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Targeting miR-29 induces apoptosis of osteosarcoma MG-63 cells via regulation of TGF-β1/PUMA signal

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Abstract. – OBJECTIVE: Recent studies have shown that high miR-29 expression was associated with poor prognosis in patients with osteosarcoma (OS). However, the exact role and mechanisms of miR-29 in human OS remains speculative. Here, we identify a connection between miR-29 and TGF-β1/PUMA signaling in this context.

PATIENTS AND METHODS: MG-63 cells were treated with anti-miR-29 for 48 h. Cell growth and apoptosis in vitro were detected by MTT, colony formation and flow cytometry assay. The effect of the miR-29 inhibitor on the growth of MG-63 cells was also evaluated in a MG-63 mouse model. Human recombinant TGF-β1 (rh TGF-β1) and PUMA siRNA transfection were used to assess the signal pathway. miR-29, TGF-β1, PUMA, and caspase-3 protein expression were detected by Western blotting assay and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays.

RESULTS: Knockdown of miR-29 resulted in 80% decrease of miR-29 compared to the negative control. Knockdown of miR-29 significantly downregulated TGF-β1 and upregulated PUMA expression, and decreased MG-63 cell growth by 70%, impaired colony formation by approximately 80%, and increased MG-63 cell apoptosis by 40%. Knockdown of PUMA reversed miR-29 silencing-induced proliferation and apoptosis of MG-63 cells. Restoration of TGF-β1 decreased PUMA expression. In murine engraftment models of MG-63, we showed that knockdown of miR-29 was able to reduce tumor growth. This was accompanied by decreased levels of TGF-β1 and increased levels of PUMA in these tumors.

CONCLUSIONS: Targeting miR-29 exhibits significant in vivo and in vitro anti-tumor activities in OS through a novel mechanism resulting in inhibition of TGF-β1 expression and inducing PUMA expression.

Key Words:
Osteosarcoma, miR-29, TGF-β1, PUMA, Apoptosis.

Introduction

Osteosarcoma is the most common primary bone cancer in children and adolescents with a poor prognosis[1]. The current treatment relies on tumor resection and nonspecific combination chemotherapy, resulting in a 5-year survival rate of 0-29% if clinically apparent metastases are present[2]. Therefore, understanding the mechanisms underlying OS as well as identifications of new molecular targets are of great importance.

MicroRNAs (miRNAs) are regulatory RNAs. It is ~22 nucleotides in length and serves to regulate post-transcriptional expression of target genes[3]. They mediate fundamental cellular processes such as proliferation, differentiation and apoptosis and are actively involved in carcinogenesis[4,5]. A single miRNA regulates hundreds of genes, which often targeting multiple components of complex intracellular networks[6]. Thus, misregulation of an individual miRNA can have a profound impact on cellular physiology that leads to disease(s)[7]. Recent reports have suggested that microRNA-29 (miR-29) family members may play important roles in human cancer by regulating cell proliferation, differentiation, apoptosis, migration, and invasion. Aberrant expression of miR-29 has been found to be associated with various types of solid malignancies, such as colorectal cancer[8], gastric cancer[9], cutaneous melanoma[10], pancreatic ductal adenocarcinoma[11], breast cancer[12], hepatocellular

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carcinoma[13] and lung cancer[14]. In clinical samples of colorectal cancer, the miR-29b expression was significantly reduced in tumor tissues compared with normal mucosa, and miR-29b expression was an independent prognostic factor for disease-free survival, lymph node metastasis, and pathological T classification[8]. In gastric cancer cells, the miR-29 family acted as tumor suppressors through targeting CCND2 and matrix metalloproteinase-2 genes[9]. The miR-29c expression was significantly downregulated in AJCC stage IV melanoma tumors compared to primary melanomas. Moreover, the expression of miR-29c was significantly associated with overall survival (OS) in AJCC stage III melanoma patients by multivariate analysis[10]. However, in breast cancer, the introduction of miR-29 into breast cancer cells resulted in decreased NMI expression and increased invasion[12]. In osteosarcoma, patients in the miR-29a-high and miR-29b-high-expression groups both showed shorter overall survival and disease-free survival[15]. Also, the serum levels of miR-29a and miR-29b in the patients with higher tumor grade, positive metastasis, and positive recurrence were both markedly higher than those with lower tumor grade, negative metastasis, and negative recurrence[15]. Therefore, the molecular details of miR-29 gene regulation appear to be disease and cell-type specific. However, their accurate expression, function, and mechanism in OS are not well known.

