

# Involvement of CHOP in activin A-induced myeloma NS-1 cell apoptosis

JINGYAN GE<sup>1</sup>, HONGYAN SUN<sup>1</sup>, JING LI<sup>2</sup>, YIDI SHAN<sup>1</sup>, YANG ZHAO<sup>2</sup>,  
FANGWEI LIAO<sup>3</sup>, YU YANG<sup>4</sup>, XUELING CUI<sup>3</sup> and ZHONGHUI LIU<sup>2</sup>

Departments of <sup>1</sup>Physiology, <sup>2</sup>Immunology, <sup>3</sup>Genetics and <sup>4</sup>Functional Laboratory, College of Basic Medical Sciences, Jilin University, Changchun, Jilin 130021, P.R. China

Received February 11, 2019; Accepted October 1, 2019

DOI: 10.3892/or.2019.7382

**Abstract.** Activin A, a multifunctional cytokine, is a member of transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily. It is associated with a variety of pathophysiological processes, including inflammation, fibrosis, and tumorigenesis. Chronic or prolonged endoplasmic reticulum (ER) stress can lead to cells apoptosis. However, whether ER stress-related proteins, such as CHOP, GADD34 are involved in activin A-induced myeloma cell apoptosis remains unknown. In the present study, it was revealed that activin A inhibited the proliferation of myeloma cell line NS-1 cells and induced NS-1 cell apoptosis. Activin A upregulated the expression of CHOP, GADD34, caspase-3, and caspase-12. Moreover, both Smad3 and p-Smad3 levels were increased with treatment of activin A. Further studies revealed that the overexpression of activin signaling protein Smad3 in NS-1 cells increased the levels of CHOP, caspase-3, and p-Smad3. These data indicated that the CHOP protein of the ER stress pathway may be involved in activin A-induced NS-1 cell apoptosis, and also indicated the potential therapy of activin A-induced apoptosis via CHOP signaling for multiple myeloma.

## Introduction

Activin is a member of transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily consisting of two inhibin  $\beta$  subunits linked by disulfide bonds. It is a type of glycoprotein initially isolated from porcine follicular fluid in 1986 and named for its ability to stimulate the release of follicle-stimulating hormone (FSH)

from the pituitary. Activin exists in three basic molecular forms composed of two inhibin  $\beta$  subunits: activin A ( $\beta A\beta A$ ), activin B ( $\beta B\beta B$ ), and activin AB ( $\beta A\beta B$ ) (1-3). Activin A is expressed widely in various tissues and cells with strong bioactivities and is the mostly studied activin (4-6). It first binds to the type II activin receptors (ActRIIA or ActRIIB) on the member surface, and then recruits and phosphorylates type I activin receptors (ActRI). The phosphorylated ActRI activates Smad2 and Smad3, which form a complex with Smad4 to translocate to the nucleus. Activin and TGF- $\beta$  share the same signaling pathway at the level of Smad2/3/4. Activin A exerts a variety of biological functions including regulation of hematopoietic cell proliferation, neuron differentiation, pituitary hormone secretion, and tissue repair. It is also involved in the process of many diseases, for example, inflammation, fibrosis and tumorigenesis (7-10).

Activin A has pro- and anti-tumorigenic functions depending on the tumor type. In breast, liver and colon cancers, activin signals were revealed to inhibit tumor cell growth. In addition, tumor tissues were revealed to express decreased levels of activin A or increased levels of activin antagonists or demonstrated the downregulation of activin receptors or Smad proteins (11). In contrast, high expression of activin A in some tumors is associated with tumor cell aggressiveness in oral squamous cell carcinoma, esophageal squamous cell carcinoma, and malignant pleural mesothelioma. In lung adenocarcinoma, high circulating levels of activin A were also associated with tumor progression and predicted poor prognosis (12).

The endoplasmic reticulum (ER) is an organelle essential for cell survival and normal functions. Various disturbances in the ER, including ischemia, hypoxia, oxidative injury, and viral infections, can induce ER stress that first tends to restore cellular homeostasis (13). However, severe or prolonged ER stress culminates in cell apoptosis. ER stress is associated with a wide range of diseases, including neurodegenerative disorders, cancer, diabetes, as well as many others (14-17). Our previous study revealed the involvement of activin A in the apoptosis of myeloma cell line NS-1 cells (18). However, whether activin A can induce the apoptosis of NS-1 cells via the ER stress pathway is still unclear. Therefore, in the present study, ER stress-related proteins such as CHOP, GADD34 and caspase-12 were examined to investigate the effects of activin A on the apoptosis of NS-1 cells.

*Correspondence to:* Professor Zhonghui Liu, Department of Immunology, College of Basic Medical Sciences, Jilin University, 126 Xinmin Street, Changchun, Jilin 130021, P.R. China  
E-mail: liuzh@jlu.edu.cn

Professor Xueling Cui, Department of Genetics, College of Basic Medical Sciences, Jilin University, 126 Xinmin Street, Changchun, Jilin 130021, P.R. China  
E-mail: cxl@jlu.edu.cn

**Key words:** activin A, apoptosis, CHOP, NS-1 cells, Smad3

## Materials and methods

**Reagents and antibodies.** RPMI-1640 medium and Iscove's modified Dulbecco's medium (IMDM) were obtained from Gibco; Thermo Fisher Scientific, Inc. Fetal bovine serum (FBS) was purchased from Biological Industries Israel Beit-Haemek Ltd. Activin A was obtained from R&D Systems, Inc. A one-step reverse transcription-polymerase chain reaction (RT-PCR) kit was provided from Takara Biotechnology Co., Ltd. Protein extraction kits were purchased from Thermo Fisher Scientific, Inc. Annexin V-FITC Apoptosis Analysis Kit, anti- $\beta$ -tubulin antibody (cat. no. KM9003T) and anti-p-Smad3 antibody (cat. no. SGAP0271) were purchased from Tingjin Sungene Biotech Co., Ltd. Anti-Smad3 antibody (cat. no. A11471) was purchased from ABclonal Biotechnology Co., Ltd. Anti-caspase-3 (cat. no. 9662), anti-caspase-12 (cat. no. 2202) and anti-CHOP (cat. no. 2895) antibodies were purchased from Cell Signaling Technology, Inc. Anti-GADD34 antibody (cat. no. ab131402) was purchased from Abcam Co. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (cat. no. A0545) or anti-mouse IgG antibodies (cat. no. A3682) were purchased from Sigma-Aldrich (Merck KGaA).

**Cell culture.** The mouse myeloma cell line NS-1 was purchased from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium with 10% FBS in a 5% CO<sub>2</sub>-humidified atmosphere at 37°C. Additionally, a second mouse myeloma cell line SP2/0 (Appendix I) was also used for confirmation. This cell line was purchased from Beijing BeNa Culture Collection and cultured with 90% IMDM+10% FBS.

**MTT assay for NS-1 cell viability.** The NS-1 cells ( $2 \times 10^4$  cells/well) were seeded in a 96-well culture plate. Then, the cells were treated with 0–10 ng/ml activin A for 12 and 24 h, and incubated with 0.5 mg/ml MTT for 4 h. After the supernatant was discarded carefully, 100  $\mu$ l DMSO per well was added to dissolve the formazan crystals. Then the absorbance values of the samples were read using a plate reader (BioTek Instruments, Inc.) at 540 nm to evaluate the cell viability.

**Flow cytometry for cell proliferation assay.** Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye that can label living cells. It can easily penetrate the cell membrane and covalently bind with intracellular proteins in living cells. The labeled fluorescence can be evenly distributed between two progeny cells, and hence the fluorescence intensity decreases step by step with cell division. In the present study, proliferating cells were counted by flow cytometry. In brief, the NS-1 cells ( $1 \times 10^7$  cells/ml) in phosphate-buffered saline (PBS) were treated with 1  $\mu$ mol/l CFSE in the dark for 10 min. Then, an equal volume of 5% FBS-RPMI-1640 medium was added to terminate the reaction. The cells were harvested by centrifugation (150  $\times$  g) and resuspended in 5% FBS-RPMI-1640 medium. The proliferating NS-1 cells in the M2 region were determined by flow cytometry (BD FACSCalibur; BD Biosciences). Data were collected and analyzed using Cell Quest software (BD Biosciences) to obtain the percentage of fluorescent cells.

