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TGF-β promotes glioma cell growth via activating Nodal expression through Smad and ERK1/2 pathways

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ABSTRACT

While there were certain studies focusing on the mechanism of TGF-β promoting the growth of glioma cells, the present work revealed another novel mechanism that TGF-β may promote glioma cell growth via enhancing Nodal expression. Our results showed that Nodal expression was significantly upregulated in glioma cells when TGF-β was added, whereas the TGF-β-induced Nodal expression was evidently inhibited by transfection Smad2 or Smad3 siRNAs, and the suppression was especially significant when the Smad3 was downregulated. Another, the attenuation of TGF-β-induced Nodal expression was observed with blockade of the ERK1/2 pathway also. Further detection of the proliferation, apoptosis, and invasion of glioma cells indicated that Nodal overexpression promoted the proliferation and invasion of tumor cells and inhibited their apoptosis, resembling the effect of TGF-β addition. Downregulation of Nodal expression via transfection Nodal-specific siRNA in the presence of TGF-β weakened the promoting effect of the latter on glioma cells growth, and transfecting Nodal siRNA alone in the absence of exogenous TGF-β more profoundly inhibited the growth of glioma cells. These results demonstrated that while both TGF-β and Nodal promoted glioma cells growth, the former might exert such effect by enhancing Nodal expression, which may form a new target for glioma therapy.

1. Introduction

Glioma is one of the common tumors seriously threatening the human health [1]. In the past 30 years, the overall therapeutic effect of glioma has not been significantly improved, and the five-years survival of glioma patients ranked third, just after that of pancreatic and lung cancers [2]. Thus glioma has long been a refractory disease, and the exploration of its etiology, pathogenesis, biological characteristics, and new treatment is a hot topic and most urgent problem to solve in the field of neurosurgery.

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide cytokine; almost all cell types in the body express TGF-β and its receptors. Current opinion for its role in tumors generally believes that TGF-β often inhibits the growth of tumor cells in the early stage of tumorigenesis; however, with tumor progression and changes of the biochemical environment, TGF-β becomes an important stimulating factor for tumor growth, invasion, metastasis, and angiogenesis. Moreover, tumor cells can secrete TGF-β to suppress the host's immune function and evade the immune surveillance [3]. In many human tumors, the expression of TGF-β was observed increasing with the increased degree of malignancy and thus used as an indicator of poor prognosis [3,4]. Studies also showed that in gliomas, TGF-β/Smad signaling pathway inhibited the growth of low-grade tumors but promoted the migration, invasion, and malignant progression of advanced tumors via reducing the cytotoxic effect of local immune cells and enhancing the release of metal matrix proteases (MMPs) [5–6]. In addition, RNA interference targeting TGF-β enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity *in vivo* [7]. Therefore, TGF-β signaling pathway forms a target of cancer therapy for its close relationship with tumor development.

The present study revealed that TGF-β promoted the growth of glioma cells via enhancing Nodal expression. Nodal is a member of the TGF-β superfamily and an important regulatory factor during the induction of embryonic tissues to form the complete body axis, in the process of embryonic stem cell development [8]. In recent years, a growing number of studies have shown that the expression of Nodal was significantly augmented in malignant tumors such as melanoma, breast cancer, endometrial cancer, and prostate carcinoma, and closely related with the degree of tumor malignancy [9–12], suggesting a possibility that Nodal protein may form a tumor marker and therapeutic target. Another recent study discovered that there was differential expression of Nodal in human

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grade-IV glioma cells, which was closely related to the invasive potential of tumor cells [13]. The secretion of MMP2 and the invasiveness of glioma cells can be promoted by either enhancing the expression of Nodal or increasing its activity in tumor cells; in contrast, knocking-down the expression of Nodal results in a significantly reduced cell invasion [13]. The mechanism of above findings may be related with the ability of Nodal to induce the expression of leukemia inhibitory factor (LIF) and Cripto-1 [13]. In the present study, we found that TGF-β1 promoted the expression of Nodal via activating Smads and MAPK pathways, and thereby promoted the growth of glioma cells.

2. Materials and methods

2.1. Cell cultures

Human U87MG (American Type Culture Collection) and GBM glioma cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum.