The transforming growth factor-beta1 (TGF-β1) signaling pathway plays a critical role in promoting tumor growth and inhibiting apoptosis. miR-29 is known to play a paramount role in the fibrotic process of several organs and provides crucial functions downstream of pro-fibrotic signaling pathways such as TGF-β1[16]. Li et al[17] have found that miR-29 inhibited endometrial fibrosis via blockade of the Sp1-TGF-β1/Smad-CTGF pathway. In retinal pigment epithelial cells, miR-29 plays an important role in TGF-β1-mediated EMT in ARPE-19 cells by targeting Akt2[18]. Luna et al[19] have reported that MiR-29 decreased the expression of TGFβ1 at the promoter, transcript, and protein levels, suggesting that TGFβ1 was regulated by miR-29. PUMA is a BH3-only member of the Bcl-2 family and a target of p53-mediated apoptosis[20]. It activates an apoptotic cascade by facilitating Bax activation, causing cytochrome C release from the mitochondria, caspase-3 activation and DNA fragmentation[21]. Ouyang et al[22] have found that miR-29 could target pro-apoptotic PUMA protein and protect against ischemia-reperfusion injury, suggesting that PUMA might be negatively regulated by miR-29. Some studies have reported that PUMA was a direct TGF-β target gene in B-cells. TGF-β induces PUMA to aid induction of the intrinsic cell death pathway[23].

The purpose of this work was to investigate the potential role of targeting miR-29 on proliferation and apoptosis of MG-63 cells *in vitro* and *in vivo*, and explore its mechanisms. We found that knockdown of miR-29 inhibits proliferation and induce apoptosis of MG-63 cells by TGF-β1/PUMA signal.

Materials and Methods

Cell Line and Culture

The MG-63 cell line was purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). The cell line was maintained in RPMI 1640 (Life Technologies, Carlsbad, CA, USA), supplemented with antibiotics (100 U/mL penicillin and100 U/mL streptomycin), 3 mmol/L L-glutamine and 10% (v/v) fetal bovine serum (Wisent, Montréal, Quebec, Canada).

Stable Anti-miR-29 Transfection

miR-29 inhibitors (anti-miR-29) and scramble control were purchased from Ambion (Applied Biosystems, Shanghai, China). MG-63 cells were transfected with anti-miR-29 or scramble control overnight according to the manufacturers' instructions. After 48hr, the cells selected with 1 μg/ml G418 for 14 days. Total RNA was extracted, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to verify the levels of miR-29.

PUMA siRNA Transfection

MG-63 cells were transfected with the PUMA siRNA or control siRNA expression plasmid using Lipofectamine2000 reagent according to manufacturer's instructions. The levels of PUMA proteins in the transfected cells were verified by Western blot.

rh TGF-β1 Treatment

The stable anti-miR-29 transfected MG-63 cells (anti-miR-29/MG-63) were treated with 10 uM recombinant TGF-β1 (rh TGF-β1) for 6 h according to manufacturer's instructions. The levels of TGF-β1 and PUMA proteins in the transfected cells were verified by Western blot.

Western Blotting

At every experimental end point, cells in different groups were lysed using Cellytic™ M cell lysis reagent (Sigma) that contained 1 mM NaF, 1 mM PMSF and 0.4 mM protease inhibitors cocktail (Roche, Hangzhou, China). Protein concentration was measured using the protein assay from Bio-Rad (Guangzhou, China). Equal amounts of protein (40 μg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on either 10% acrylamide gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membranes (Amersham) and blocked with 5% nonfat dry milk, the membranes were incubated with anti-TGF-β1, PUMA, activated-caspase-3 and anti-β-actin antibodies (Santa Cruz, Shanghai, China) at 4°C overnight followed by incubation with an horseradish peroxidase-conjugated goat anti-rabbit serum (BD Biosciences, Franklin Lakes, NJ, USA) and rabbit anti-goat serum (DAKO, Carpinteria, CA, USA), and visualized via chemiluminescence detection (Amersham Biosciences). Representative blots are shown from 3 independent experiments.

qRT-PCR Analysis

At every experimental end point, cells were collected and washed twice with ice-cold PBS and lysed with QIAzol reagent to isolate total RNA. miRNA was isolated using a miRNeasy kit (Qiagen, Hilden, Germany) from the total RNA above. Single-stranded cDNA was synthesized using TaqMan Fast System and reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. U6 RNA was used as an endogenous reference for normalizing the expression levels of miR-29. PCR cycle threshold (Ct) values were recorded for each target gene and normalization controls. Then, they were averaged across three independent runs. All reactions were performed in triplicate, and the results were analyzed using the $2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$ method.