**Giemsa staining.** The NS-1 cells ( $2 \times 10^4$  cells/well) were seeded in a 96-well culture plate, and treated with 0–10 ng/ml activin A for 24 h. The cells were washed three times with PBS, and then incubated with 50  $\mu$ l of Giemsa for 5 min. After washing twice with PBS, the cell morphology was observed under an IX71 microscope and images were captured using a microscope digital camera system (Olympus Optical Co. Ltd.).

**RT-PCR.** The NS-1 cells ( $2 \times 10^5$  cells/well) were seeded in a 12-well culture plate and incubated with 0–5.0 ng/ml activin A for 12 h. Then, total RNA was extracted by using TRIzol reagent in accordance with the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA was amplified by using a one-step RT-PCR kit according to the manufacturer's protocols (Takara Biotechnology Co., Ltd.). PCR was performed using the following reaction conditions: 94°C for 30 sec, 56°C for 20 sec, 72°C for 40 sec (35 cycle) and final extension was 72°C for 10 min. The primer sequences are listed in Table I. PCR products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide. The specific bands were visualized using ImageMaster VDS (Pharmacia Biotech; GE Healthcare). The densitometric quantification of mRNA was normalized to the internal control GAPDH.

**Flow cytometry for cells apoptosis assay.** The NS-1 cells ( $1 \times 10^6$  cells/well) were seeded in a 12-well culture plate. They were treated with 0–10 ng/ml activin A for 24 h, collected, and re-suspended in 100  $\mu$ l of FACS buffer followed by the addition of 1  $\mu$ l FITC-Annexin V and 1  $\mu$ l 7-AAD for 5 min in the dark. Then, the labeled cells were analyzed by flow cytometry (BD FACSCalibur). Data were collected and analyzed using the Cell Quest software (BD Biosciences) to obtain the percentage of fluorescent cells.

**Western blotting.** The NS-1 cells ( $1 \times 10^6$  cells/well) were treated with activin A for 12 h, harvested, and lysed in protein lysis buffer (M-PER; Thermo Fisher Scientific, Inc.). Then the protein concentration was determined by the Pierce BCA Protein Assay (Thermo Fisher Scientific, Inc.). The proteins (30  $\mu$ g each sample) were separated by electrophoresis with 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane. Then the membrane was blocked with 2% BSA for 1 h at room temperature and incubated in primary antibodies against CHOP (1:1,000 dilution), caspase-3 (1:1,000 dilution), caspase-12 (1:500 dilution), GADD34 (1:500 dilution), and  $\beta$ -tubulin (1:1,000) at 4°C overnight, respectively. Following washing with 1X TBST with 0.1% Tween-20 three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1:160,000) or anti-mouse IgG antibodies (1:40,000) for 1 h at room temperature. Finally, the labeled proteins were detected by chemiluminescence (ECLPlus; Amersham Pharmacia Biotech; GE Healthcare) and analyzed using ImageJ software (v1.43; National Institutes of Health). The protein levels were normalized to  $\beta$ -tubulin.

**Establishment of tumor-bearing mice with NS-1 cells.** Male Balb/c mice (18–20 g) provided by Vital River Laboratories Technology Co. Ltd. were housed in a temperature-controlled room with a 12-h light/dark cycle. All animal experiments

Table I. Primer sequences used in RT-PCR.

Gene	Primer	Sequence (5'-3')	Fragment size (bp)	T (°C)
ActRIIA	F	ATTGGCCAGCATCCATCTCTTG	295	56
	R	GCCACCATCATAGACTAGATTC		
ActRIIB	F	TGCTGAAGAGCGACCTCAC	535	58
	R	AGCAGGTCCACATTGGTGAC		
Smad3	F	CTCCTACTACGAGCTGAACCA	572	58
	R	AAGACACACTGGAACAGCGGA		
Caspase-3	F	TGGTGATGAAGGGGTCAATTATG	105	56
	R	TTCGGCTTTCCAGTCAGACTC		
Caspase-12	F	TGCTGACAGCTCCTCATGGAC	342	56
	R	ATGTGCTGTCTGAGGACTGGTG		
CHOP	F	TAGCTTGGCTGACAGAGGAG	331	56
	R	GTTCAATGCTTGGTGCAGGCT		
p53	F	CCTCCAGAAGATATCCTGCCAT	275	56
	R	CACATAACAGACTTGGCTGTCC		
p21	F	GCCTTGTCGCTGTCTTGCACT	297	56
	R	GAGAGGGCAGGCAGCGTATATA		
GAPDH	F	GATTGTTGCCATCAACGACC	372	56
	R	GTGCAGGATGCATTGCTGAC		

F, forward; R, reverse.

were conducted in accordance with the Jilin University guidelines for the care and use of animals. A laboratory animal ethics review form was approved by the Laboratory Animal Ethics Committee of the College of Basic Medical Sciences of Jilin University (No. 2018-018). The NS-1 cells in the logarithmic growth phase were resuspended in saline. Then,  $2.0 \times 10^6$  NS-1 cells in 100  $\mu$ l of saline were inoculated on the back of the mouse via subcutaneous injection and the tumor size was measured every day until the volume was  $\sim 105 \pm 25$  mm<sup>3</sup>. The tumor-bearing mice with NS-1 cells were randomly divided into control and activin A groups. Furthermore, 20 ng activin A in 1  $\mu$ l of saline was injected with a microsyringe into each tumor center of mice in the activin A group, and the same volume of saline was injected in the control group. Before injection, a cotton ball fully soaked with 0.5 ml ether was placed into an anesthesia box, then the mouse was quickly placed into the anesthesia box for  $\sim 30$  to 60 sec in order to relieve mouse suffering and distress. The administration was repeated once 12 h later. The tumor was measured in the following six consecutive days. On the sixth day, all the mice were sacrificed by anesthesia with intraperitoneal injection of 3% pentobarbital sodium overdose at 90 mg/kg until the collection of the blood from the heart and when breathing and a heartbeat disappeared. The lethal dose was in general approximately three times the anesthetic dose. Then the tumors were dissected, and the volume of the tumors was measured. Activin A was injected directly into tumors. It was observed that after injection of activin A, the volume of the tumors had significantly decreased. However, the volume of the tumors in the control group grew too fast, and as time progressed, the tumors in the control group began to ulcer and rupture. Therefore, only 6 days were observed.

*Smad3 overexpression in NS-1 cells.* The NS-1 cells were transfected with Smad3-expressing plasmid pcDNA-Smad3 and control empty plasmid pcDNA3, respectively. Briefly, the pcDNA-Smad3 plasmids or control pcDNA3 plasmids (0.3  $\mu$ g) were enfolded with Lipofectamine 2000 (1:2) according to the manufacturer's protocol and then transfected into NS-1 cells.

*Statistical analysis.* Data were repeated at least three times. All results were expressed as the means  $\pm$  standard deviation (SD). The data were analyzed using Student's t-test for comparison of two groups and ANOVA with post hoc Dunnett's test for multiple group comparisons. Statistical analysis was performed using the software SPSS17.0 (SPSS, Inc.). Data were considered as statistically significant at  $P < 0.05$ .

