Panax notoginseng saponins elicits anti-cancer effect by modulating miR-760/SMAD4 signaling in osteosarcoma

Type

Research paper

Keywords

Apoptosis, osteosarcoma, SMAD4, Panax notoginseng saponins, miR-760

Abstract

Introduction
Osteosarcoma (OS) is one of the most common malignant bone tumors, with an incidence of 4-5 per million among children and teenagers. Panax notoginseng saponins (PNS) a derivatives from Panax notoginseng, are potent drugs that have many biological activities including antitumor effects. However, there have been no reports focused on the effect of PNS on OS development.

Material and methods
MTT and flow cytometry was used to detect the proliferation and apoptosis of OS cells treated by PNS. The expression of miR-760 was identified by qPCR. Luciferase assay was performed to verify the target of miR-760. Western blot was used to detect the expression of target proteins. In vivo analysis was employed to confirm the antitumor effect of PNS.

Results
We tested the PNS effect on a large numbers of microRNAs (miRs) in OS cells, we found that PNS significantly reduced miR-760. Also, luciferase assay has shown SMAD4 to be the target gene of miR-760 in OS cells. Rescue experiments were carried out to verify the relation between SMAD4 and miR-760. we found that overexpression of miR-760 can reverse the effect of PNS. PNS proven to exerts its effect through miR-760. Moreover, SMAD4 can reverse the effect of miR-760, indicating that miR-760 targets SMAD4 in OS cells.

Conclusions
This study extends our understanding of the effect of PNS in OS cell. we revealed a novel signaling pathway involved in the PNS mode of action, miR-760/SMAD4, this new pathway might be feasible as a target for the treatment of OS.

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Running title:

PNS inhibits osteosarcoma

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Introduction

Osteosarcoma (OS) is one of the most common malignant bone tumors which accounts for approximately 60% of bone malignancies[1,2]. It has become the most frequent cause of cancer-related death in children[3]. At present, the standard treatment for OS is surgery along with chemotherapy using doxorubicin and cisptatin[4]. Despite great advances, the efficacy of OS treatment remains unsatisfactory, high recurrence and drug resistance make the 5-year survival rate of OS less than 30-40%[5,6]. Therefore, understanding the molecular mechanisms underlying the initiation and recurrence of OS is highly essential for developing novel therapeutic methods.

Over the past few decades, numerous dietary and botanical natural compounds or synthesized compounds have been studied on there their properties that can prevent tumorigenesis[7,8]. *Panax notoginseng* saponins (PNS), mainly derived from *Panax notoginseng*, are patent medicines that are commonly used as a treatment for cardiovascular disorders, such as strokes, atherosclerosis, coronary artery disease, and heart failure[9,10,11]. For instance, Su et al elucidated the mechanisms and potential advantages of PNS on nervous system diseases such as alzheimer's disease, parkinson's disease, ischermic cerebral apoplexy and depressive disorder. Zhang et al proposed that PNS may function as potential antidepressant-like candidate by intestinal bacteria into active derivatives. Xiang et al

pointed out that total PNS extracted from the caudexes and leaves have been used for improving mental function, treating insomnia and alleviating anxiety. Other studies also demonstrated its antioxidant, anti-inflammation, and anti-apoptosis effects[12,13]. For instance, Hu et al illustrated that PNS may function as an extrinsic regulator by mitigating the oxygen-glucose deprivation/reperfusion-induced cell injury in a dose-dependent manner. Zhou et al demonstrated that PNS could attenuate cell inflammation by modulating CCL2-induced intracellular oxidative stress; meanwhile, PNS pretreatment could inhibit apoptotic pathways by reducing Bax/Bcl-2 ratio and Caspase 3,8,9 expressions. As in cancer therapy, PNS has been determined to attenuate lung cancer growth through Met/miR-222 axis, and breast cancer metastasis via modulating miR-18a-regulated Smad2 expression[14]. Meanwhile, another study reported that PNS treatment could inhibit cell migration and invasion of in metastatic mouse breast cancer cell line[15]. Hence, PNS may function as a potential tumor-suppressor in various cancers. However, the effect of PNS in the progression of OS has not been studied.

MicroRNAs (miRNA/miR) are 20-25 nucleotides small non-coding RNAs which induce degradation of target mRNAs or suppress translation of them through binding the targeting sequences in the 3'-untranslated regions (UTR) of the target genes. Thus, microRNAs are considered to participate in the development of various tumors, including osteosarcoma, and regulate cell proliferation, apoptosis, and tumorigenesis through multiple signaling pathways[16,17,18,19].

In the present study, we explored the effect of PNS in the progression of OS and the underlying mechanism. We found that PNS inhibits the growth and induces apoptosis of OS cells through the miR-760/SMAD4 pathway.

Methods and materials

Cell culture and transfection

Osteosarcoma cell lines MG63 and HOS were obtained from the Chinese Academy of Science and were maintained in RPMI1640 (Gibco, NY, USA) medium containing 10% FBS (Gibco, NY, USA) with 100U/mL penicillin-streptomycin (Invitrogen, CA, USA). Cells were cultured in RPMI1640 medium at 37°C in 5% CO_2. PNS was obtained from Sigma Chemical Co (St. Louis, MO, USA). Pre-miR-760 oligonucleotide, pcDNA3.1/SMAD4 vector, and their negative control were synthesized by Genepharma (Shanghai, China). 5×10^5 NSCLC cells were seeded into 6 well plates. 24 hours later, the oligonucleotides were transfected into MG63 and HOS cells (200 nM) using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. In the rescue experiments exploring whether SMAD4 can reverse the effect of miR-760, OS cells were transfected with 400nM of miR-760 mimic. Cell line stably expressing miR-760 was selected by G418 administration.