MTT Assay

At every experimental end point, cells from each group were collected and plated in 96-well plates at a density of 1.0×10^4 cells/well for methylthiazol tetrazolium (MTT) assay. The absorbance was measured at 570 nm. Each assay was performed in triplicate. Cell growth (mean absorbance ± standard deviation) was plotted versus time.

Clonogenic Assay

At every experimental end point, cells were seeded in 6-well plates at a density of 600 cells/well and incubated for 2 weeks at 37°C in a humidified atmosphere of 5% CO_2. The colonies were fixed with in 4% paraformaldehyde at room temperature for 20 min, stained with 0.1% crystal violet for 10 min, and finally, positive colony formation (more than 50 cells/colony) was counted and colony formation rate was calculated.

In vitro Apoptosis Assay

At every experimental end point, the cells were harvested and washed twice with cold PBS; then, the cells were stained with Annexin V-FITC and 10 μl propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (KEYGEN, Shanghai, China). The percentage of apoptotic cells was detected using FACSCalibur Flow Cytometer (BD, San Jose, CA, USA). All analyses were performed in triplicate.

Tumor Xenograft Studies

Four-week-old male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). For each mouse, 3×10^6 miR-29 inhibitor/ MG-63 or control/MG-63 cells in 100μ l PBS were subcutaneously inoculated in the left and right flank areas (N = 6). The mice were observed over 8 weeks for tumor formation.The mice were sacrificed, and tumors were dissected after 8 weeks. The tumors were excised. Half of each tumor was fixed in 4% buffered formaldehyde, paraffin-embedded and processed for histological examination, while the second half was frozen in liquid nitrogen and stored at −80 °C for immunoblot analysis, qRT-PCR and TUNEL assay.

In vivo TUNEL Staining

Tissue sections were prepared and treated according to manufacturer's recommendations (TACS XL; Guangzhou, China).

Statistical Analysis

All data are presented as mean ± SE. Statistical analyses were performed using SPSS.17 Software (SPSS Inc., Chicago, IL, USA). The significance was determined by two-tailed Student's *t*-test or one-way analysis of variance with Bonferroni post-test where applicable. The experiments were performed in triplicate. A *p*-value less than 0.05 was considered statistically significant.

Results

Targeting miR-29 Inhibits Growth of MG-63 cells

After transfection of anti-miR-29, the expression level of miR-29 decreased more than 80% compared with transfection of a negative control (Figure 1A). To investigate the influence of anti-miR-29 on cell proliferation, the MTT assay was used. MTT assays illustrated significant decreases in MG-63 cells after transfection of anti-miR-29 (Figure 1B). Clonogenic survival assay also showed a 10-fold reduction of colony numbers in anti-miR-29-infected cells (Figure 1C). Next, we examined whether the inhibition of cell growth was also accompanied by the apoptosis induced by anti-miR-29. Annexin V/PI staining was employed to investigate the degree of apoptosis induced by anti-miR-29.

Targeting miRNA-155 Induces Apoptosis of MG-63 cells

To investigate the effect anti- miR-29 on apoptosis, PI and Annexin V double staining assays were performed (Figure 1 D). At 48 h post-transfection, a significantly high apoptotic rate was observed in the anti-miR-29-transfected MG-63 cells compared with the controls. These results suggest that anti-miR-29 could inhibit cell growth and increase the induction of apoptosis.

Targeting miR-29 Induces PUMA in MG-63 cells

We first investigated the effects of miR-29 silencing on PUMA expression in MG-63 cells. Targeting miR-29 led to a rapid increase of PUMA protein within 12 h of transfection and reached the peak at 48 h (Figure 2A). Further-