## Results

*Activin A affects the expression of ActRIIs and Smads in NS-1 cells.* Activin A first binds to activin type II receptors (ActRIIA or ActRIIB) and then activates downstream signaling molecules Smad2 and Smad3. To confirm whether activin A exerted an effect on NS-1 cells, the mRNA expression of ActRIIs and Smads were examined in NS-1 cells after treated with activin A. The results revealed that not only was the mRNA expression of ActRIIA, ActRIIB, Smad2 and Smad3 detectable in NS-1 cells, but ActRIIA, Smad2 and Smad3 were also upregulated in NS-1 cells treated with activin A for 12 h. Conversely, activin A did not alter the mRNA expression of ActRIIB. In addition, the protein levels of Smad3 and p-Smad3 were increased (Fig. 1), confirming that activin A may exert an effect on NS-1 cells via the ActRIIA/Smad3 signaling pathway.

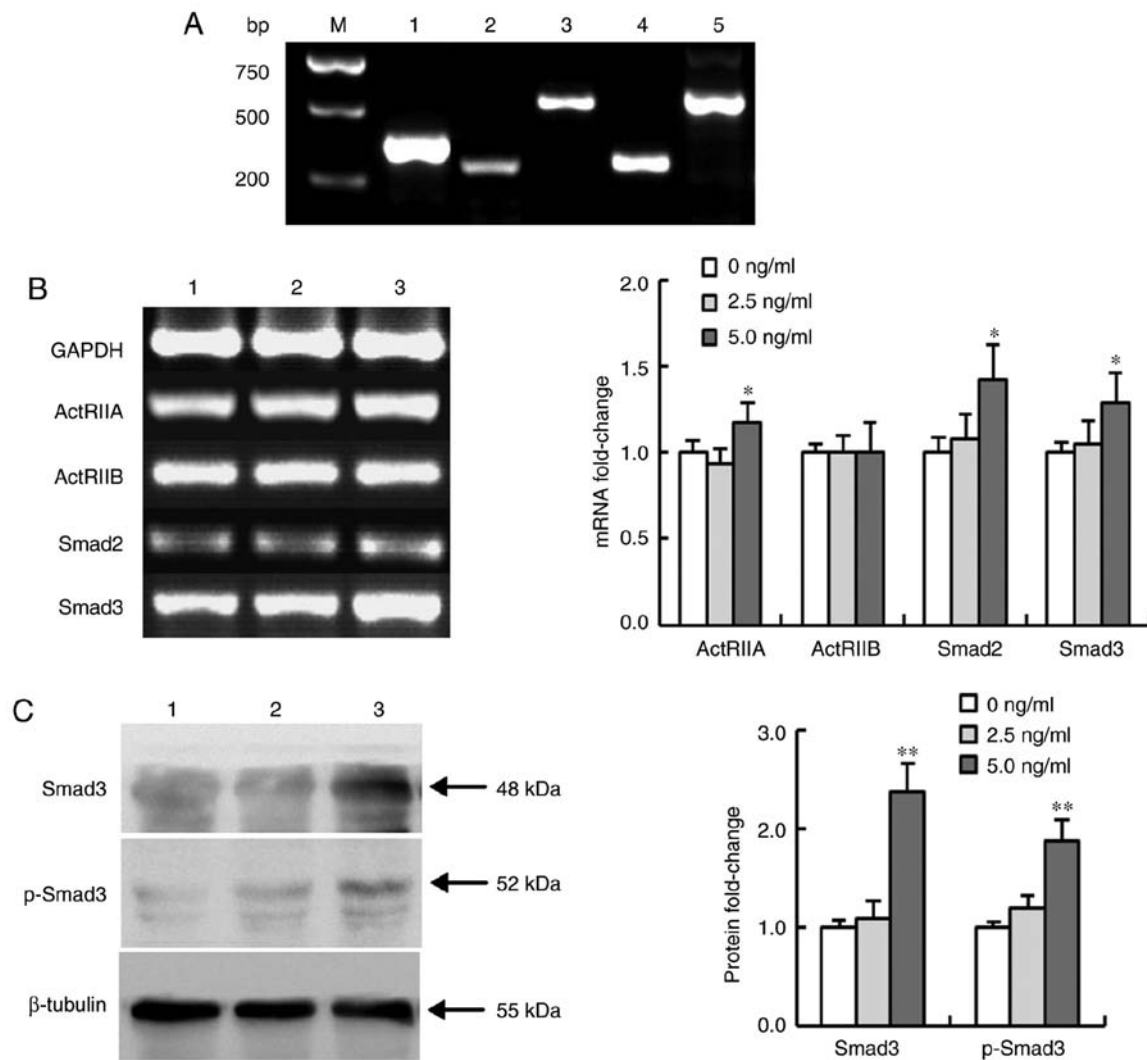


Figure 1. Effect of activin A on the expression of ActRIIs and Smads in NS-1 cells. (A) The mRNA expression of ActRIIA, ActRIIB, Smad2 and Smad3 in NS-1 cells was examined by RT-PCR. M, molecular weight (bp); Lane 1: GAPDH; Lane 2: ActRIIA; Lane 3: ActRIIB; Lane 4: Smad2; Lane 5: Smad3. (B) The mRNA expression of ActRIIA, ActRIIB, Smad2, and Smad3 in NS-1 cells treated with activin A was examined by RT-PCR. Lane 1: 0 ng/ml activin A; Lane 2: 2.5 ng/ml activin A; Lane 3: 5 ng/ml activin A. The graph reveals the fold change in mRNA expression normalized to GAPDH from three independent experiments. \* $P < 0.05$ , compared with the 0-ng/ml group. (C) Levels of Smad3 and p-Smad3 proteins were examined by western blotting. The graph reveals the fold change in protein expression normalized to  $\beta$ -tubulin from three independent experiments. \*\* $P < 0.01$  compared with the 0-ng/ml group.

#### Activin A inhibits the viabilities and proliferation of NS-1 cells.

To demonstrate the effect of activin A on NS-1 cells, cell viability was first examined by MTT assay. After treatment with activin A for 12 and 24 h, the viability of NS-1 cells was inhibited in a dose-dependent manner, compared with untreated NS-1 cells in the control group (Fig. 2A). Then, the proliferation of NS-1 cells was further evaluated by flow cytometry after the cells were labeled with CFSE and treated with activin A for 24 h. The results revealed that the percentage of proliferating cells (M2) significantly decreased with the treatment of activin A, compared with that in the control group (Fig. 2B). Additionally, the second mouse myeloma cell line SP2/0 (Appendix I) was used to verify the findings, and it was revealed that activin A also inhibited the viability and proliferation of SP2/0 cells (Fig. S1). These results indicated that activin A could suppress NS-1 cell proliferation.

**Activin A alters the morphology of NS-1 cells.** In order to further evaluate the effect of activin A, the morphological changes of NS-1 cells were examined by Giemsa staining

after treatment with activin A for 24 h. In the control group, the cells were identical in size and the nuclear membrane was smooth. However, the morphology of cells treated with activin A was evidently different in size and shape. The cells shrunk and the number of cells with chromatin condensation was markedly increased (Fig. 3A). The percentage of viable cells also decreased with the treatment of activin A (Fig. 3B). These data indicated that activin A may induce NS-1 cell apoptosis. According to Fig. 3, a greater effect of reduction of cell viability was observed than Fig. 2A. Cells in Fig. 3 were stained by Giemsa. Some cells lost the ability of adhesion although they were not dead. These cells detached from the culture plate when they were washed with PBS repeatedly. Therefore, the ratio of cell viability by MTT detection was different from that of Giemsa staining living cells.