Cell proliferation measured by MTT assay

After transfection, 6×10^3 MG63 and HOS cells were seeded onto 96 well plates. MTT experiment was performed to detect cell proliferation. After different concentrations of PNS treatment, 1% MTT (Beyotime, Shanghai, China) was added to the culture medium and incubated at 37°C. 4 h later, the supernatant was removed and DMSO was added into each well. After that, the OD value was detected with a microplate reader (Bio-Rad, USA) at 490 nm.

Quantitative real-time PCR

Total RNAs were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Total RNAs in the amount of 1 μg were reversely transcribed to cDNA using an RNA PCR Kit (Takara Biotechnology, Japan) which was used as a PCR template. To detect gene expression, quantitative real-time PCR (qRT-PCR) was performed using an iCycler iQ System with the iQ SYBR Green SuperMix (BioRad, USA) according to the manufacturer's instructions. Small endogenous nuclear U6 snRNA was used as the internal control for the normalization of miRNA and GAPDH for mRNAs. The relative gene expression levels were calculated using ($2^{-\Delta\Delta Ct}$) method.

Western blot

The protein samples were extracted from the MG63 and HOS cells using a lysis buffer. The protein concentrations were measured by BCA reagent kit (Thermo Fisher, Shanghai, China). 40 μg protein was separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transferring to the polyvinylidene fluoride (PVDF) membranes. The blots were then incubated in the 5% skimmed milk for 2 h at room temperature. Thereafter, they were incubated with the primary antibodies overnight at 4°C. After wash, the blots were incubated with horseradish peroxidase-conjugated (HRP) labeled goat anti-rabbit secondary antibody at room temperature for 2 h. Finally, the blot was treated with an ECL plus reagent kit (Pierce, Rockford, IL, USA) and visualized

using charged-coupled device LAS 4000 (Fujifilm, Valhalla, NY, USA). GAPDH was used as an internal control.

Cell apoptosis measured by flow cytometry analysis

MG63 and HOS cells were washed with PBS followed by digesting with trypsin. Then, the cells were resuspended and stained with AnnexinV FITC/propidium iodide following the manufacturer's protocols. Cell apoptosis was assessed in a flow cytometry system (Verse, BD, USA).

Luciferase assay

The wild-type and mutant 3'UTR of SMAD4 were synthesized and subcloned into the pGL3 vector (Promega, Shanghai, China). MG63 and HOS cells were co-transfected with miR-760 mimics or mimic control and the pGL3 vector carrying wild-type or mutant SMAD4-3'UTR. Subsequently, 0.15 μg PRL-TK was transfected to MG63 and HOS cells as the internal control. Finally, the luciferase activity was evaluated 48 h after transfection.

In vivo analysis

Zhejiang University approved all animal studies conducted in the current study (No. 2017056285). 15 Balb/c nude male mice aged 4-6 weeks were randomly divided into three groups including the control group, PNS treatment group, PNS along with the miR-760 transfection group and housed within a constant temperature of 18-23℃ and 60% humidity. Normal MG63 cells in the amount of 1×10^6 were subcutaneously injected into the flank region of the mice in the control and PNS group.

And miR-760 stably expressed MG63 cells were injected into the mice in the PNS+miR-760 group in the same way. PNS was dissolved in normal saline was administered orally to these mice once a day at the dose of 50 mg/kg for 10 days before tumor inoculation until the end of the study. The tumors size were measured every two days using a caliper. The mice were sacrificed at day 30 under anesthesia and tumors were excised. Mice were sedated with 10% 60 mg/kg ketamine hydrochloride (Ketasol; Richter Pharma, Weis, Austria) and 2% 10 mg/kg xylazine (Rompun; Bayer Health Care, Whippany, NJ, USA). A part of the issues was placed in 10% formalin for histological and the remaining was frozen at -80°C. The animal experiments were approved by the Ethics Committee of Zhejiang Hospital (No. 2017056285).

Immunohistochemistry

After mice were anesthetized, the brain was quickly removed and hippocampus was rapidly dissected in 4% paraformaldehyde for 2 h. Then, the formalin-fixed and paraffin-embedded tissues were cut into 5 μm sections on a cryostat (Leica CM1510, Leica Miceosystems, Heidelberg, Germany). Then, the sections were microwaved in 0.01 M sodium citrate (pH 6.0) for antigen retrieval. Afterward, the sections were blocked via incubation in PBS containing 10% goat serum for 2 h at room temperature. Therefore, these sections were incubated with the primary anti-SMAD4 antibody (Abcam, England) at room temperature overnight. The HRP-labelled secondary antibody was then added and the positive staining cells were detected using diaminobenzidine (DAB) according to the manufacturer's protocol.

Statistical analysis

GraphPad Prism version 6.0 software (GraphPad Software, San Diego, CA, US) and SPSS version 17.0 software (SPSS Inc., Chicago, IL, US) were used to analyze the data. All the data are presented as the means ± SD. A student's t-test was used to analyze the differences between the two groups. One-way ANOVA was used to analyze the difference between multiple groups. $P<0.05$ was considered as statistical significance.

Results

PNS repressed cell proliferation of MG63 and HOS

MTT assay was used to investigate the effects of PNS on the proliferation of MG63 and HOS cells. As shown in Figure 1A and B, after treatment for 24 hours with control or different concentrations of PNS, the proliferation of MG63 and HOS cells were reduced by 0.5, 1, 5 and 10 μM of PNS. Also, 12, 18, and 24 but not 6 hours of 5 μM PNS treatment inhibited the proliferation of OS cells (Figure 1B). Furthermore, MG63 and HOS cells were treated with 0.5, 1, and 5 μM PNS for over 3 days, respectively. 0.5, 1, and 5 μM of PNS exerts a significant inhibitory effect on both MG63 and HOS cells on days 2 and 3 (Figure 1 C, D).

PNS induced apoptosis of MG63 and HOS cell

Cell proliferation is always associated with apoptosis. Thus, we next evaluated the cell apoptosis of MG63 and HOS cells treated with PNS using flow cytometry. PNS induced apoptosis of MG63 and HOS cells at the concentration of 1 and 5 μM (Figure 2A, B). We further detected the apoptosis-related protein expression such as bax, cyto-c, bcl-2, and cleaved caspase3. The results

indicated that 1 and 5 μM PNS notably elevated the level of bax, cyto-c, and cleaved caspase3, however, reduced the expression of bcl-2 (Figure 2C).