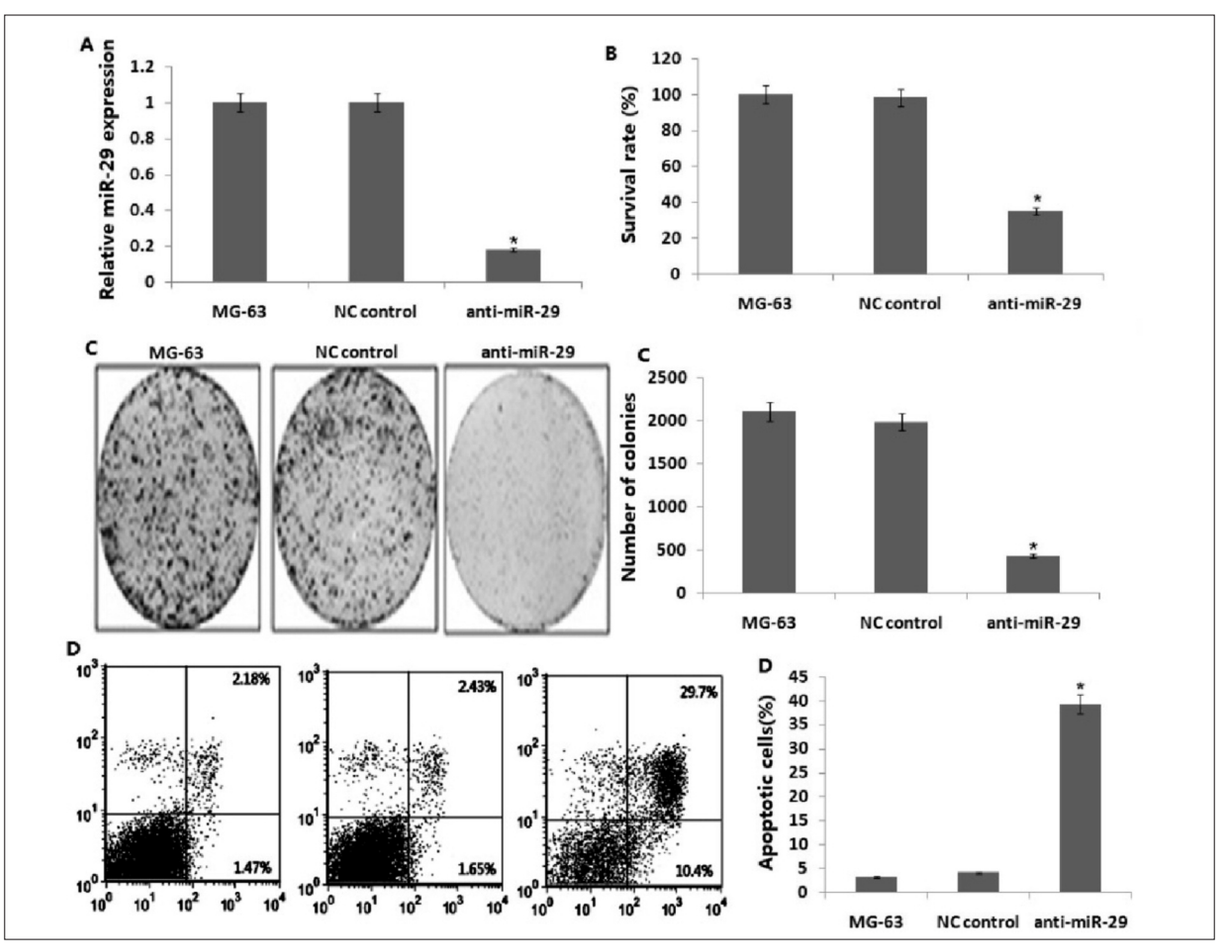

Figure 1. Targeting miR-29 induces apoptosis and inhibits growth and colony formation of MG-63 cells in vitro. MG-63 cells was transfected with anti-miR-29. ***A,*** miR-29 mRNA expression was detected by qRT-PCR assay; ***B,*** Cell proliferation by MTT assay; ***C,*** Cell growth by colony formation assay. ***D,*** Cells apoptosis was detected by FCM. The data are shown as the mean ± SD. The experiments were all repeated at least 3 times to confirm the reproducibility of the results. $*p < 0.05$ versus the control.

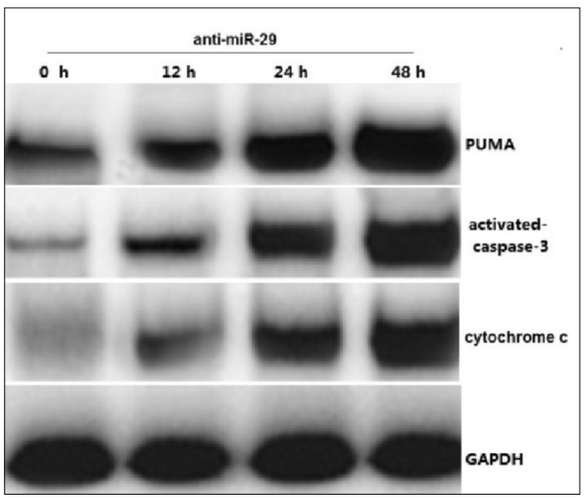

Figure 2. Targeting miR-29 induces PUMA expression in MG-63 cells. Western blot analysis of the expression of PUMA at indicated time points in MG-63 cells transfected with anti-miR-29.

more, the caspase-3 and cytochrome c downstream of PUMA was also activated by anti-miR-29 (Figure 2).