**Activin A increases the apoptotic ratio of NS-1 cells.** Chromatin condensation is one of the hallmarks of apoptosis. Therefore, the apoptotic ratio of NS-1 cells stained with

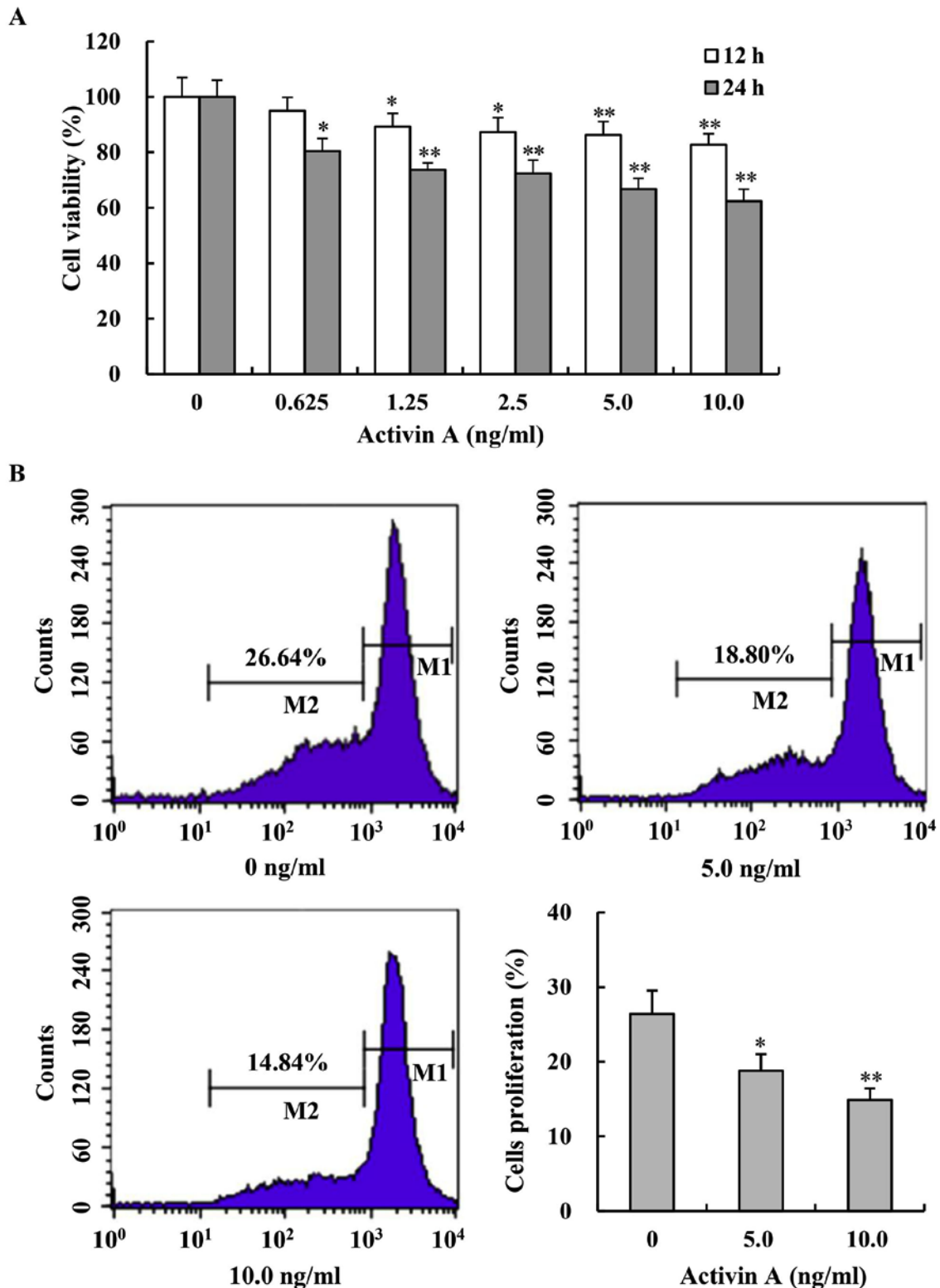


Figure 2. Effect of activin A on the viability of NS-1 cells. (A) Cell viability was determined by MTT assay after treatment with activin A for 12 and 24 h. The values were expressed as the means  $\pm$  SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the 0-ng/ml group. (B) Proliferation of NS-1 cells was examined after treatment with activin A for 24 h by staining with CFSE. Then the proliferating cells were counted in the M2 region by flow cytometry. The graph reveals the percentage of proliferating NS-1 cells from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the 0-ng/ml group.

FITC-Annexin V and 7-AAD was further examined by flow cytometry. After treatment with activin A for 24 h, the apoptotic ratio of NS-1 cells significantly increased compared with that in the control group (Fig. 4). These data further confirmed that activin A induced NS-1 cell apoptosis.

*Activin A inhibits the growth of solid tumors of NS-1 cells in mice.* After the solid tumors of NS-1 cells were formed in mice, exogenous activin A was injected into the solid tumors and the same volume of saline was injected in the control group. Then, the volume of the tumors was assessed for the following



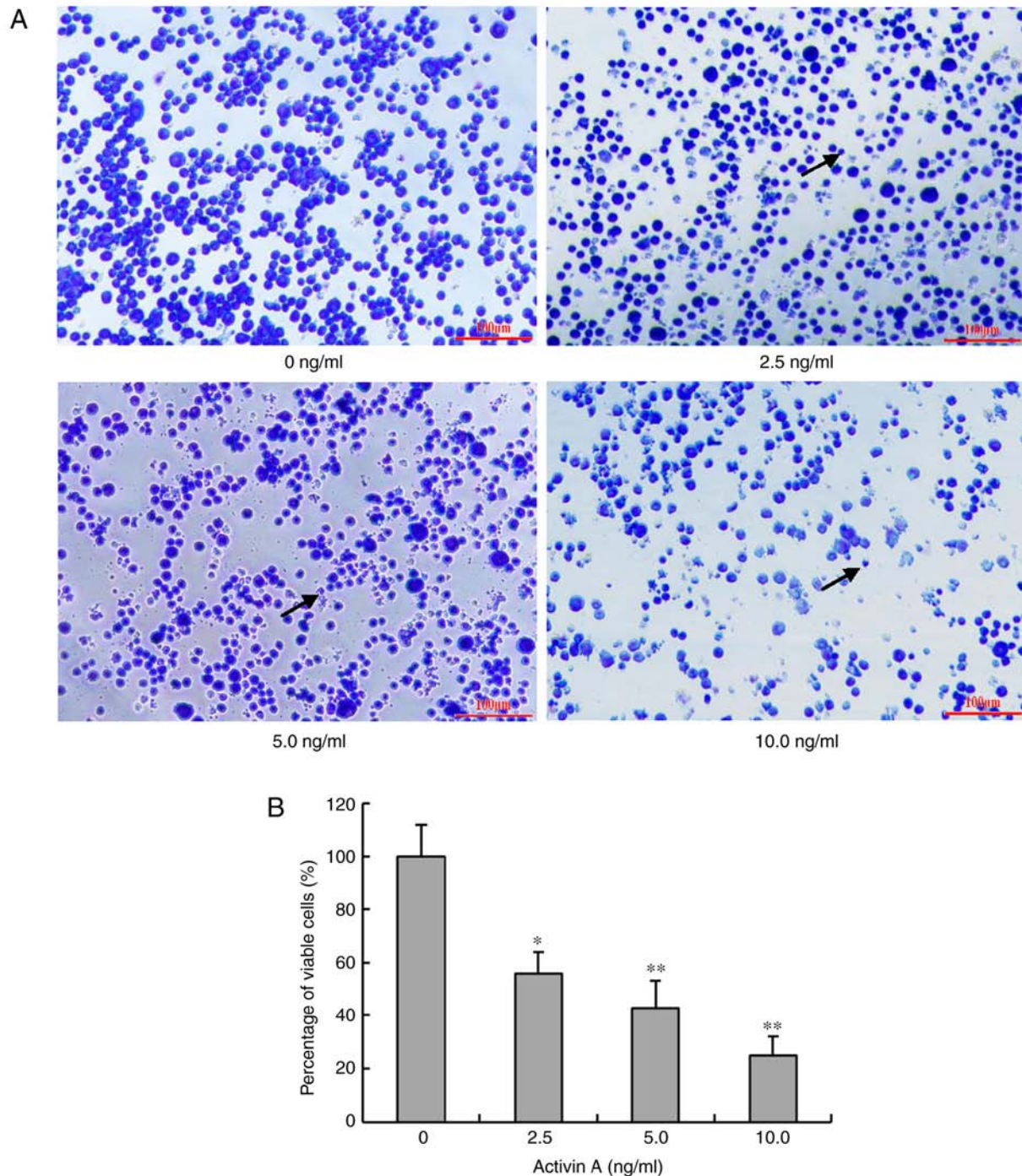


Figure 3. Effect of activin A on the morphology of NS-1 cells. (A) Cell morphological changes were examined by Giemsa staining. The arrows represent apoptotic cells. (B) The graph reveals the percentage of viable cells from three independent experiments. The group of untreated cells was adjusted to 100%. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the 0-ng/ml group.