2.2. Nodal overexpression vector construction and siRNA design

U87MG cells were collected, and RNA was extracted with Trizol (Invitrogen) according to the instructions of the manufacturer, followed by reverse transcription PCR to amplify Nodal coding region. The primers were shown as follow:

Nodal-F: 5′-GGGGTACC**GCCACC**ATGCACGCCCACTGCCTG-3′; Nodal-R: 5′-CGGAATTCTCAGAGGCACCCACATTCTTC-3′. Then the product was digested with *Kpn* I and *Eco*R I (TaKaRa), cloned into pcDNA3.1 vector, sequenced and verified. Meanwhile, Nodal siRNA (sense: 5′-AGACAUGAUCGUGGAAGAAtt-3′, antisense: 5′-UUCUUCCACGAUCAUGUCUtt-3′) [14], Smad2 siRNA (sense: 5′-GUCCCAUGAAAAGACUUAAtt-3′, antisense: 5′-UUAAGUCUUUUCAUGGGACtt-3′) [15], Smad3 siRNA (sense: 5′-CUGUGUGAGUUCGCCUUCAtt-3′, antisense: 5′-UGAAGGCGAACUCACACAGtt-3′) [16] and control siRNA (sense: 5′-UUCUCCGAACGUGUCACGUtt-3′, antisense: 5′-ACGUGACACGUUCGGAGAAtt-3′) sequences were taken from publications and synthesized.

2.3. Transfection and signaling pathway blockage

The cells were seed into 6-well plate at 1×10^5 cells/mL and incubated for 24 h. Plasmid and siRNAs transfection were carried out following the instruction of Lipofectamine 2000 (Invitrogen), when the cell confluence reached about 70%. The concentration of the transfection plasmid was 2 μg/mL, and the final concentration of siRNA was 50 nM. The medium was changed within 4–6 h after transfection. After 24 h of transfection, whether FR180204 (20 μM, ERK Inhibitor II, Santa Cruz Biotechnology, Inc), SP600125 (a JNK inhibitor), SB 203580 (the P38 inhibitor) and LY294002 (a PI3K inhibitor) (Calbiochem, Merck Biosciences, Darmstadt, Germany) were added or not and cells were cultured for 6 h, then added 10 ng/mL TGF-β (R&D Systems, Minneapolis, Minn) for culturing 24 h.

2.4. Fluorescence quantitative PCR

Cells were collected and total RNA was extracted with Trizol. After quantification, 1 μg RNA was used for reverse transcription, and quantitative PCR was performed using SYBR-Green PCR Master Mix (TOYOBO, Japan), with 100 ng of cDNA contained in 20 μL of reaction mixture. The primer sequences are shown as follow: Nodal-QTF: 5′-GCGAGTGTCCTAATCCTGTTG-3′ and Nodal-QTR: 5′-CAGCGGCTTGGTCTTCAC-3′; GAPDH-F: 5′-GGTATCGTGGAAGGACTC-3′ and GAPDH-R: 5′-GTAGAGGCAGGGATGATG-3′. The reaction was performed at one cycle of 95 °C for 5 min and 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Three independent experiments were conducted for each sample. Data were analyzed by comparing the $2^{-\Delta\Delta Ct}$ value.

2.5. Western blot

Total cellular proteins were extracted by incubating cells in RIPA buffer (1X PBS, 1% NP-40, 0.1% sodium dodecylsulfate (SDS), 5 mM EDTA, 0.5% sodium deoxycholate, and 1 mM sodium orthovanadate) with protease inhibitors. The protein concentrations were determined by BCA Protein Assay (Reagent Kit, Pierce). SDS–PAGE was done in 8% glycine gels (Bio-rad) loading equal amount of proteins per lane. After electrophoresis, separated proteins were transferred to PVDF membrane and blocked with 5% non-fat milk in TBST buffer for 1 h. After that, the membranes were incubated with Nodal (Santa Cruz Biotech, 1:200), Smad2/3 (Cell Signaling Technology, 1:800), p-Smad2/3 (Cell Signaling Technology, 1:600), Erk1/2 (Cell Signaling Technology, 1:1000), Phospho-Erk1/2 (Cell Signaling Technology, 1:1000) and GAPDH (Novus Biologicals, 1:1000) overnight at 4 °C, and then anti-rabbit IgG monoclonal antibody conjugated with horseradish peroxidase (Cell Signaling Technology) at 1:2000 dilution for 1 h at room temperature. Protein bands were detected using the West Femto system (PIERCE).