PUMA is Essential for Anti-miR-29 Induced Apoptosis in MG-63 Cells

Next, we determined the role of PUMA in anti-miR-29-induced apoptosis and growth inhibition. *PUMA* knockdown with siRNA led to reduced apoptosis in MG-63 cells following anti-miR-29 transfection (Figure 3A) with little or no activation of caspases-3 and cytochrome c release at 48 h of anti-miR-29 transfection (Figure 3B). In addition, PUMA siRNA transfection reversed anti-miR-29 induced growth inhibition (Figure 3C). Collectively, these results demonstrate an essential role of PUMA and the mitochondrial pathway in anti-miR-29-induced apoptosis and growth inhibition in MG-63 cells.

TGF-β1-dependent Induction of PUMA by miR-29

MG-63 cells were transfected with anti-miR-29 for 48 h; TGF-β1 protein expression was significantly decreased by Western blot assay (Figure 4A). We treated the MG-63 cells with 10 uM rhTGF-β1 for 6 h, then transfected with anti-miR-29 for 48 h, and found TGF-β1 protein expression was significantly increased, and PUMA, caspase-3 and cytochrome c release was significantly decreased (Figure 4A). In addition, rhTGF-β1 treatment inhibited apoptosis in anti-miR-29 transfected MG-63 cells (Figure 4B). Moreover, rhTGF-β1 treatment reversed anti-miR-29 induced growth inhibition (Figure 4C).

Anti-miR-29 Inhibits the Growth of MG-63 Cells Xenografted in Mice

Having validated our cell model, we next evaluated the *in vivo* effect of miR-29 silencing on MG-63 tumor xenograft growth. Stably anti-miR-29 and control transfected MG-63 cell suspensions were injected s.c. into the flanks of 6-week-old immunodeficient mice, and tumor growth was evaluated and registered periodically, to plot tumor growth curves. Tumors arose ~2 weeks after the subcutaneous administration, and tumorigenicity studies ended 6 weeks after the cell implantation. Our results demonstrated that the anti-miR-29 transfection suppressed the growth of MG-63 tumors by 70% compared to control transfected xenografts, from 5 weeks after implantation until the end of the experiment, at 6 weeks (Figure 5A).

We next evaluated the effect of targeting miR-29 on apoptosis of MG-63 xenografts in mice. In stably anti-miR-29 transfected xenografts, miR-29 (Figure 5B) and TGF-β1 (Figure 5C) were significantly decreased, whereas PUMA, caspase-3 and cytochrome c release were significantly increased (Figure 5C). As expected, the frequency of TUNEL-positive cells were increased in anti-miR-29 *versus* control xenograft tumoral tissue ($p < 0.01$, Figure 5D).

Discussion

Members of the miR-29 family have gained interest as tumor suppressors because they are silenced or downregulated in several types of cancer[24,25]. Several patent applications and clinical studies involving miR-29 as diagnostic targets, as well as miR-29 mimics, as treatment options are under way[26]. Although anticancer therapies with miR-29 analogues are being considered, there is some evidence suggesting that in some instances miR-29 might have tumor-promoting activities and antiapoptotic behavior[27]. These variations in the miR-29 regulation of cell survival are because of the fact that the miR-29 family has been reported to potentially regulate more than 4000 gene products, which are likely to differ between tissues and immediate cellular milieu.

The data presented here demonstrated that targeting *miR-29* in MG-63 cells inhibited cell growth and induced apoptosis. The proapoptotic