PNS down-regulated miR-760 expression in MG63 and HOS cells

Quantitative real-time PCR was used to evaluate the expression of miRNAs. First, we screened the expression of several miRs in MG63 and HOS cells after PNS treatment. These miRs have been proved to be involved in the progression of OS. We found that PNS significantly down-regulated miR-128 and miR-760 expression at the dosage of 1 μM (Figure 3A). Then, we selected miR-760 for further research. The results indicated that 1 and 5 μM PNS notably inhibited the miR-760 expression (Figure 3B). Moreover, we found that 6 hours treatment of 1 μM PNS didn't change the expression level of miR-760 while 12, 18, and 24 hours of treatment of 1 μM PNS inhibited that (Figure 3C).

MiR-760 reversed the effect of PNS on MG63 and HOS cells

To confirm whether PNS exerts the anti-tumor effect through targeting miR-760, we carried out a rescue experiment. MTT and flow cytometry was used to evaluate the cell proliferation and apoptosis of MG63 and HOS cells. As suggested by the findings, PNS decreased the expression of miR-760. miR-760 transfection notably increased the expression of miR-760 compared to the PNS+miR-nc group (Figure 4A). Also, PNS treatment along with miR-760 transfection increased cell proliferation (Figure 4B) and reduced cell apoptosis compared to the PNS+miR-nc group (Figure 4C).

MiR-760 directly targets SMAD4

To investigate the mechanism through which miR-760 reversed the effect of PNS, potential target genes of miR-760 were predicted by bioinformatic algorithms targetscan7.2 and SMAD4 was selected (Figure 5A). To further confirm whether miR-760 directly targets SMAD4 and suppresses its expression, a firefly luciferase reporter was constructed containing a wild type or mutated type fragment of the 3'-UTR of SMAD4 mRNA. The wild type or mutated luciferase reporters were co-transfected into MG63 and HOS cells with miR-760 or miR-nc. The data showed that the co-expression of miR-760 with wild type 3'UTR but not with mutant 3'UTR significantly inhibited the luciferase activity (Figure 5B). Western blot was carried out to verify the prediction, the protein expression of SMAD4 was notably inhibited by miR-760 overexpression and was promoted by miR-760 knockdown (Figure 5C, D).

SMAD4 reversed the effect of miR-760

As miR-760 targeted SMAD4 directly, we next investigated whether SMAD4 was involved in the effect of miR-760 on cell proliferation or apoptosis. We found that PNS significantly up-regulated SMAD4 expression at the dosage of 1 and 5 μM (Figure 6A). miR-760 reversed the effect of PNS on cell proliferation and apoptosis, while SMAD4 overexpression reversed this effect of miR-760 (Figure 6B, C). Flow cytometry results showed miR-760 could decrease the apoptotic rates of MG63 and HOS cells compared to the PNS+miR-760 group, while SMAD4 overexpression restored it (Figure 6D). The western blot assay results revealed that miR-760 significantly inhibited the expression of pro-apoptosis such as cyto-C, bax, and cleaved-caspase3, meantime promoted that of anti-apoptosis protein such as bcl-2 compare to PNS group. Again, SMAD4 overexpression significantly increased

the level of cyto-C, bax, and cleaved-caspase3 while reduced that of bcl-2 compare to the PNS+miR-760 group (Figure 6E).

PNS inhibited the OS tumor growth which could be reversed by miR-760

As Figure 7 A and B showed, PNS treatment significantly inhibited the tumor growth of MG63 cells in mice. However, the pre-miR-760 transfected OS cell exerts a faster growth compared to the PNS group. Moreover, PNS reduced the tumor weight while miR-760 reversed this effect of PNS (Figure 7C). To verify whether PNS and miR-760 modulate the expression of SMAD4 in vivo, we detected the expression of SMAD4 in the tumor tissue. As expected, PNS notably promoted the expression of SMAD4 while miR-760 reversed this elevation (Figure 7D).

Discussion

In the present study, we evaluated the anti-tumor effect of PNS on OS cells. First, we demonstrated that PNS inhibit cell proliferation and promoted apoptosis in MG63 and HOS cells *in vitro* and *in vivo*. Next, we investigated PNS mode of action in OS cells. Previous studies have shown that PNS exerts vast and complex biological effects. For instance, PNS modulates the HIF-1α/mitochondria/ROS pathway in cisplatin-induced mitochondrial injury[20]. Also, PNS alleviates skeletal muscle insulin resistance via regulating GLUT4 expression and IRS1-PI3K-AKT signaling[21]. Moreover, Panax notoginsenoside saponins Rb1 regulates the expressions of Akt/ mTOR/PTEN signals in the hippocampus after focal cerebral ischemia in rats[22].

MiRs participate in the regulation of various cell physiological processes such as proliferation, apoptosis, and tumorigenesis for its critical inhibitory effect on their target genes. For instance, For instance, Zhao et al proposed that miR-345-5p could suppress tumorigenesis of papillary thyroid carcinoma by targeting SETD7[23]. Previous studies have determined the interaction between PNS and miRs. PNS inhibit lung cancer growth via modulating miR-222[24]. It also promotes miR-181b expression and the downstream mTOR/Akt/caspase-3 pathway to alleviate severe acute pancreatitis[25]. Also, by targeting miR-155, PNS inhibited oxygen-glucose deprivation/reoxygenation-induced injury in human SH-SY5Y cells[26]. The mechanism of PNS on miR in the OS has not been reported yet. We first determined that PNS inhibits the expression of miR-760 in OS cells. Ectopic expression of miR-760 can reverse the effect of OS which further proved their interaction.