2.6. Cell proliferation

A BrdU colorimetric immunoassay kit (Cell Proliferation ELISA, Roche Diagnostics, Germany) was used for quantification of cell proliferation according to the protocol provided by the manufacturer. Cell proliferation was expressed as the mean percentage of the control values (set at 100%).

2.7. Annexin-V-FLUOS apoptosis analysis

Cells were collected after transfection for 72 h, and the translocation of phosphatidylserine in treated cells was detected using the Annexin-V-FLUOS staining kit (Roche Applied Science). Briefly, cells were suspended in 500 μL of binding buffer and incubated at room temperature in the dark for 15 min after labeled with 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of propidium iodide. The stained cells were then analyzed by flow cytometry.

2.8. Transwell matrigel invasion assay

Invasion of cells was evaluated by Transwell matrigel invation assay. Briefly, 200 μL of cells after transfection (1×10^6 cells/mL) and 600 μL of the complete medium were added to the upper and lower compartments of the chamber, respectively. After incubation of 48 h, cells migrating to the lower side of the filter were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS, stained with crystal violet, and then observed under a confocal microscope.

2.9. Statistical analysis

Experiments were carried out at least in triplicate and results were expressed as mean ± S.D. Statistical analysis was performed using SPSS statistical program version 17 (SPSS Inc., Chicago, IL). Difference with $P < 0.05$ (*) or $P < 0.01$ (**) was considered statistically significant.

3. Results

3.1. TGF-β promoted Nodal expression in glioma cells

TGF-β can promote the growth of malignant gliomas. Although certain progresses have been made, the specific mechanism of this action remains not fully understood. In the present study, After 24 h of addition TGF-β into U87MG and GBM cells, Nodal expression was found to be significantly enhanced at both mRNA and protein levels (Fig. 1). Recent studies have already shown that Nodal is highly expressed in most tumors, closely related with the malignancy of tumor cells, and thus considered as a potential biomarker and therapeutic target of tumors. Therefore, we propose that a possible new mechanism of TGF-β promoting glioma growth is that TGF-β upregulates the expression of Nodal, which in turn facilitates the growth of tumor cells.

3.2. TGF-β promoted Nodal expression via activating Smad and ERK1/2 pathways

The signal transduction of TGF-β is mainly achieved depending on Smad proteins, but it has been shown that in different cell lines, TGF-β signal transduction can also be completed via activating ERK1/2, p38, or c-Jun N-terminal kinase (JNK), independent of the participation of Smad proteins [17]. Since Smad2 and Smad3 are the main downstream molecules in TGF-β signaling, this study first detected whether TGF-β regulated Nodal expression through Smad pathway. After transfection siRNAs targeting these two genes respectively into glioma cells, we found that the effect of TGF-β on induction Nodal expression was suppressed, and such suppressive effect was more evident with Smad3 knockdown (Fig. 2A). Study on other signaling pathways revealed that ERK1/2 and PI3K pathways blockade attenuated the TGF-β-induced Nodal expression, and the effect of ERK1/2 pathway was more significant; however, inhibiting JNK and p38 pathways did not affect the TGF-β-induced Nodal expression (Fig. 2A). Our results indicated that TGF-β promoted Nodal expression via activating Smad and ERK1/2 pathways.

3.3. The effect of TGF-β-promoted Nodal expression on the proliferation and apoptosis of glioma cells

Studies have shown that Nodal can promote the growth of glioma cells and TGF-β can promote Nodal expression in glioma cells, implying that TGF-β may promote the growth of glioma cells via induction of Nodal expression. In this study, we transfected glioma cells with Nodal overexpression vector and found that Nodal overexpression indeed promoted the proliferation of glioma cells and inhibited the apoptosis, resembling the effect of TGF-β addition (Figs. 2B, 3). We further transfected siRNA targeting Nodal in the presence of exogenous TGF-β and found that Nodal downregulation attenuated the promoting effect of TGF-β on the growth of glioma cells, and transfection of Nodal siRNA in the absence of exogenous TGF-β more profoundly inhibited the growth of glioma cells (Figs. 2B, 3). These results proved that both TGF-β and Nodal exerted promoting effect on the growth of glioma cells, and the former might achieve such effect via promoting the expression of the latter.