6 days. The results revealed that the volume of the tumors treated with exogenous activin A was significantly reduced with time, compared with that in the control group (Fig. 5A). On the sixth day, the tumors were dissected. The gross morphology of solid tumors is presented in Fig. 5B. Although there was no significant difference in the body weight of the mice between the control group and activin A group, the ratio of tumor weight/body weights of mice treated with exogenous activin A was significantly lower than that in the control group (Fig. S2). These data revealed that activin A inhibited the growth of solid tumors of NS-1 cells *in vivo* in mice.

*Activin A influences the expression of apoptosis-related genes in NS-1 cells.* To assess whether activin A promoted NS-1 cell apoptosis via the ER stress pathway, the expression of certain apoptosis-related genes was examined after treatment with activin A for 12 h. The results revealed that the mRNA expression of caspase-3, caspase-12 and CHOP was significantly upregulated, whereas no change was observed in the mRNA expression of p53 and p21 (Fig. 6A). Furthermore, western blotting results revealed that activin A significantly upregulated the protein expression of CHOP, caspase-3, cleaved-caspase-3, caspase-12 and GADD34 (Fig. 6B). These data indicated

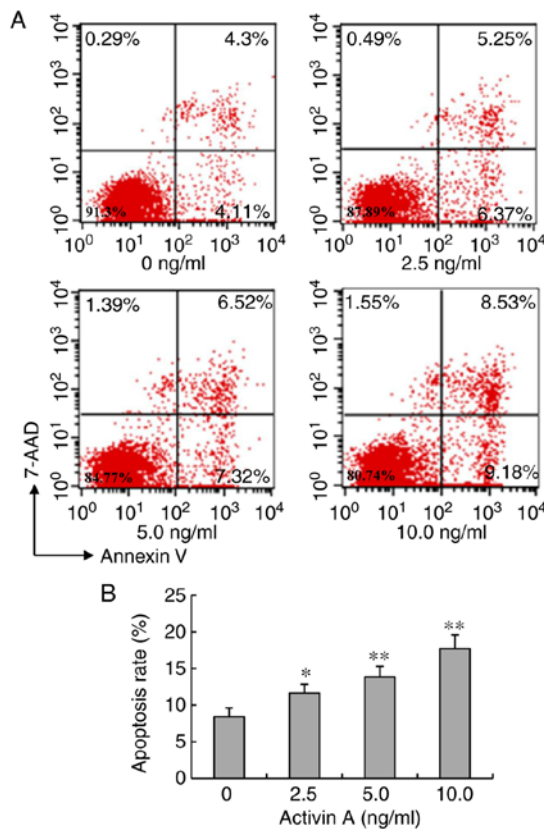


Figure 4. Effect of activin A on the apoptotic ratio of NS-1 cells. (A) The apoptosis of cells labeled with FITC-Annexin V and 7-AAD was determined by flow cytometry. (B) The graph reveals the percentage of apoptotic cells treated with activin A for 24 h. \*P<0.05, \*\*P<0.01, compared with the control group.

the involvement of the ER stress pathway proteins in activin A-induced NS-1 cell apoptosis.

*Smad3-overexpression regulates the expression of apoptosis-related proteins in NS-1 cells.* Smad3 plays an important role in activin signaling transduction. In Fig. 1 it was revealed that activin A promoted Smad3 expression. Thus, the role of Smad3 in NS-1 cells was further investigated. Fig. 7A and B revealed that the mRNA and protein expression of Smad3 were overexpressed in NS-1 cells transfected with Lipofectamine 2000. In addition, the level of p-Smad3 was also increased. Furthermore, the results revealed that Smad3 overexpression significantly increased the expression of caspase-3, cleaved-caspase-3 and CHOP protein of the ER stress pathway, compared with the pcDNA3 empty plasmid control group (Fig. 7B). These data further confirmed the involvement of CHOP in activin A-induced apoptosis of myeloma NS-1 cells.

## Discussion

Activin A binds with high affinity to activin type II receptors, which recruit type I receptors and are necessary for the activation of Smad2/3 signaling. It is of greater clinical significance to study human tumors. However, tumors can also occur in animals, thus the effect of activin A was first analyzed on mouse NS-1 cells. The present study revealed the expression of ActRIIs and Smad2/3 in NS-1 cells. Activin A

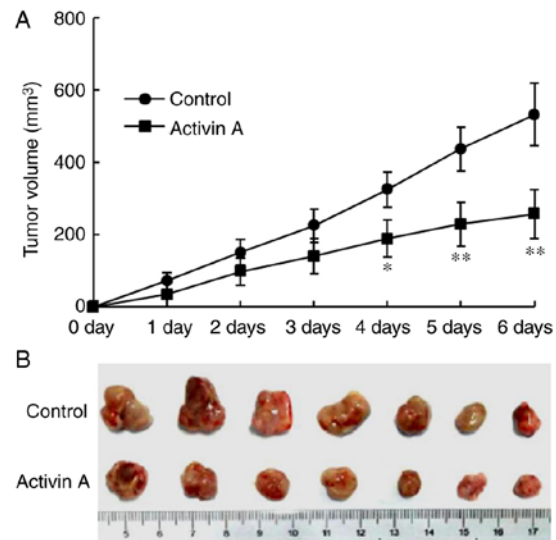


Figure 5. Effect of activin A on the growth of solid tumors of NS-1 cells in mice. (A) The change in the volume of solid tumors of NS-1 cells in mice was examined for 6 days following treatment with saline and activin A. The growth volume of the tumor = the tumor volume at dn - tumor volume at d0. (B) Gross morphology of solid tumors of NS-1 cells in mice on the sixth day after treatment with saline and activin A. \*P<0.05, \*\*P<0.01, compared with the control group.

first binds with either ActRIIA or ActRIIB, however not both receptors are increased in one cell at the same time. In addition, in the present study, activin A induced the increase in the mRNA expression of ActRIIA, Smad2 and Smad3 but not ActRIIB. Activin A also increased the levels of Smad3 and p-Smad3 proteins. These results imply that activin A may play an important role in NS-1 cells by promoting ActRII/Smad3 signaling.

Activin A plays a pro- or anti-tumor role according to the tumor type (11). Circulating activin A levels are elevated in patients with advanced multiple myeloma, and MSCs and osteoblasts are the main sources of activin A (19,20). Previous studies have revealed that activin A induces apoptosis in mouse myeloma NS-1 cells via the mitochondrial pathway by increasing the expression of cleaved caspase-3 and Cyt c proteins and the ratio of Bax/Bcl-2 (18). However, whether activin A can induce NS-1 cell apoptosis via the ER stress pathway is still unclear. In the present study, it was revealed that activin A inhibited the viability and proliferation of NS-1 cells using MTT assays and CFSE staining. Hence, the anti-tumor effect of activin A on NS-1 cells *in vivo* was examined. This study demonstrated that activin A inhibited the growth of solid tumors of NS-1 cells in mice. The aforementioned findings indicated that activin A could directly exert a suppressive effect on the proliferation of myeloma cells.