MicroRNAs inhibit the translation of mRNAs or leads to its degradation by targeting the mRNAs. Bioinformatics analysis and luciferase assay confirmed that miR-760 targeted SMAD4 in both MG63 and HOS cells. SMAD4 was initially known as “deleted in pancreatic carcinoma locus 4 (DPC4)” which was identified as a candidate tumor suppressor gene[27,28,29]. It is a pivotal transducer of the TGF-β pathway and participates in the complex pathological process during tumorigenesis[30,31]. Increasing evidence indicates that abnormal Smad4 expression is closely associated with different human cancers including OS[32,33]. We assessed the expression of SMAD4 after the treatment of different concentrations of PNS and revealed that PNS promotes the expression of SMAD4 in OS cells. Next, we performed rescue experiments and found that SMAD4 overexpression reversed the effect of miR-760 on cell proliferation and apoptosis.

Apoptosis is a kind of programmed cell death that plays a crucial role in cancer development[34]. Pro-apoptotic Bcl-2 family proteins such as Bax or Bak promotes the release of mitochondrial cytochrome-C into the cytoplasm to activate caspase-9 then activates caspase-3, finally induce apoptosis[35,36]. For instance, caspase-3 was found to be related to cell apoptosis in mesangial cell and ischemia/reperfusion injury progress[37,38]. Also, Zhang et al found that Bcl-2 and Bax expressions were related to cell apoptosis in human lung cancer cells[39]. We found that PNS could attenuate the miR-760 expression and accordingly elevate the SMAD4 level. The aberrant expression of miR-760 and SMAD4 subsequently promoted the expression of apoptotic protein such as bax, cytoC, caspase3, and reduced anti-apoptotic protein bcl-2 level, finally initiate apoptosis.

In a previous study, the authors found that miR-760 was up-regulated in OS and could be sponged by long non-coding RNA ASB16-AS1 to functions as a tumor suppressor. Sometimes, the contradictive results will be found due to the difference in the experimental methods or regents. We will confirm it in our future studies.

In conclusion, we firstly demonstrated that PNS inhibited cell proliferation and induced apoptosis of MG63 and HOS cells by regulating the expression of miR-760. Moreover, miR-760 regulated the apoptotic protein expression by directly targeting SMAD4. These findings extended our understanding of the effect of PNS in cancer progression. However, more work should be carried out for the clinical use of PNS in cancer therapy.

Declaration of absence of conflicts of interest

No conflicts of interest exist in the present study.

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Figure legends

Figure 1. PNS inhibited the cell proliferation of MG63 and HOS cells at the dose-dependent or time-dependent manner. (A) MTT was used to evaluate the cell proliferation of MG63 and HOS cells after different concentrations of PNS treatment for 24 h. (B) MTT was used to evaluate the cell

proliferation of MG63 and HOS cells after 5 μM PNS treatment at the indicated time. (C) The growth curve of MEG63 cell line was made under PNS treatment for a period of 3 days. (D) The growth curve of HOS cell line was made under PNS treatment for a period of 3 days. The results were presented as the mean ± SD, n = 6, *P<0.05 vs normal saline (NS).

Figure 2. PNS induced apoptosis of MG63 and HOS cells. (A) Flow cytometry was carried out to detect the apoptosis of MG63 cell line treated with 0.1, 1, 5μM PNS. (B) Flow cytometry was carried out to detect the apoptosis of HOS cell line treated with 0.1, 1, 5μM PNS. (C) Western blot was used to investigate the apoptosis-related protein expressions in MG63 and HOS cells. The results were presented as the mean ± SD, n = 3, *P<0.05 vs NS.

Figure 3. PNS inhibited the expression of miR-760. (A) The expression of several miRs was evaluated in OS cells after PNS treatment by qPCR. (B) qPCR was performed to detect the expression level of miR-760 in OS cells after different concentrations of PNS treatment. (C) qPCR was performed to detect the expression level of miR-760 in OS cells after different periods of PNS treatment. The results were presented as the mean±SD, n = 6, *P<0.05 vs NS.

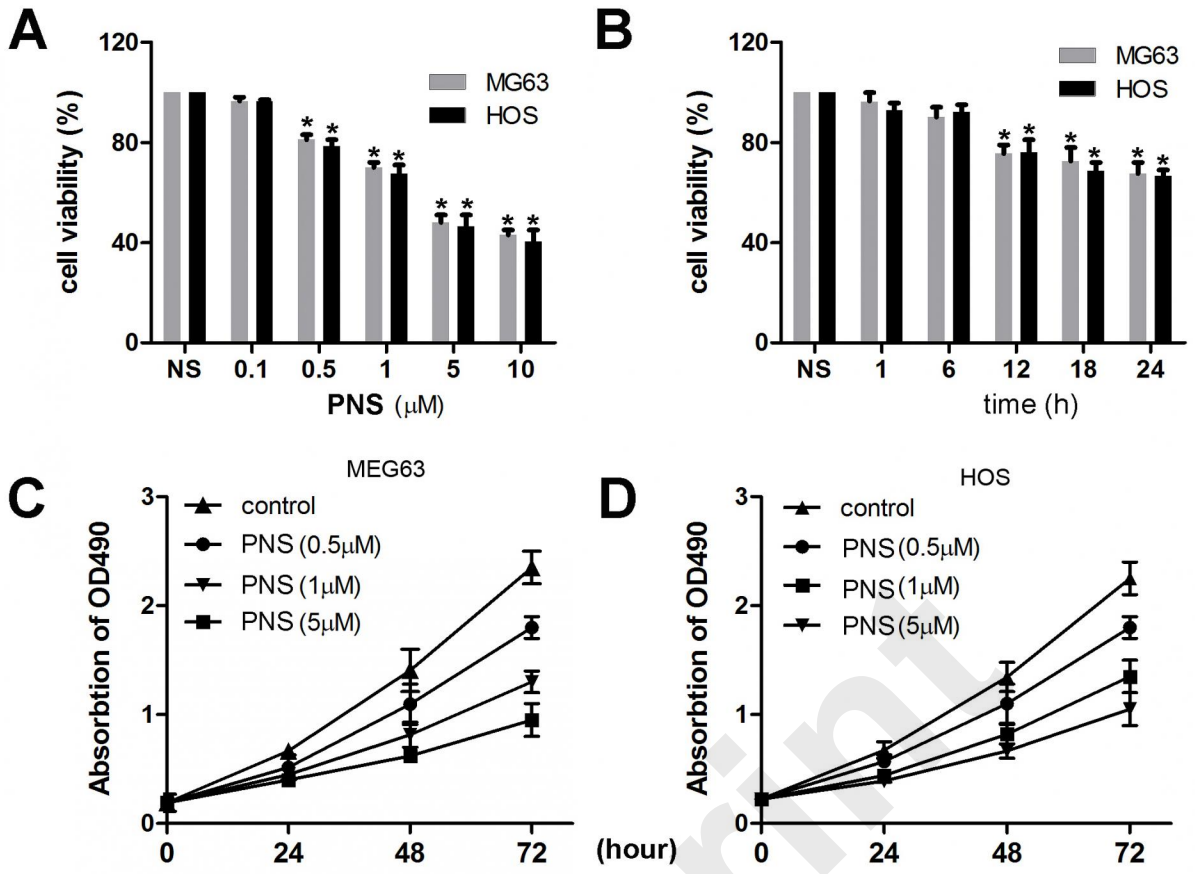
Figure 4. MiR-760 overexpression reversed the effect of PNS. (A) qPCR was used to detect the expression level of miR-760 after PNS treatment or miR-760 transfection. (B) MTT was used to investigate the cell proliferation of MG63 and HOS cells after PNS treatment or the transfection of miR-760. (C) Flow cytometry was used to investigate the cell apoptosis of MG63 and HOS cells after