3.4. The effect of TGF-β-promoted Nodal expression on glioma cell invasion

Studies have shown that Nodal is highly expressed in metastatic melanoma cell lines C8131, WM278, and 1205Lu but is low or

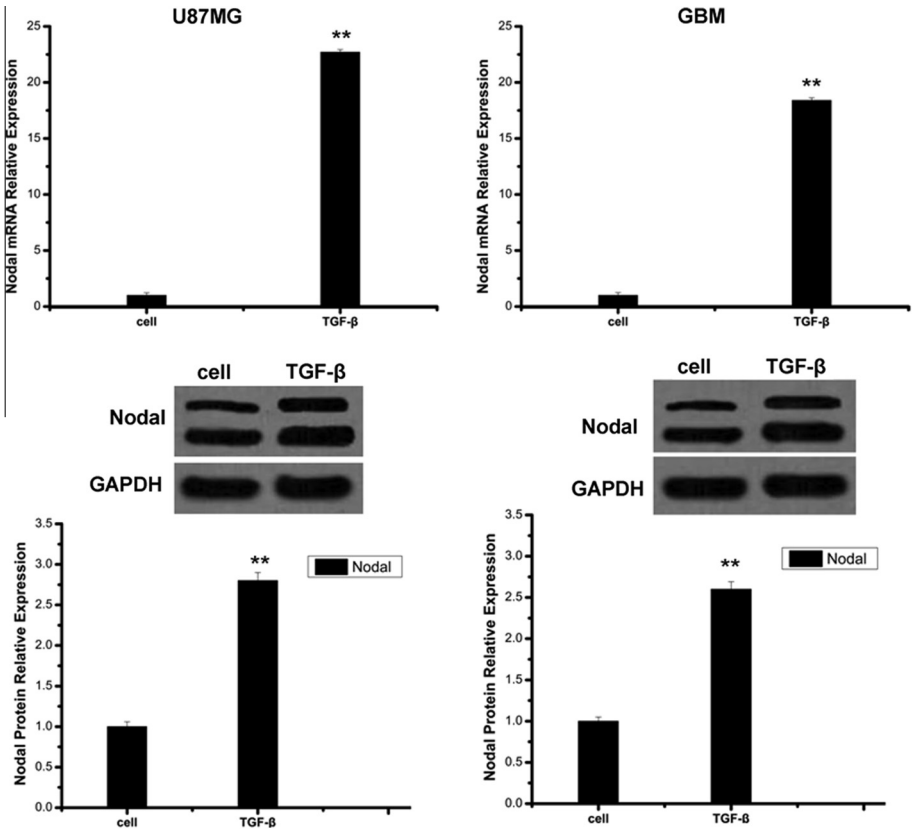

Fig. 1. Expression of Nodal in glioma cells. 10 ng/mL TGF-β were added into human U87MG and GBM glioma cells and cells were cultured for 24 h, and Nodal expression as measured by Real-time qPCR and Western blot analysis. Each bar represents the mean ± SD from three samples (**$P < 0.01$ vs. the control).