Apoptosis is recognized as a programmed cell death involving complex signaling cascades and stress responses. It is closely correlated with tumor cell proliferation and drug resistance in tumor therapy (21), and can occur through three major pathways: The Fas death receptor pathway, mitochondrial pathway, and ER stress pathway (22-24). The ER is a membranous compartment present in eukaryotic cells, which controls the synthesis, folding, and trafficking of proteins to be secreted, as well as calcium storage and synthesis of

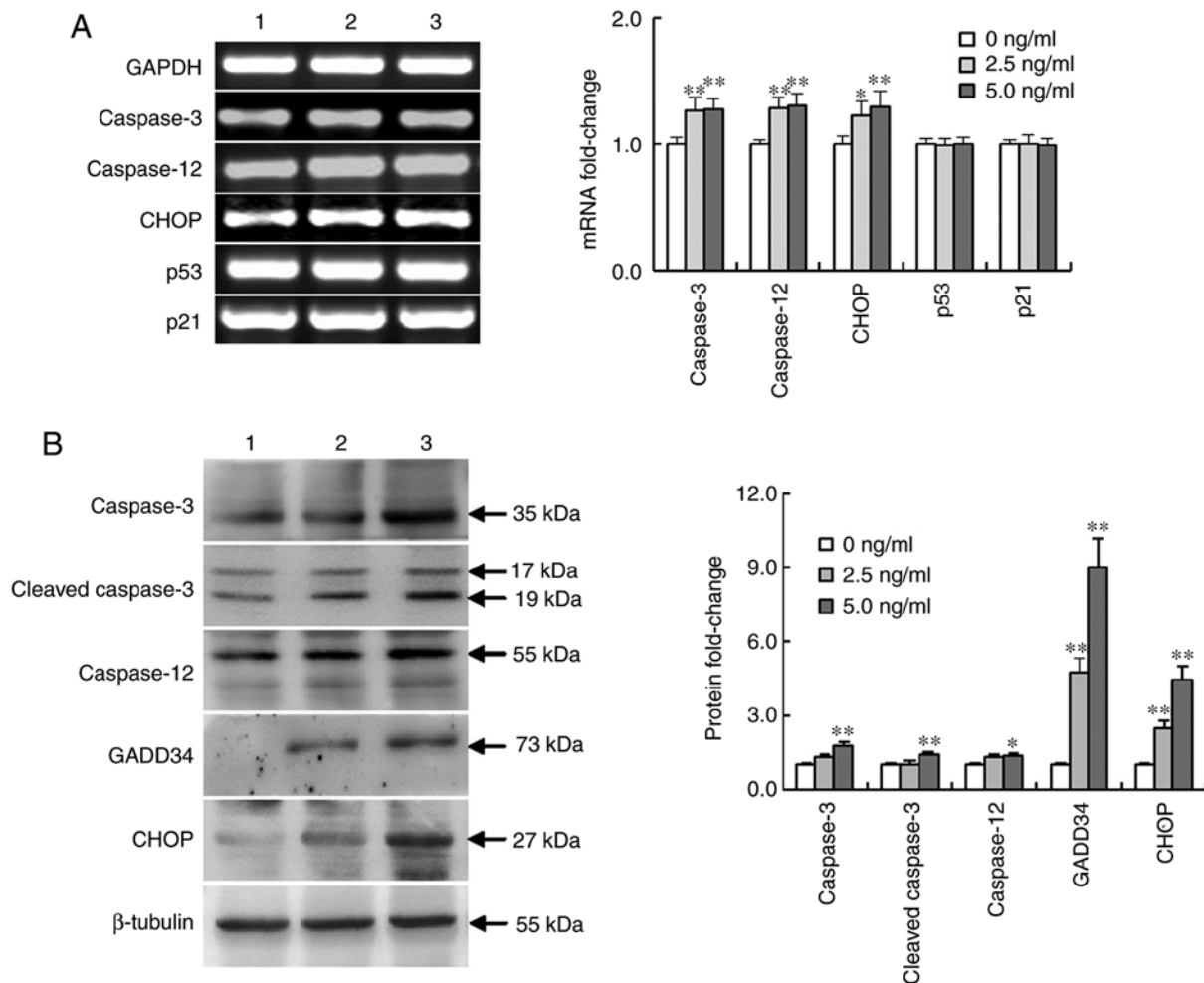


Figure 6. Effect of activin A on the expression of apoptosis-associated proteins in NS-1 cells. (A) The mRNA expression of apoptosis-associated proteins was detected by RT-PCR. The graph represents the levels of relative mRNA from triplicate determinations and the fold change in mRNA expression normalized to GAPDH. Lane 1: 0 ng/ml activin A; Lane 2: 2.5 ng/ml activin A; Lane 3: 5 ng/ml activin A. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the 0-ng/ml group. (B) The expression of apoptosis-associated proteins was examined by western blotting. The graph reveals the fold change in protein expression normalized to  $\beta$ -tubulin from three independent experiments. Lane 1: 0 ng/ml activin A; Lane 2: 2.5 ng/ml activin A; Lane 3: 5 ng/ml activin A. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the 0-ng/ml group.

membranes. Previous studies reported that activin A can induce B-cell apoptosis associated with Smad3 (25), and induce NS-1 cell apoptosis mediated by the mitochondrial pathway (18). However, whether the ER stress pathway is involved in activin A-induced NS-1 cell apoptosis is still unclear.

Apoptosis induced by ER stress is accompanied by the induction of pro-apoptotic CHOP (26,14). CHOP-induced apoptosis in ER stress has been involved in numerous human metabolic diseases. As a key marker of ongoing ER stress, CHOP not only leads to the downregulation of the anti-apoptotic protein Bcl-2 expression, but also can exacerbate ER stress by inducing the expression of GADD34 involved in a pro-apoptotic mechanism (28,29). The present study revealed an upregulation of the mRNA and protein expression of CHOP in NS-1 cells after treatment with activin A, and an increase of GADD34. Cell death triggered by CHOP-mediated apoptosis is also closely correlated with the suppression of cell cycle regulator protein 21 (p21), which inhibited the cell cycle in the G1 phase. Tumor suppressor p53 (p53) upregulates p21 to suppress the cell cycle at the G1 phase (30-32). However, no effect of activin A on the expression of p21 and p53 was

observed in the present study. Smad3 has been reported to alter the expression of p21. However, activin A may also induce activation of non-Smad3 dependent pathways. Smad3 induces changes in p21 expression in other cells, while in NS-1 cells, there is no significant change in p21 expression after activin A treatment. Therefore, p21 expression was not detected in NS-1 cells in subsequent experiments. These results indicated that activin A elicited a downstream apoptosis response mediated by CHOP/GADD34 signaling. The ER pathway contains many proteins, however CHOP and GADD34 are important proteins in this pathway. Therefore, the results of this study are only a first step in suggesting that activin A may induce NS-1 cell apoptosis via the ER stress pathway and more studies are required with further experiments.