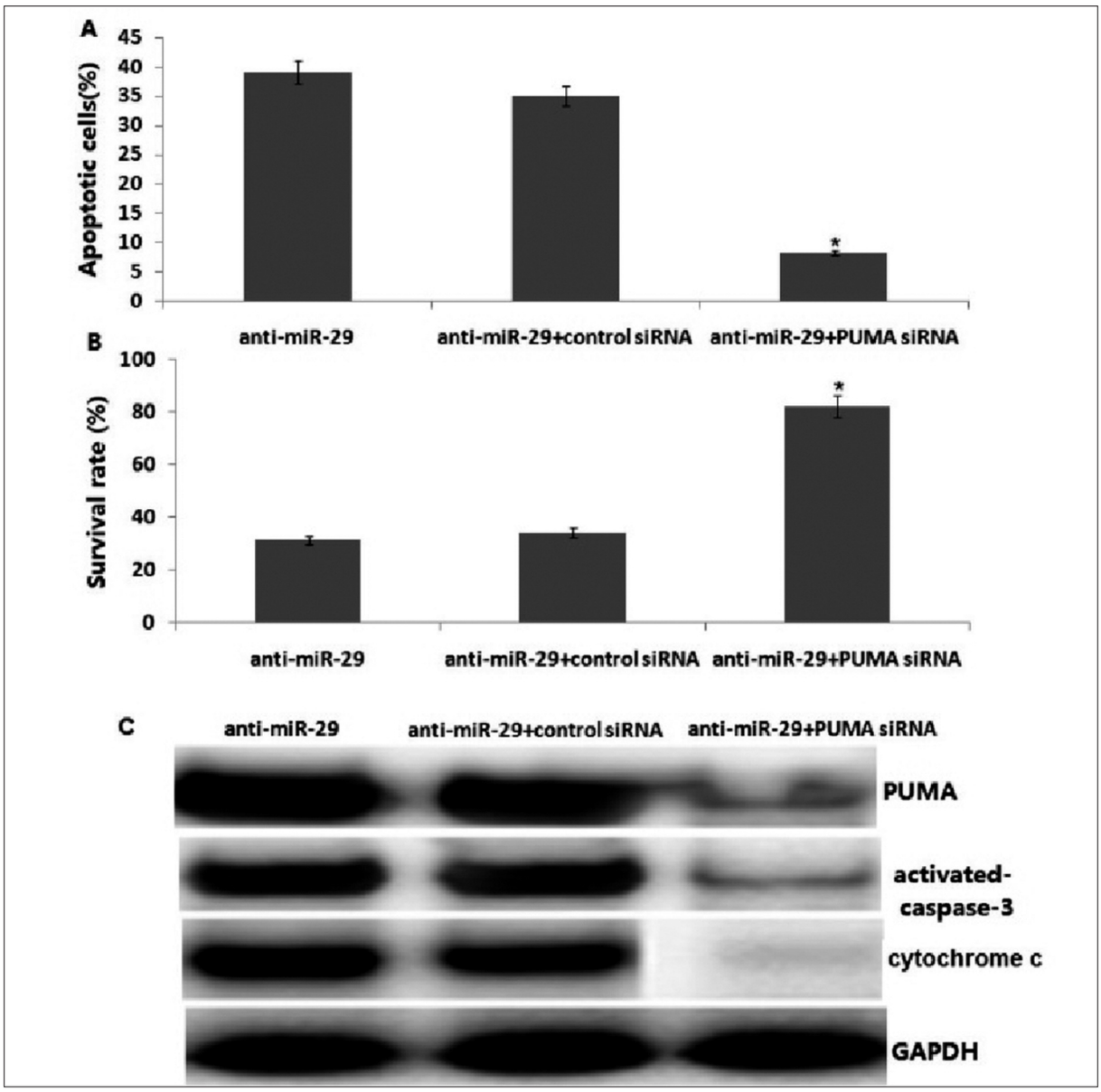

Figure 3. Targeting PUMA inhibited anti-miR-29-induced apoptosis in MG-63 cells. MG-63 cells were transfected with anti-miR-29 or/and PUMA siRNA. ***A,*** Cells apoptosis was detected by FCM. ***B,*** Western blot analysis of the expression of PUMA, caspase-3 and cytochrome C in MG-63 cells transfected with anti-miR-29/PUMA siRNA. ***C,*** Cell proliferation by MTT assay. The data are shown as the mean ± SD. The experiments were all repeated at least 3 times to confirm the reproducibility of the results. $*p < 0.05$ versus the control.

and antiproliferative effect of anti-*miR-29* in MG-63 cell lines was observed after 48 hours of transfection. These results indicate that anti-*miR-29*-dependent cell antiproliferation effects are the result of apoptosis and likely to contribute to the tumor suppressor activity of anti-miR-29. The tumor suppressor effect of anti-*miR-29* in MG-63 cells was further demonstrated *in vivo*. However, the exact mechanism of how anti-miR-29 functions remains unclear.

In our study, targeting *miR-29* inhibited the *TGF-β1* levels, followed by increased cell apoptosis and cell growth inhibition in MG-63 cells. However, the restoration of *TGF-β1 levels with* human recombinant TGF-β1 (rh TGF-β1) could block the anti-*miR-29*-induced apoptosis in MG-63 cells, suggesting that targeting miR-29-induced apoptosis was achieved by inactivation of *TGF-β1* signaling. Although targeting *miR-29* signaling affects cellular growth and apoptosis