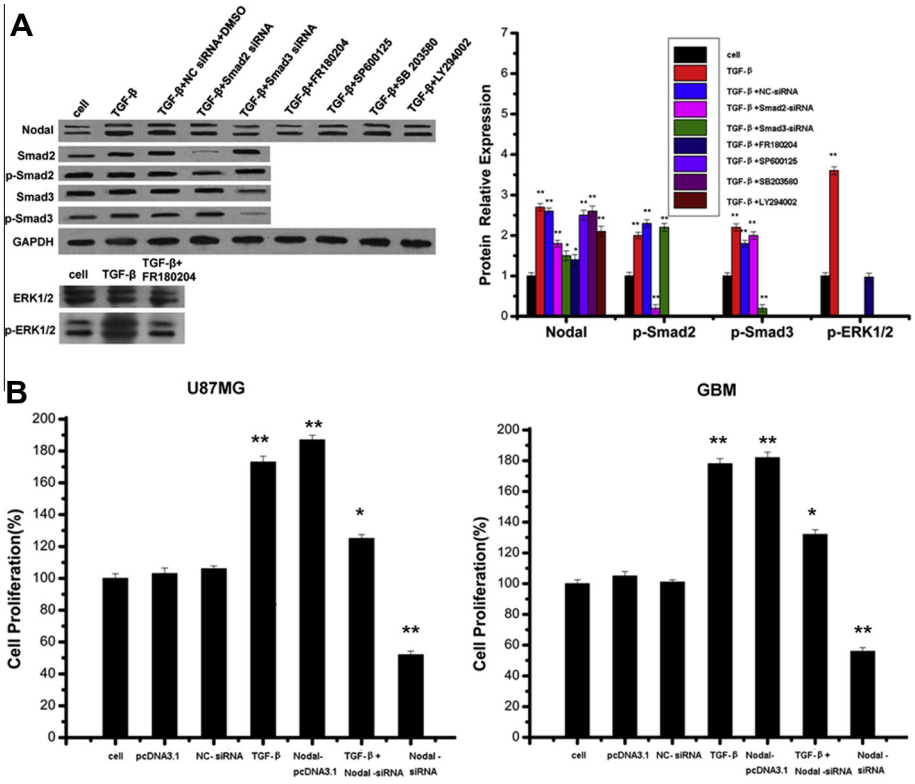

Fig. 2. TGF-β promoted Nodal expression via activating Smad and ERK1/2 pathways and roles of Nodal in glioma cells proliferation. (A) U87MG cell was transfected with siRNA and cultured for 24 h, FR180204, SP600125, SB 203580 and LY294002 were added or not respectively; after cultured for 6 h, 10 ng/mL TGF-β were added and cells were cultured for 24 h. Nodal, Smad2/3, p-Smad2/3, ERK1/2, p-ERK1/2 expression as measured by Western blot analysis. (B) After pretreatments of the cells with siRNA or vector transfection for 24 h, 10 ng/mL TGF-β was added or not, and cells were cultured for 24 h. The cell proliferation was examined by BrdU assay. The results are presented as mean ($n = 3$) ± SD, $^{*}P < 0.05$ vs. the control, $^{**}P < 0.01$ vs. the control.

absent in non-invasive melanoma cell line C81-61 [9,18], suggesting that Nodal may exert promoting effect on the invasion and metastasis of tumor cells. Transwell assay revealed that Nodal overexpression promoted the invasion of glioma cells, while inhibiting Nodal expression weakened the TGF-β-promoted glioma cell invasion (Fig. 4).

4. Discussion

Recent studies found that the expression of TGF-β in serum and tissue of cancer patients is increased significantly with the degree of tumor malignancy, suggesting that TGF-β may be closely related with the malignant development of tumors [19–20]. Studies have also shown that TGF-β plays a dual role in the occurrence and development of tumor, i.e., it behaves as a suppressor in the early stage of tumorigenesis, but promotes the malignant transformation of late tumor in autocrine and paracrine manners [19]. Wick et al. [20] have found that TGF-β is highly expressed in patients with glioma, especially malignant glioma, which is closely related to the prognosis of treatment.

TGF-β was shown to promote glioma growth through crosstalk with platelet-derived factor-β (PDGF-β) signaling pathway [21]. TGF-β can increase the expression levels of both PDGF-β mRNA and protein, upregulate the phosphorylation level of PDGF receptor, thereby enhance the proliferation of U373MG glioblastoma cells. Downregulation of PDGF-β expression in tumor cells by transfection short hairpin RNA evidently inhibited TGF-β-induced cell proliferation. Guo et al. [22] showed that the growth of U87MG cells stably transfected with vector overexpressing PDGF-β was significantly accelerated in a mouse model of intracranial tumor, further supporting the above speculation. Sciumè et al. [23] found that endogenously expressed CX3CL1 negatively regulates glioma invasion likely by promoting tumor cell aggregation, and that TGF-β inhibition of CX3CL1 expression might contribute to glioma cell invasive properties. Another study suggested that TGF-β may promote glioma cell migration and invasion through TNF-α converting enzyme (ADAM17) [24]. TGF-β can also induce the migration and invasion of glioma cells by upregulating the expression of integrin [25]. Integrin β8 is an essential regulatory factor of tumor-induced angiogenesis and glioma invasion, while TGF-β is the protein ligand of integrin β8 in the extracellular matrix. Inhibiting either integrin β8 or TGF-β signaling pathway can reduce the invasiveness of tumor cells [25].