Chronic or excessive ER stress can also lead to the activation of caspase-dependent apoptosis. Caspase-12 is an ER protease typically activated under ER stress conditions. Previous studies revealed the role of caspase-12 in ER stress-induced cell death (33-35). Caspase-12 activation was also detected in NS-1 cells treated with activin A, indicating that the induction of apoptosis caused by activin A was also mediated by the upregulation



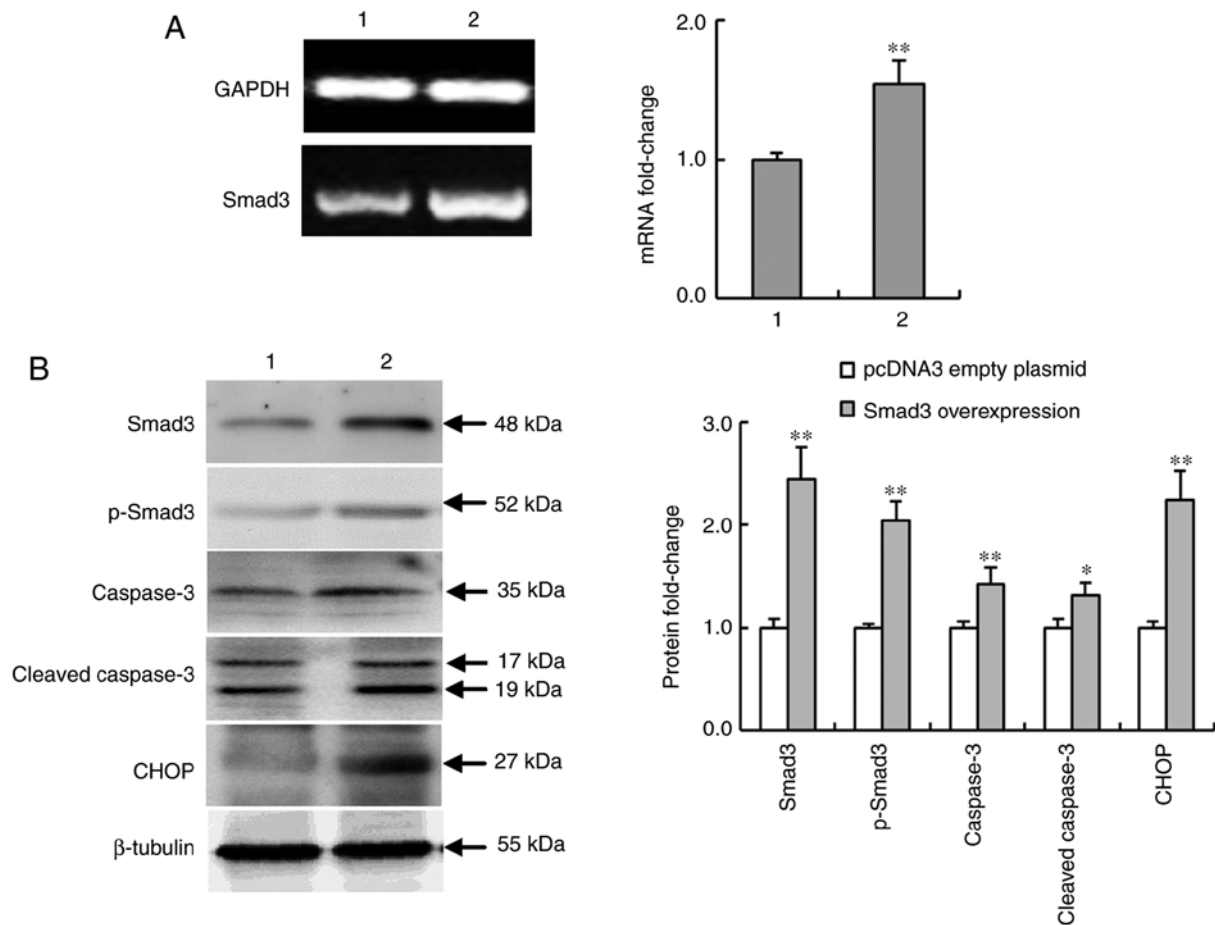


Figure 7. Effect of Smad3 overexpression on the expression of CHOP and caspase-3. (A) The mRNA expression level of Smad3 in Smad3-overexpressed NS-1 cells was examined by RT-PCR. Lane 1, pcDNA3 empty plasmid group; lane 2, Smad3-overexpression group. The graph represents the fold change in mRNA expression normalized to GAPDH. (B) The protein expression of Smad3, p-Smad3, caspase-3, cleaved caspase-3 and CHOP protein expression was assessed by western blotting. The graph reveals the fold change in protein expression normalized to β-tubulin from three independent experiments. \*P<0.05, \*\*P<0.01, compared with the pcDNA3 empty plasmid group. Lane 1, pcDNA3 empty plasmid group; lane 2, Smad3-overexpression group.

of caspase-12. Furthermore, caspase-12 can activate caspase-3 to induce apoptosis directly (36). We previously demonstrated that activin A increased the expression of cleaved caspase-3 protein in NS-1 cells (18). In the present study, activin A enhanced the expression of both caspase-3 and cleaved caspase-3, which further confirmed that the activation of caspases-3 is involved in activin A-induced NS-1 cell apoptosis.

The activin A signaling pathway is mediated by the serine/threonine kinase receptor on the cell surface and intracellular Smad proteins. Activin A binds to ActRIIA/IIB, which recruits ActRI to form a signal-transducing heterodimer, leading to the phosphorylation of cytoplasmic Smad2/3 proteins. The activated Smad2/3 associates with Smad4, which translocates into the nucleus and drives downstream transcriptional targets (37). A number of studies have revealed the involvement of ActRII/Smad3 signaling in the pathophysiological processes of various diseases. Therefore, the levels of ActRIIA/IIB and Smads were assessed after treatment with activin A. The RT-PCR results revealed that the mRNA expression of ActRIIA and Smad3 and the protein levels of Smad3 and p-Smad3 were increased in NS-1 cells treated with activin A. A high concentration of activin A could induce cardiomyocyte apoptosis via the ER stress pathway by enhancing the expression of CHOP, and the apoptotic rates were significantly

reduced by Smad3 inhibitor (38). Smad3/ATF4 signaling was revealed to be involved in ER stress-induced apoptosis of brown adipocytes *in vivo* and *in vitro* (39). Therefore, the present study further explored the relationship between Smad3 and CHOP. It was revealed that Smad3 overexpression could promote the expression of pro-apoptotic proteins CHOP, caspase-3 and p-Smad3, indicating that activin A inhibited NS-1 cell proliferation and promoted apoptosis via Smad3/CHOP signaling.

In conclusion, the activation of activin A/Smad3 signaling may inhibit NS-1 cell proliferation and induce apoptosis by activating CHOP in the ER stress pathway. Therefore, these results supported the therapeutic implications on targeting activin A-induced apoptosis via CHOP signaling for multiple myeloma therapy.

#### Acknowledgements

Not applicable.

#### Funding

The present study was funded by the National Basic Research Program of China (2015CB943300), the Health Commission

Foundation and the International Cooperation Foundation of Jilin Province, China (20180414040GH, 2018J063 and 2016J066), and the Foundation of Bethune Project of Jilin University (2018B05).

#### Availability of data and materials

The datasets described in this study are available from the corresponding author on reasonable request.

#### Authors' contributions

ZL designed the study and reviewed the manuscript. JG was responsible for writing the manuscript and analyzing the data. XC contributed to the study design and performed the statistical analysis. HS contributed the animal model experiments. JL, YS, YZ and FL performed the experiments and analyzed the data. YY cultured the cell lines. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

#### Ethics approval and consent to participate

All animal experiments were conducted in accordance with the Jilin University guidelines for the care and use of animals. A laboratory animal ethics review form was approved by the Laboratory Animal Ethics Committee of the College of Basic Medical Sciences of Jilin University (No. 2018-018).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no conflicts of interest.