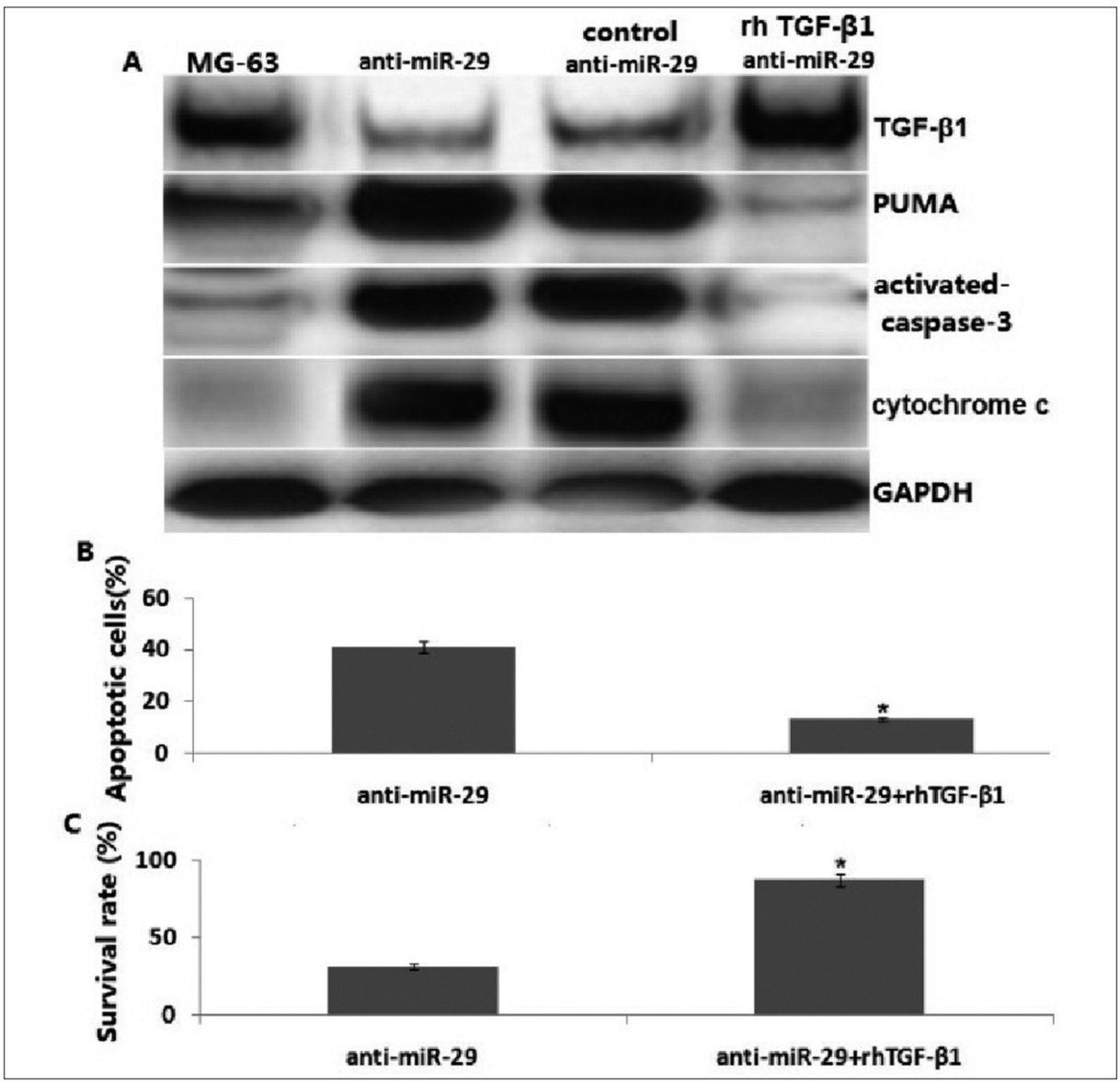

Figure 4. rhTGF-β1 inhibited anti-miR-29-induced apoptosis in MG-63 cells. MG-63 cells were transfected with anti-miR-29 or/and treated with rhTGF-β1. ***A,*** Western blot analysis of the expression of TGF-β1, PUMA, caspase-3 and cytochrome C in MG-63 cells transfected with anti-miR-29/rh TGF-β1. ***B,*** Cells apoptosis was detected by FCM. ***C,*** Cell proliferation by MTT assay. The data are shown as the mean ± SD. The experiments were all repeated at least 3 times to confirm the reproducibility of the results. *$p < 0.05$ versus the control.

by regulation of TGF-β signal, but the central signaling pathway, acting downstream of TGF-β and leading to cell death is not clear.