Our results indicated that TGF-β can promote the expression of Nodal in glioma cells. Other recent literatures have also reported the expression of Nodal protein in malignant melanoma, testicular cancer, breast cancer, glioma, and its expression levels are closely related with the invasion, metastasis, and poor prognosis of tumors [9–12]. Some existing data suggest that Nodal is likely to develop into a biomarker of the malignant progression of tumor and a target molecule of clinical intervention in the future [26]. Research also revealed that Nodal is a regulatory factor of tumor cell plasticity; downregulation of Nodal expression abolishes the plasticity and the formation of vasculogenic mimicry in tumor [26]. Using *in situ* hybridization technique, McAllister et al. [27] confirmed that Nodal mRNA only expressed in the dendritic network (the vasculogenic mimicry) in implanted tumors but not in other tissues in nude mice, indicating the important role of Nodal expression in the formation of vasculogenic mimicry. Studies have also shown that there is a certain correlation between Nodal gene and the malignancy of human melanoma [28], and that reduced Nodal

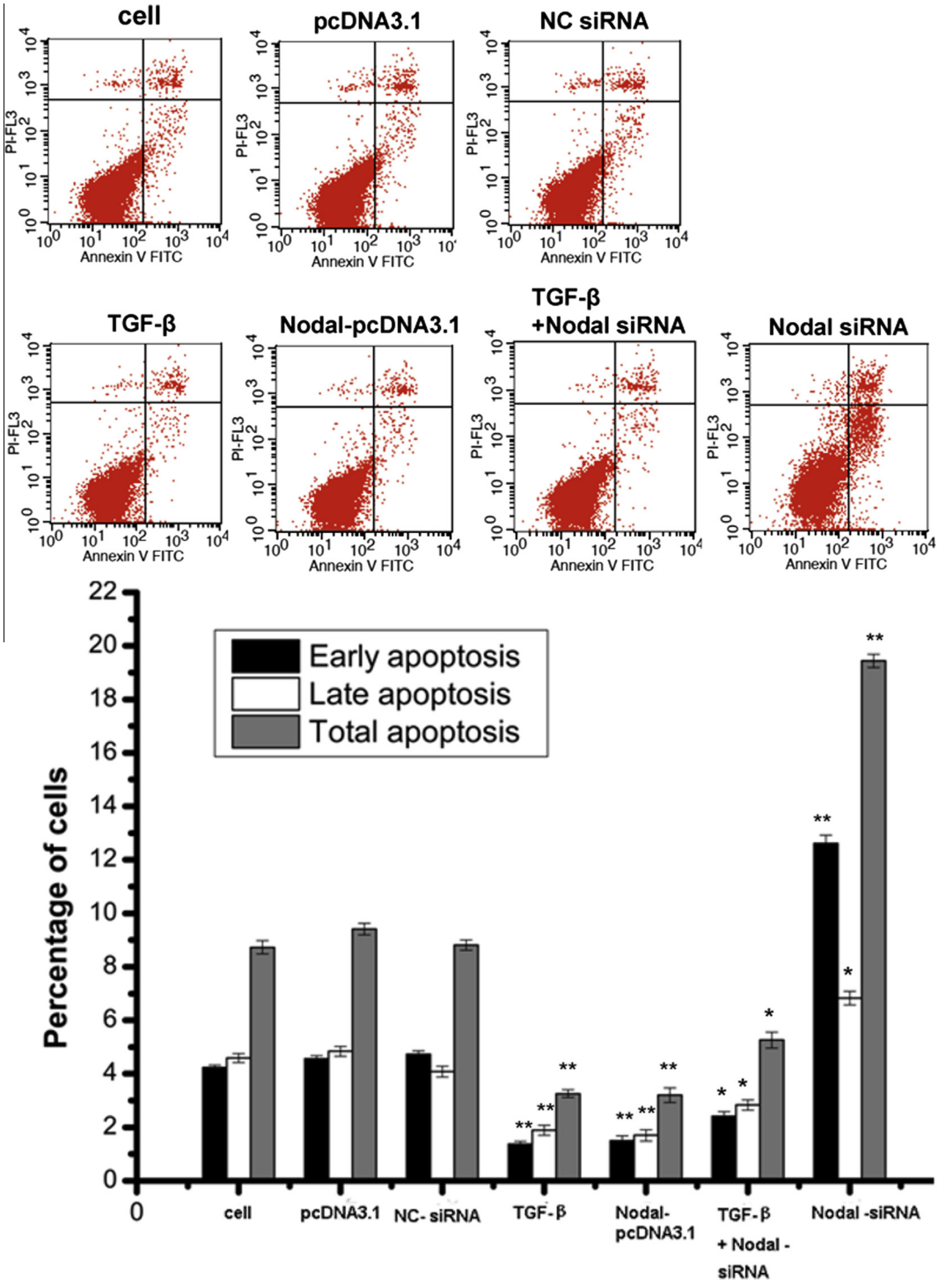