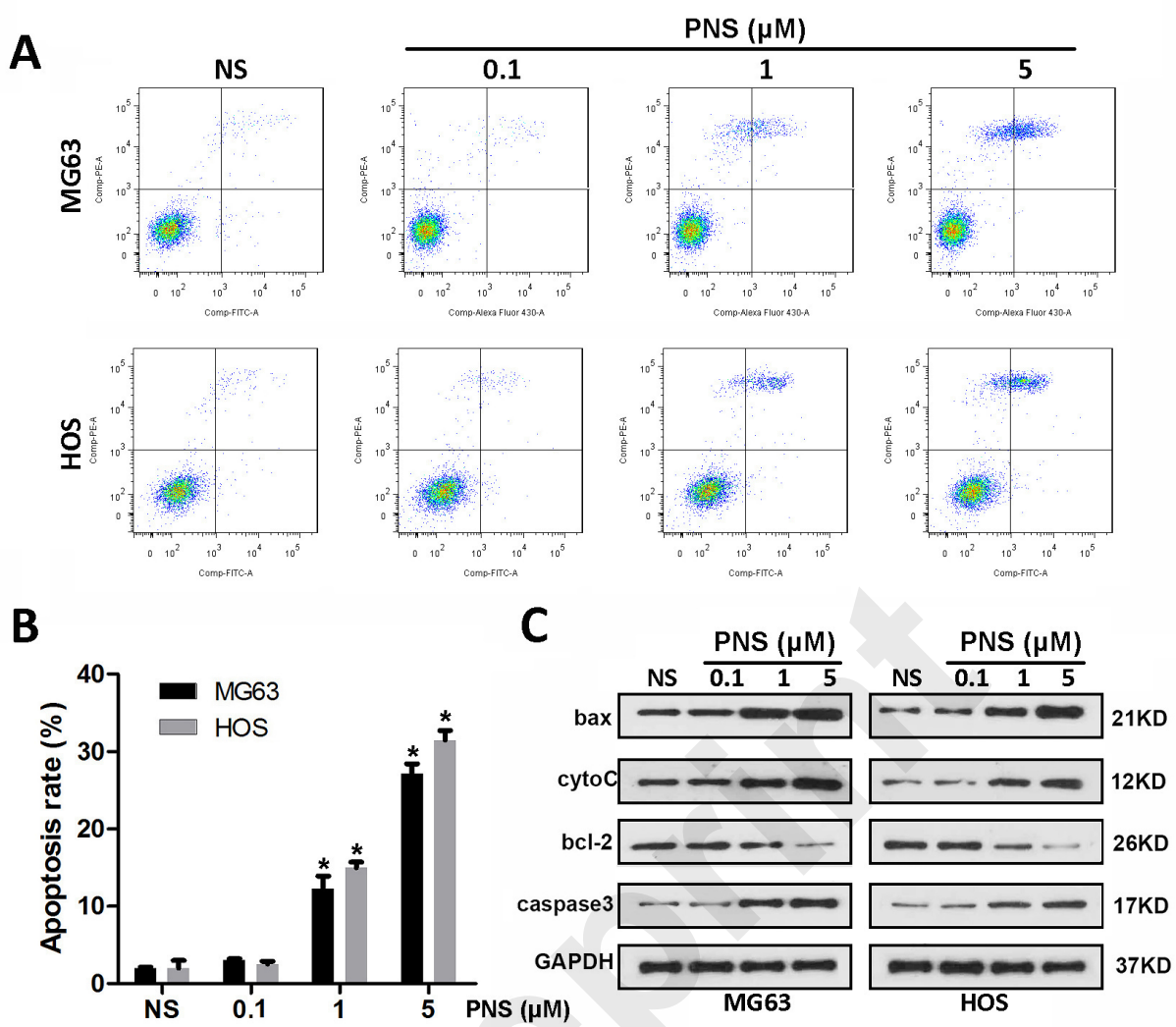
PNS treatment or the transfection of miR-760. The results were presented as the mean±SD, n = 6, $^{*}P<0.05$ vs control, $^{\#}P<0.05$ vs PNS+miR-nc.

