Relative REGγ mRNA level
Adjacent normal
Tumour
D
hFOB1.19
MG-63
SaoS-2
REGγ
β- actin
E
Relative REGγ mRNA level
hFOB1.19
MG-63
SaoS-2
F
PSME3 Expression in Barretina Sarcoma
Leiomyosarcoma vs. Normal
Dedifferentiated Liposarcoma vs. Normal
Myxofibrosarcoma vs. Normal
Barretina Sarcoma Statistics
Legend
1. Adipose Tissue (9)
2. Leiomyosarcoma (26)
2. Dedifferentiated Liposarcoma (46)
2. Myxofibrosarcoma (31)

Figure 2

Si- REG γ reduce the expression of REG γ at mRNA (A, B) and protein level (C, D) in OS cells.

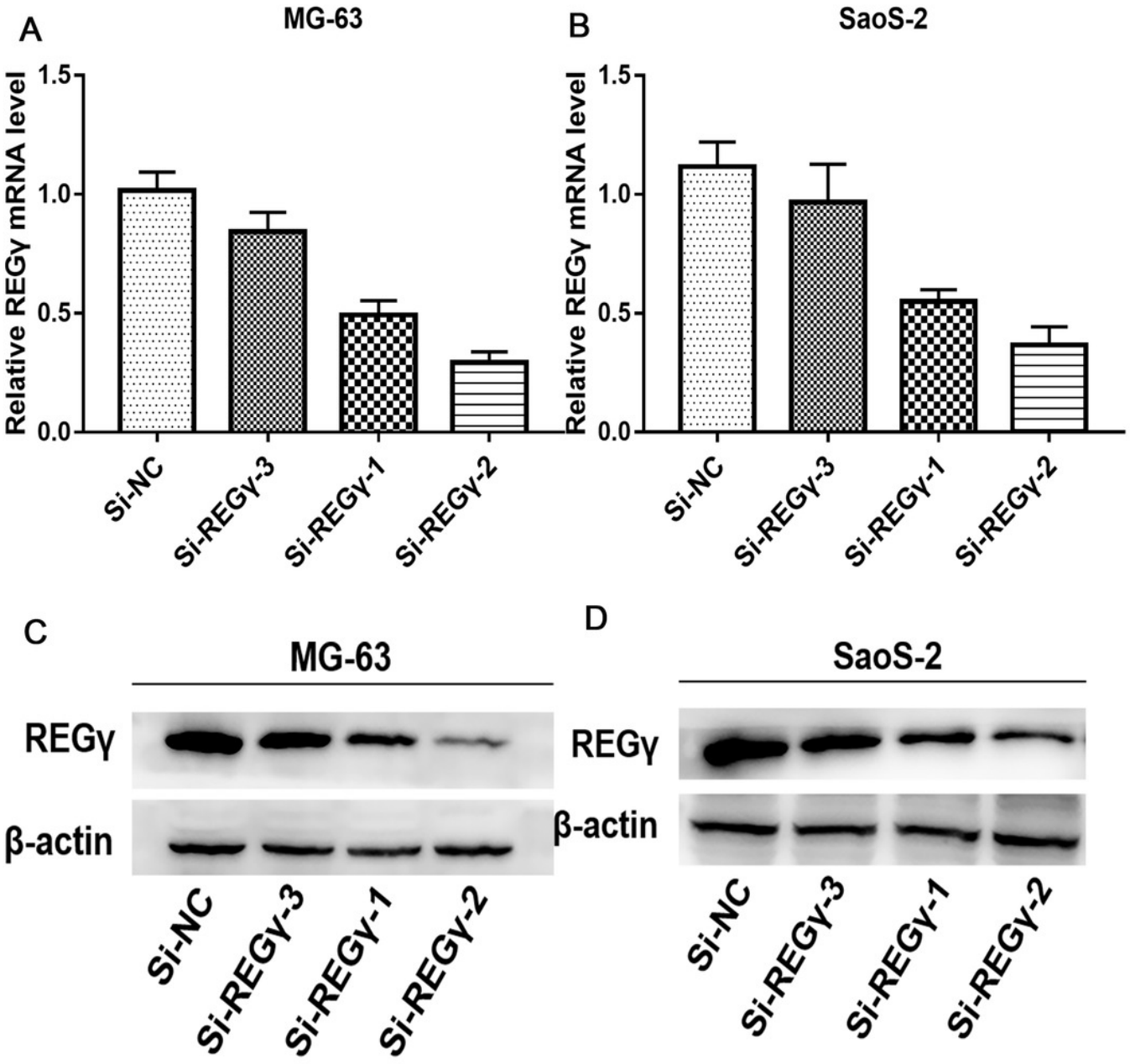

Figure 3