Fig. 3. Effect of Nodal on U87MG cell apoptosis. U87MG cell apoptosis was measured by Annexin V-FITC-propidium iodide flow cytometric analysis. The results are presented as mean ($n = 3$) ± SD, $^{*}P < 0.05$ vs. the control, $^{**}P < 0.01$ vs. the control.

expression inhibits the formation of vasculogenic mimicry, induces apoptosis, and thereby inhibits the growth of melanoma [26]. These results suggest that TGF-β may promote the growth of glioma cells via enhancing the expression of Nodal.

Our further studies showed that TGF-β promoted Nodal expression via activating Smad and ERK1/2 pathways. Transfection of siRNAs targeting TGF-β downstream signaling molecules Smad2 and Smad3, suppressed the effect of TGF-β on inducing Nodal expression, with a more significant inhibitory effect observed with Smad3 knockdown. Blockade of the ERK1/2 pathway most significantly attenuated the TGF-β-induced expression of Nodal; TGF-β also regulated the expression of Nodal through PI3K pathway, though to a lesser extent compared with ERK1/2 pathway. The TGF-β-induced Nodal expression was not affected by blocking the JNK and p38 pathways. Further detection of the proliferation, apoptosis, and invasion of glioma cells showed that Nodal overexpression promoted the proliferation and invasion and inhibited the apoptosis of tumor cells, resembling the effect of TGF-β addition. Downregu-