Proapoptotic protein PUMA mediates death signals primarily through the mitochondria. Through interaction with Bcl-2 family members Bax and/or Bak, PUMA induces mitochondrial dysfunction and caspase activation, then, inducing cell apoptosis[28]. To explore the effect of PUMA on anti-*miR-29*-induced apoptosis in MG-63 cells, PUMA siRNA was transfected into the MG-63 cells, only to find that PUMA is required for anti-*miR-29*-induced apoptosis in MG-63 cells. In our study, inactivity of *TGF-β1* and activation of PUMA were all necessary for anti-*miR-29*-induced apoptosis of MG-63 cells. Furthermore, TGF-β activation blocked anti-*miR-29*-induced PUMA upregulation. We, therefore, suggested that TGF-β/PUMA pathway was necessary for anti-*miR-29*-induced apoptosis and growth inhibition in MG-63 cells.

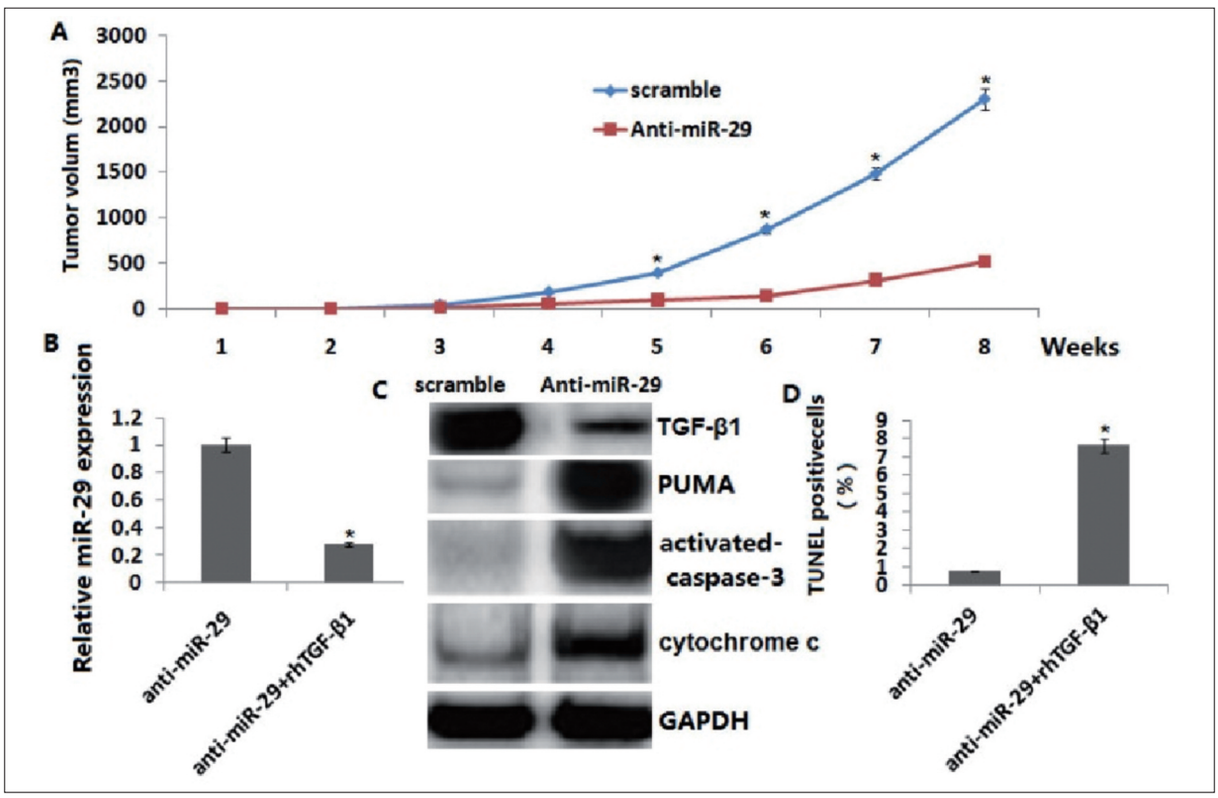

Figure 5. *In vivo* antitumor activity of anti-miR-29 against s.c. xenografted tumors of MG-63 cells in mice. ***A,*** Subcutaneous tumors were seeded in immunodeficient mice using MG-63 cells, as described in the Materials and Methods section. ***B,*** miR-29 mRNA was detected by qRT-PCR; ***C,*** TGF-β1, PUMA, caspase-3 and cytochrome C expression was detected by Western blot assay. ***D,*** TUNEL staining in the tumors. Significant differences between the anti-miR-29 groups and scramble group are indicated by $*p < 0.01$.

Conclusions

In this study, we report the identification and functional characterization of miR-29 as a regulator of growth and tumor progression in MG-63 cells by targeting PUMA via regulation of *TGF-β1 levels*. We demonstrate that targeting miR-29 effectively induce apoptosis and reduce tumor burden, thereby providing the first proof of concept that systemically administered anti-miR-29 can inhibit OS progression.

Funding

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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