#### References

- Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M and Guillemin R: Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. *Nature* 321: 779-782, 1986.
- Woodruff TK, Besecke LM, Groome N, Draper LB, Schwartz NB and Weiss J: Inhibin A and inhibin B are inversely correlated to follicle stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. *Endocrinology* 137: 5463-5467, 1996.
- Thompson TB, Cook RW, Chapman SC, Jardetzky TS and Woodruff TK: Beta A versus beta B: Is it merely a matter of expression? *Mol Cell Endocrinol* 225: 9-17, 2004.
- Qi Y, Ge J, Ma C, Wu N, Cui X and Liu Z: Activin A regulates activation of mouse neutrophils by Smad3 signalling. *Open Biol* 7: 160342, 2017.
- Hoda MA, Rozsas A, Lang E, Klikovits T, Lohinai Z, Torok S, Berta J, Bendek M, Berger W, Hegedus B, *et al*: High circulating activin A level is associated with tumor progression and predicts poor prognosis in lung adenocarcinoma. *Oncotarget* 7: 13388-13399, 2016.
- Wei Q, Liu H, Liu M, Yang C, Yang J, Liu Z and Yang P: Ramipril attenuates left ventricular remodeling by regulating the expression of activin A-follistatin in a rat model of heart failure. *Sci Rep* 6: 33677, 2016.
- Jana A, Krett NL, Guzman G, Khalid A, Ozden O, Staudacher JJ, Bauer J, Baik SH, Carroll T, Yazici C and Jung B: NFkB is essential for activin-induced colorectal cancer migration via upregulation of PI3K-MDM2 pathway. *Oncotarget* 8: 37377-37393, 2017.
- Arber C, Precious SV, Cambray S, Risner-Janiczek JR, Kelly C, Noakes Z, Fjodorova M, Heuer A, Ungless MA, Rodríguez TA, *et al*: Activin A directs striatal projection neuron differentiation of human pluripotent stem cells. *Development* 142: 1375-1386, 2015.
- de Kretser DM, O'Hehir RE, Hardy CL and Hedger MP: The roles of activin A and its binding protein, follistatin, in inflammation and tissue repair. *Mol Cell Endocrinol* 359: 101-106, 2012.
- Li N, Cui X, Ge J, Li J, Niu L, Liu H, Qi Y, Liu Z and Wang Y: Activin A inhibits activities of lipopolysaccharide-activated macrophages via TLR4, not of TLR2. *Biochem Biophys Res Commun* 435: 222-228, 2013.
- Jeruss JS, Sturgis CD, Rademaker AW and Woodruff TK: Down-regulation of activin, activin receptors, and smads in high-grade breast cancer. *Cancer Res* 63: 3783-3790, 2003.
- Loomans HA and Andl CD: Intertwining of activin A and TGFβ signaling: Dual roles in cancer progression and cancer cell invasion. *Cancers (Basel)* 7: 70-91, 2014.
- Rutkowski DT and Kaufman RJ: A trip to the ER: Coping with stress. *Trends Cell Biol* 14: 20-28, 2004.
- Tabas I and Ron D: Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 13: 184-190, 2011.
- Doyle KM, Kennedy D, Gorman AM, Gupta S, Healy SJ and Samali A: Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. *J Cell Mol Med* 15: 2025-2039, 2011.
- Catena V, Bruno T, De Nicola F, Goeman F, Pallocca M, Iezzi S, Sorino C, Cigliana G, Floridi A, Blandino G and Fanciulli M: Depror transcriptionally regulates endoplasmic reticulum homeostasis in multiple myeloma cells. *Oncotarget* 7: 70546-70558, 2016.
- Clark AL and Urano F: Endoplasmic reticulum stress in beta cells and autoimmune diabetes. *Curr Opin Immunol* 43: 60-66, 2016.
- Zhang Y, Qi Y, Zhao Y, Sun H, Ge J and Liu Z: Activin A induces apoptosis of mouse myeloma cells via the mitochondrial pathway. *Oncol Lett* 15: 2590-2594, 2018.
- Garcia-Gomez A, Sanchez-Guijo F, Del Cañizo MC, San Miguel JF and Garayoa M: Multiple myeloma mesenchymal stromal cells: Contribution to myeloma bone disease and therapeutics. *World J Stem Cells* 6: 322-343, 2014.
- Terpos E, Kastritis E, Christoulas D, Gkotzamanidou M, Eleutherakis-Papaioakovou E, Kanellias N, Papaioakou A and Dimopoulos MA: Circulating activin-A is elevated in patients with advanced multiple myeloma and correlates with extensive bone involvement and inferior survival; no alterations post-lenalidomide and dexamethasone therapy. *Ann Oncol* 23: 2681-2686, 2012.
- Cubillos-Ruiz JR, Bettigole SE and Glimcher LH: Tumorigenic and immunosuppressive effects of endoplasmic reticulum stress in cancer. *Cell* 168: 692-706, 2017.
- Fernández A, Ordóñez R, Reiter RJ, González-Gallego J and Mauriz JL: Melatonin and endoplasmic reticulum stress: Relation to autophagy and apoptosis. *J Pineal Res* 59: 292-307, 2015.
- Wachmann K, Pop C, van Raam BJ, Drag M, Mace PD, Snipas SJ, Zmasek C, Schwarzenbacher R, Salvesen GS and Riedl SJ: Activation and specificity of human caspase-10. *Biochemistry* 49: 8307-8315, 2010.
- Su J, Zhou L, Xia MH, Xu Y, Xiang XY and Sun LK: Bcl-2 family proteins are involved in the signal crosstalk between endoplasmic reticulum stress and mitochondrial dysfunction in tumor chemotherapy resistance. *BioMed Res Int* 2014: 234370, 2014.
- Yamakawa N, Tsuchida K and Sugino H: The ras GAP-binding protein, Dok-1, mediates activin signaling via serine/threonine kinase receptors. *EMBO J* 21: 1684-1694, 2002.
- Dong Y, Kalueff AV and Song C: N-methyl-d-aspartate receptor-mediated calcium overload and endoplasmic reticulum stress are involved in interleukin-1β-induced neuronal apoptosis in rat hippocampus. *J Neuroimmunol* 307: 7-13, 2017.
- Mc Cullough KD, Martindale JL, Klotz LO, Aw TY and Holbrook NJ: Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* 21: 1249-1259, 2001.

28. Huang J, Fairbrother W and Reed JC: Therapeutic targeting of Bcl-2 family for treatment of B-cell malignancies. *Expert Rev Hematol* 8: 283-297, 2015.
29. Sano R and Reed JC: ER stress-induced cell death mechanisms. *Biochim Biophys Acta* 1833: 3460-3470, 2013.
30. Mihailidou C, Papazian I, Papavassiliou AG and Kiaris H: CHOP-dependent regulation of p21/waf1 during ER stress. *Cell Physiol Biochem* 25: 761-766, 2010.
31. Wu YM, Chen ZJ, Jiang GM, Zhang KS, Liu Q, Liang SW, Zhou Y, Huang HB, Du J and Wang HS: Inverse agonist of estrogen-related receptor  $\alpha$  suppresses the growth of triple negative breast cancer cells through ROS generation and interaction with multiple cell signaling pathways. *Oncotarget* 7: 12568-12581, 2016.
32. Kapur A, Felder M, Fass L, Kaur J, Czarnecki A, Rath K, Zeng S, Osowski KK, Howell C, Xiong MP, *et al*: Modulation of oxidative stress and subsequent induction of apoptosis and endoplasmic reticulum stress allows citral to decrease cancer cell proliferation. *Sci Rep* 6: 27530, 2016.
33. Duclos C, Lavoie C and Denault JB: Caspases rule the intracellular trafficking cartel. *FEBS J* 284: 1394-1420, 2017.
34. García de la Cadena S and Massieu L: Caspases and their role in inflammation and ischemic neuronal death. *Focus on caspase-12. Apoptosis* 21: 763-777, 2016.
35. Lu XS, Qiao YB, Li Y, Yang B, Chen MB and Xing CG: Preclinical study of cinobufagin as a promising anti-colorectal cancer agent. *Oncotarget* 8: 988-998, 2017.
36. Nakagawa T and Yuan J: Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 150: 887-894, 2000.
37. Miyazawa K, Shinozaki M, Hara T, Furuya T and Miyazono K: Two major Smad pathways in TGF-beta superfamily signaling. *Genes Cells* 7: 1191-1204, 2002.
38. Liu M, Mao C, Li J, Han F and Yang P: Effects of the activin A-follistatin system on myocardial cell apoptosis through the endoplasmic reticulum stress pathway in heart failure. *Int J Mol Sci* 18: E374, 2017.
39. Liu Z, Gu H, Gan L, Xu Y, Feng F, Saeed M and Sun C: Reducing Smad3/ATF4 was essential for Sirt1 inhibiting ER stress-induced apoptosis in mice brown adipose tissue. *Oncotarget* 8: 9267-9279, 2017.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.