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 CCK-8 assay. B Representative OS cell colony formation images after transfection of Si-REG γ versus Si-NC. C, D Representative images of the EdU incorporation assay after transfection of Si-REG γ compared to after transfection of Si-NC in Mg-63 (C) and in SaoS-2 □ D. Data are shown as the mean ± SD. *P <0.05.

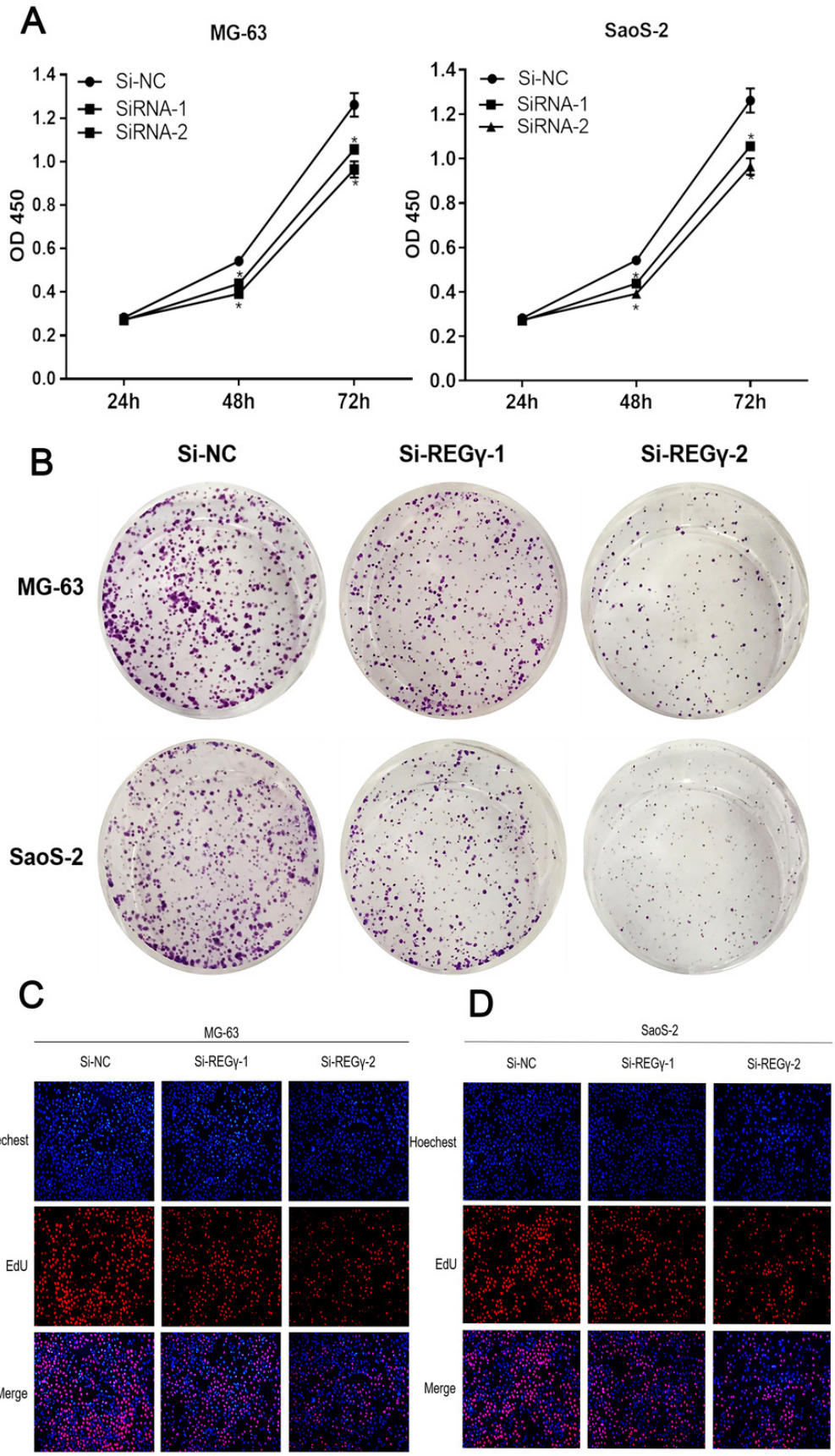
A
MG-63
SaoS-2
Si-NC
SiRNA-1
SiRNA-2
OD 450
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24h
48h
72h
B
Si-NC
Si-REGγ-1
Si-REGγ-2
MG-63
SaoS-2
C
D
MG-63
SaoS-2
Si-NC
Si-REGγ-1
Si-REGγ-2
Hoechest
EdU
Merge