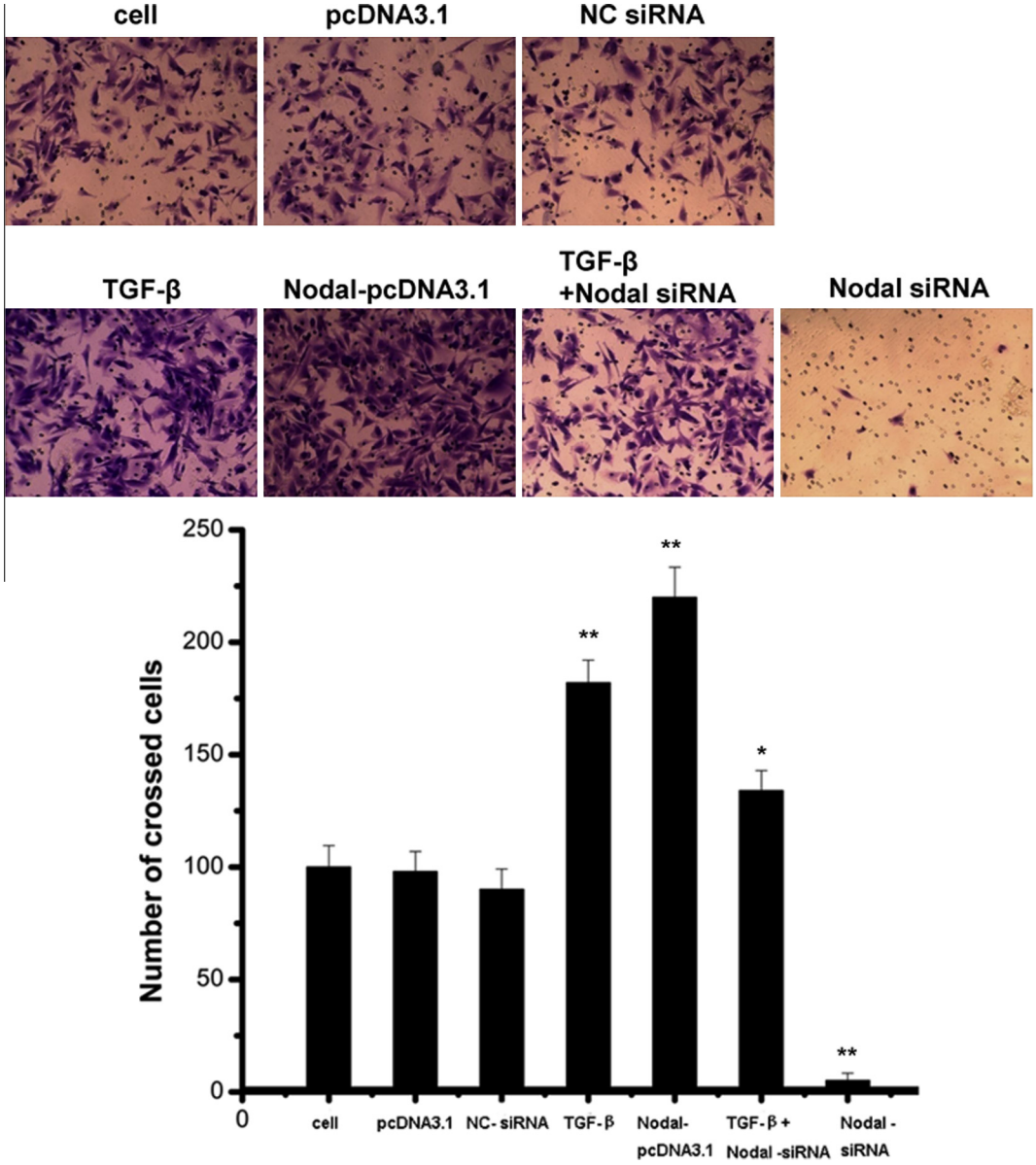

Fig. 4. Effect of Nodal on U87MG cell invasion. Each bar represents the mean ± SD from three samples ($^{*}P < 0.05$ vs. the control, $^{**}P < 0.01$ vs. the control).

lation of Nodal expression by transfecting Nodal-specific siRNA in the presence of TGF-β attenuated the promoting effect of the latter on the growth of glioma cells, while transfection of Nodal siRNA alone in the absence of exogenous TGF-β more profoundly inhibited the growth of glioma cells. These results demonstrated that while both TGF-β and Nodal promoted the growth of glioma cells, the former might exert such effect through promoting the expression of the latter. Lee et al. [13] showed that overexpression of Nodal not only increased MMP-2 secretion, enhanced cell invasiveness, and promoted cell proliferation *in vitro*, but also increased the tumor growth *in vivo*. A subsequent report indicated that Nodal stimulated the angiogenesis of U87 glioblastoma, and ERK1/2-HIF-1α signaling pathway was involved in this process [29]. De Silva et al. [30] found than stable transfection of U87 cells with plasmid overexpressing Nodal strongly induced Smad2 phosphorylation and significantly enhanced the cell growth. Overexpression of Nodal also resulted in tight spheroid formation and the tumor-promoting effect of Nodal on glioblastoma cells were mediated by ALK4, ALK7, and Smad3 [30].

In summary, the present study demonstrated that promoting Nodal expression is one of the mechanisms underlying the effect of TGF-β on promoting the growth of glioma cells, i.e., TGF-β promotes the tumor cell growth via upregulating the expression of Nodal. Therefore, our finding may provide a new gene target for tumor chemotherapy.

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