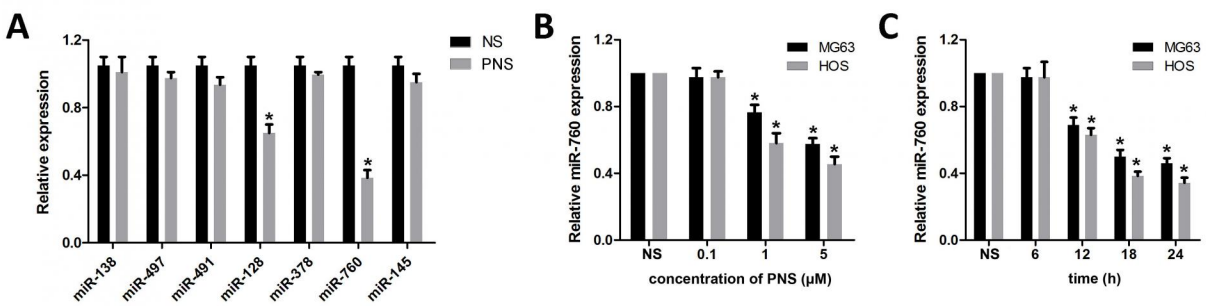
Figure 5. SMAD4 is a direct target of miR-760. (A) Schematic representation of the miR-760 targeting sequences within the 3'-UTR of SMAD4. (B) Luciferase reporter assay was conducted in MG63 and HOS cells following transfection with miR-760 or miR-nc, and together with Wt or Mut SMAD4 3'UTR luciferase reporter plasmid. (C, D) The protein expression of SMAD4 in MG63 and HOS cells transfected with miR-760, miR-nc, miR-760 inhibitor, or inhibitor control was detected by western blotting. The results were presented as the mean ± SD, n = 6, *P<0.05 vs miR-nc, $^{\#}P<0.05$ vs inhibitor control.

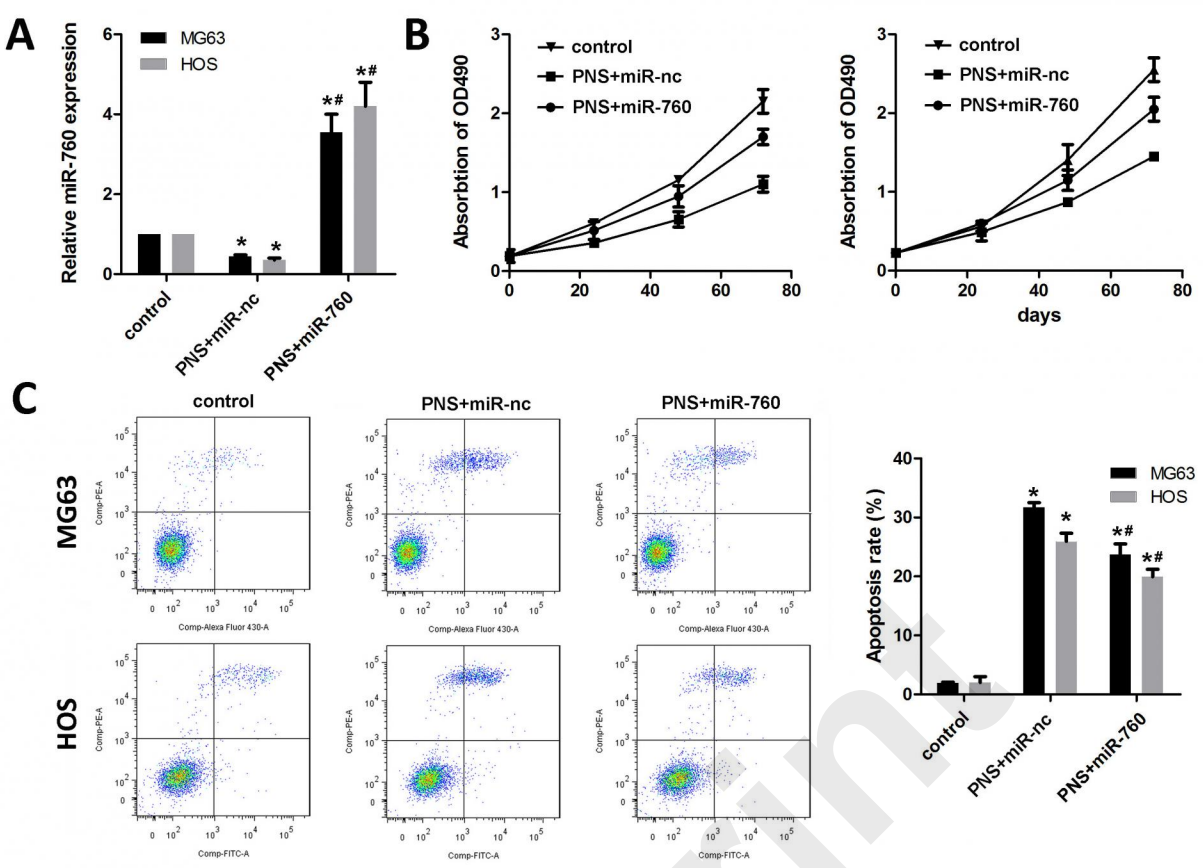
Figure 6. SMAD4 overexpression reversed the effect of miR-760. OS cells were transfected with 200nM miR-760 mimic and the SMAD4 overexpressing vectors. (A) Western blot was used to detect the expression level of SMAD4 after the treatment of PNS and the quantified results of western blot assay was presented. (B, C) MTT was used to investigate the cell proliferation of MG63 and HOS cells after PNS treatment or overexpression of SMAD4. (D) Flow cytometry was used to investigate the cell apoptosis of MG63 and HOS cells after PNS treatment or overexpression of SMAD4. (E) Western blot was used to evaluate the apoptotic protein expressions of MG63 and HOS cells after PNS treatment or overexpression of SMAD4. The results were presented as the mean ± SD, n = 6, *P<0.05 vs PNS+miR-nc, $^{\#}P<0.05$ vs PNS+miR-760.