Figure 4

REG γ deficiency induce apoptosis and cell cycle arrest and alters multiple cell apoptosis and cell cycle related genes in MG-63 and SaoS-2.

A, B Representative fl ow cytometry analysis of the cell cycle distribution of MG-63 and SaoS-2 cells transfected with Si- REG γ and Si-NC. C, D Apoptosis rate of MG-63 and SaoS-2 cells after transfection with Si-REG γ and Si-NC, as determined by fl ow cytometry. E, F Si- REG γ alters apoptosis and cell cycle related genes at protein levels. Data are shown as the mean ± SD, *P <0.05.

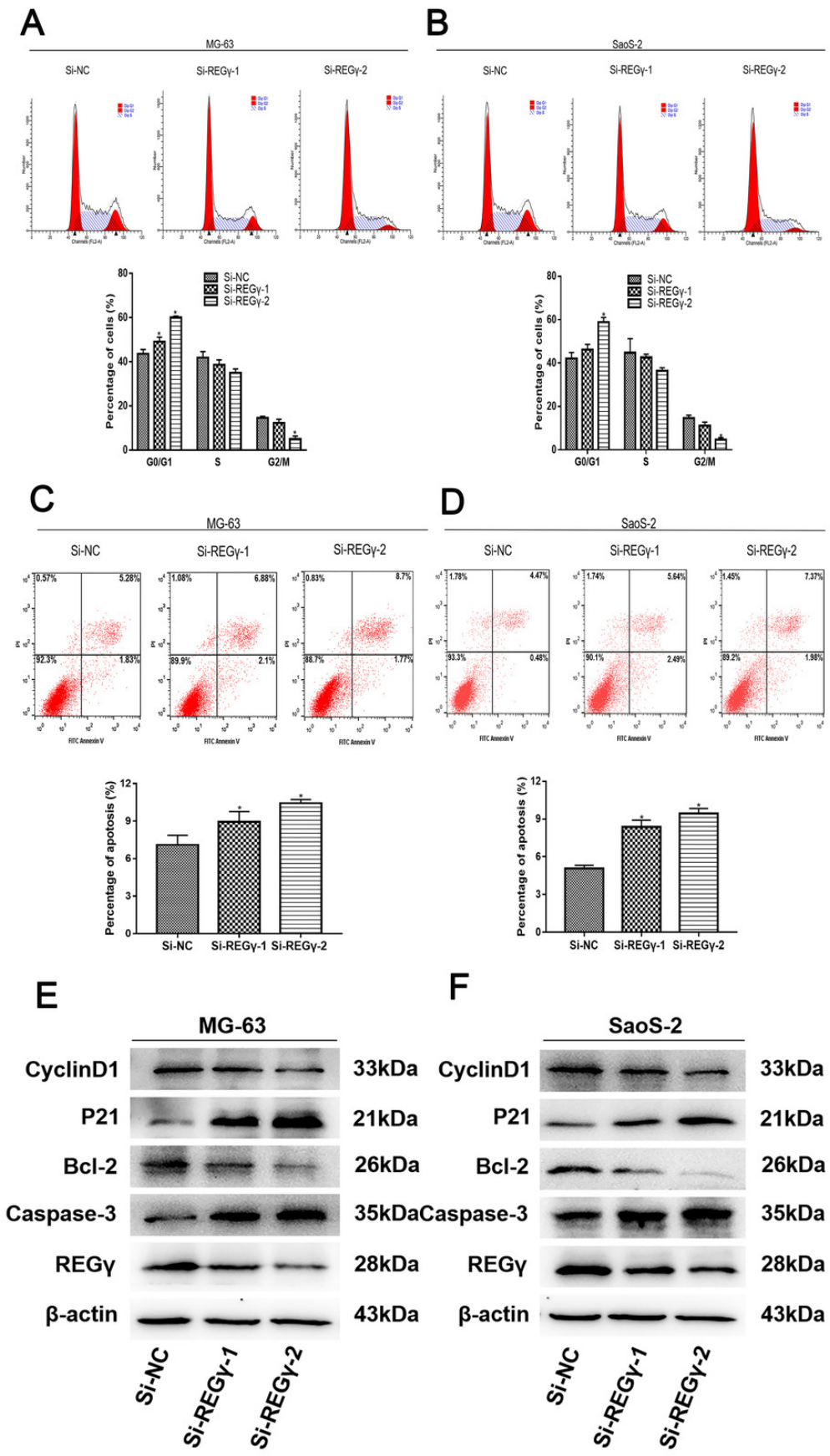
A
MG-63
Si-NC
Si-REGγ-1
Si-REGγ-2
B
SaoS-2
Si-NC
Si-REGγ-1
Si-REGγ-2
Percentage of cells (%)
G0/G1
S
G2/M
C
MG-63
Si-NC
Si-REGγ-1
Si-REGγ-2
10.57%
5.28%
92.3%
1.83%
11.08%
6.88%
89.9%
2.1%
10.83%
8.7%
88.7%
1.77%
FITC Annexin V
PI
Percentage of apotosis (%)
D
SaoS-2
1.78%
4.47%
93.3%
0.48%
1.74%
5.64%
90.1%
2.49%
1.45%
7.37%
89.2%
1.98%
E
MG-63
CyclinD1
33kDa
P21
21kDa
Bcl-2
26kDa
Caspase-3
35kDa
REGγ
28kDa
β-actin
43kDa
F
SaoS-2

Table 1(on next page)

Sequences of Si-RNA and primers

1 **Table 1: Sequences of Si-RNA and primers:**

Name:	Sequence:
Si-negative control	Sense:5’-UUCUCCGAACGUGUCACGUTT-3’
	Antisense: 5’-ACGUGACACGUUCGGAGAATT-3’
Si-REGγ-1	Sense: 5’-GCAGAAGACUUGGUGGCAATT-3’
	Antisense:5’-UUGCCACCAAGUCUUCUGCTT
Si-REGγ-2	Sense: 5’-CCAAGGAACCAAGGUGUUUTT-3’
	Antisense:5’-AAACACCUUGGUUCCUUGGTT-3’
Si-REGγ-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’

2