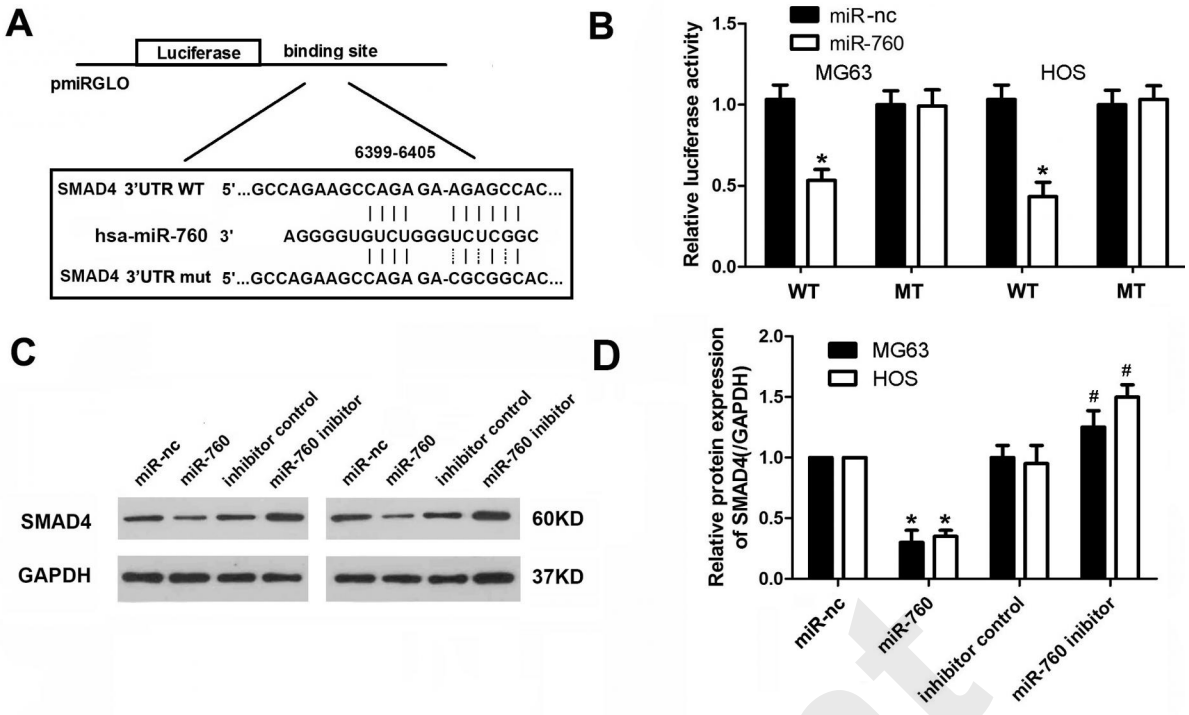
Figure 7. PNS attenuated the tumor growth of OS cells and miR-760 reversed this effect of PNS. (A) The mice in each group were sacrificed and the tumors were excised and pictured. (B) Tumor sizes of the mice in each group were measured and the growth curve was shown. (C) Tumor weight was measured in each group. (D) IHC was performed to evaluate the expression of SMAD4 in the tumor tissues. The results were presented as the mean ± SD, n = 3, *P<0.05 vs control, $^{\#}$P<0.05 vs PNS.

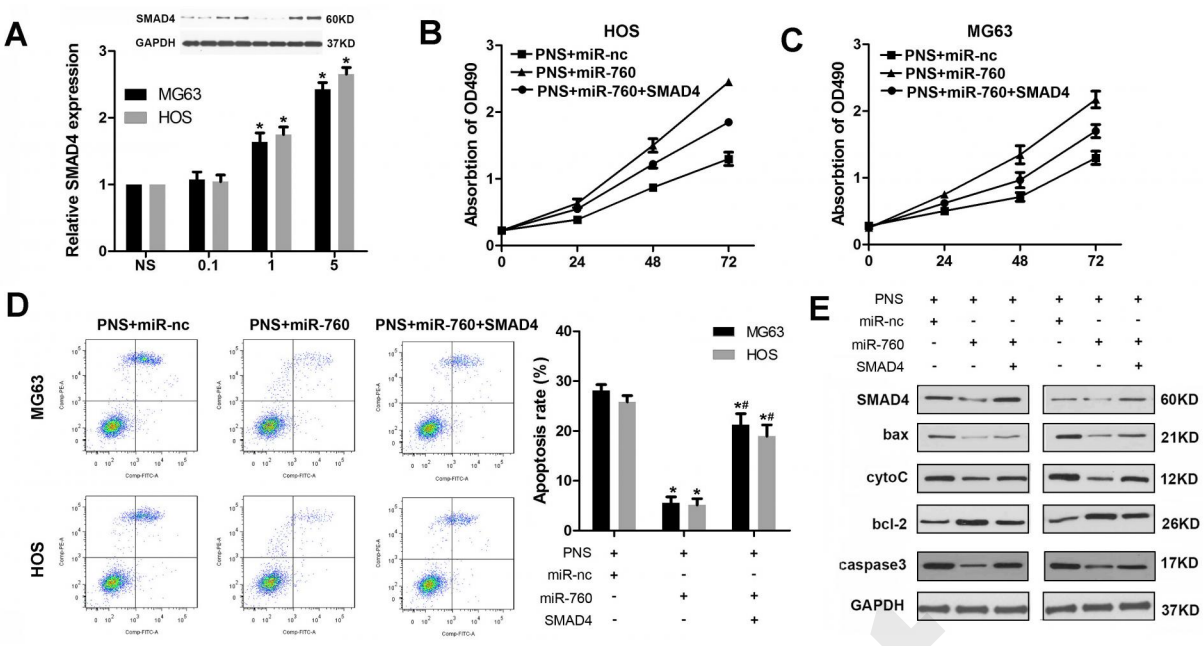

A
cell viability (%)
120
80
40
0
MG63
HOS
NS
0.1
0.5
1
5
10
PNS (μM)
B
cell viability (%)
MG63
HOS
NS
1
6
12
18
24
time (h)
C
MEG63
Absorbtion of OD490
control
PNS (0.5μM)
PNS (1μM)
PNS (5μM)
0
24
48
72
D
HOS
Absorbtion of OD490
control
PNS (0.5μM)
PNS (1μM)
PNS (5μM)
(hour)
0
24
48
72

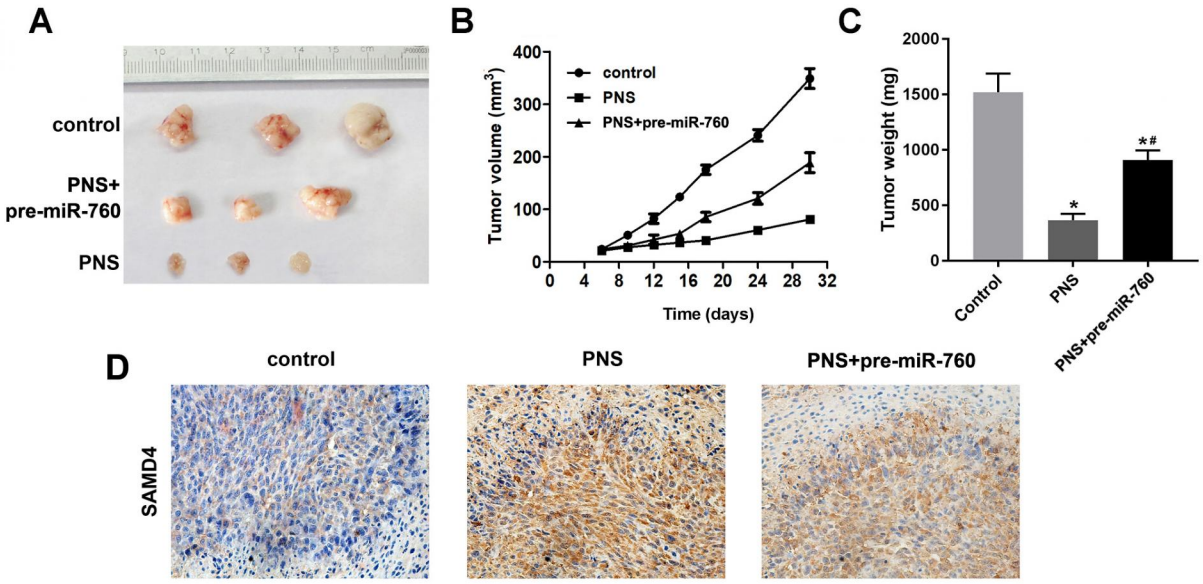

A
PNS (μM)
NS
0.1
1
5
MG63
HOS
Comp-PE-A
Comp-FITC-A
Comp-Alexa Fluor 430-A
B
Apoptosis rate (%)
MG63
HOS
NS
0.1
1
5
PNS (μM)
C
PNS (μM)
NS
0.1
1
5
bax
cytoC
bcl-2
caspase3
GAPDH
21KD
12KD
26KD
17KD
37KD
MG63
HOS


A
Relative expression
1.2
0.8
0.4
0.0
NS
PNS
miR-138
miR-497
miR-491
miR-128
miR-378
miR-760
miR-145
B
Relative miR-760 expression
MG63
HOS
NS
0.1
1
5
concentration of PNS (μM)
C
Relative miR-760 expression
MG63
HOS
NS
6
12
18
24
time (h)


A
Relative miR-760 expression
MG63
HOS
control
PNS+miR-nc
PNS+miR-760
B
Absorbtion of OD490
control
PNS+miR-nc
PNS+miR-760
days
C
control
PNS+miR-nc
PNS+miR-760
MG63
HOS
Comp-PE-A
Comp-Alexa Fluor 430-A
Comp-FITC-A
Apoptosis rate (%)


A
Luciferase
binding site
pmiRGLO
6399-6405
SMAD4 3'UTR WT 5'...GCCAGAAGCCAGA GA-AGAGCCAC...
hsa-miR-760 3' AGGGGUGUCUGGGUCUCGGC
SMAD4 3'UTR mut 5'...GCCAGAAGCCAGA GA-CGCGGCAC...
B
miR-nc
miR-760
MG63
HOS
Relative luciferase activity
1.5
1.0
0.5
0.0
*
*
WT
MT
WT
MT
C
miR-nc
miR-760
inhibitor control
miR-760 inibitor
miR-nc
miR-760
inhibitor control
miR-760 inibitor
SMAD4
GAPDH
60KD
37KD
D
MG63
HOS
Relative protein expression of SMAD4(/GAPDH)
2.0
1.5
1.0
0.5
0.0
*
*
#
#
miR-nc
miR-760
inhibitor control
miR-760 inibitor


A
SMAD4
60KD
GAPDH
37KD
Relative SMAD4 expression
MG63
HOS
NS
0.1
1
5
B
HOS
PNS+miR-nc
PNS+miR-760
PNS+miR-760+SMAD4
Absorbtion of OD490
C
MG63
D
PNS+miR-nc
PNS+miR-760
PNS+miR-760+SMAD4
Apoptosis rate (%)
E
PNS
miR-nc
miR-760
SMAD4
bax
cytoC
bcl-2
caspase3
GAPDH
60KD
21KD
12KD
26KD
17KD
37KD


A
control
PNS+
pre-miR-760
PNS
B
control
PNS
PNS+pre-miR-760
Tumor volume (mm3)
Time (days)
C
Tumor weight (mg)
Control
PNS
PNS+pre-miR-760
*
*#
D
control
PNS
PNS+pre-miR-760
SAMD4