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Original Article

NRSN2 promotes osteosarcoma cell proliferation and growth through PI3K/Akt/MTOR and Wnt/β-catenin signaling

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Abstract: Osteosarcoma is the most common bone cancer in children and adults. However, its pathogenesis, especially molecular mechanisms remain elusive. In current study, we screened GEO Database and found a poorly studied protein Neurensin-2 (NRSN2), which is highly expressed in osteosarcoma tissues. Neurensin-2 (NRSN2) is a small neuronal membrane protein and localized in small vesicles in neural cells, previous study found that it has been implicated in hepatocellular carcinoma (HCC) and non-small cell lung cancer (NSCLC). We here report that the expression of NRSN2 is more commonlyelevated in 18 fresh osteosarcoma tissues. Furthermore, both loss- and gain-functions assays revealed that NRSN2 could promote osteosarcoma cell proliferation and growth both in vitro and in vivo. In addition, we further found that those effects on osteosarcoma by NRSN2 are associated with the dysregulated PI3K/AKT/mTOR signaling and Wnt/β-catenin signaling. In conclusion, our study found a novel oncogenic protein, NRSN2, which promotes osteosarcoma cell proliferation and as a membrane protein, NRSN2 also could be a potential treatment target for osteosarcoma.

Keywords: Neurensin-2, osteosarcoma, proliferation and growth, PI3K/Akt/GSK3β, Wnt/β-catenin

Introduction

Osteosarcoma is the most common bone malignancy and is most frequently occurred in adolescents and young adults [1]. Although the advances in diagnosis and treatment have improved the survival rate of patients, the overall clinical outcomes remain unsatisfactory, especially for patients in advanced stages [2]. Pathological studies have revealed that osteosarcoma is derived from primitive bone-forming mesenchymal cells [3]. However, the detailed mechanisms in the tumorigenesis of osteosarcoma remain elusive.

Recently, we observed that Neurensin 2 (NRSN2) has been reported plays contradictory roles in hepatocellular carcinoma (HCC) and non-small cell lung cancer (NSCLC) [4-6]. NRSN2 had been screened in HCC [7] and reported that it is down-regulated in HCC and the down-regulation of NRSN2 promotes HCC cell apoptosis and senescence [4]. While Zhang et al. reported that NRSN2 is elevated in NSCLC and promotes NSCLC cell proliferation [6]. These reports prompted us to investigate its role in osteosarcoma for its role in osteosarcoma hasn't been reported to date.

PI3K/Akt signaling has been reported plays essential roles in the progression of many tumors including osteosarcoma [8, 9]. A large body of evidence suggests that numerous oncogenic genes including TGFα through PI3K/Akt pathways to promote the malignant progression of osteosarcoma [10]. And PI3K/Akt almost participates in all malignant phenotypes including tumorigenesis, cancer cell proliferation, metastasis, angiogenesis and chemoresistance [11]. And previous reports suggest that NRSN2 could inhibit or promote PI3K/Akt pathway to suppress or promote cancer cell apoptosis or proliferation [4, 6]. These clues remind us to explore the significance of NRSN2 and PI3K/

Table 1. sequence of primers and sh RNAs

Name	Sequence (5'-3')
NRSN2 primer	F: GAGGACGTGCTGGGGCT
	R: CTGAGTCCATGTCCCGAATC
GAPDH primer	F: AGCCTCAAGATCATCAGCAATGCC
	R: TGTGGTCATGAGTCCTTCCACGAT
CCND1 primer	F: TCCTCTCCAAAATGCCAGAG
	R: GGCGGATTGGAAATGAACTT
c-myc primer	F: TTTCGGGTAGTGGAAAACCA
	R: CACCGAGTCGTAGTCGAGGT
Sh-#1	CCGGCCTCACTGTGTTGGAAGATCACTCGAGTGATCTTCCAACACAGTGAGGTTTTTTG
Sh-#2	CCGGGCAGGATGAGTGAAGACGTTTCTCGAGAAACGTCTTCACTCATCCTGCTTTTTTG

Akt in osteosarcoma. In addition, PI3K/Akt axis could suppress the activation of GSK3β [12], which plays an essential role in the degradation of β-catenin [13], a renowned tumor promoter which plays a central role in Wnt canonical signaling [14]. As we known, Wnt/β-catenin signaling also contributes to osteosarcoma progression, we given the hypothesis that NRSN2 might through PI3K/Akt/GSK3β axis to activate Wnt/β-catenin in osteosarcoma.

Materials and methods

Tissue samples and ethic statements

18 fresh tumor tissues and matched adjacent tissues were collected from patients with pathologically and clinically confirmed osteosarcoma. All human tumor tissues were obtained with written informed consent from patients. The Institutional Review Board of the First Affiliated Hospital, Xinjiang Medical University approved the use of the tumor sample in this study.

Immunohistochemical staining

For Immunohistochemical (IHC) staining assays, the procedure were according to a previous study [15].

Cell culture

hFOB 11.9, Saos2 and U2OS cells were purchased from ATCC. All those cells were maintained under standard culture conditions (37°C, 5% CO_2) in culture medium recommended by ATCC or Cell Bank of the Chinese Academy of Sciences.

RNA isolation and quantitative real-time PCR

Total RNA was purified from OSTEOSARCOMA and adjacent tissues or cells using TRIzol (Invitrogen) following the manufacturer's protocol. RNA (1 μg) was reverse transcribed using SuperScript Reverse Transcriptase III (TAKARA). Quantitative real time PCR was performed using SYBR green Supermix (ABI) in ABI 7500 PCR system. Housekeeping gene GAPDH was used as an internal control. Primers using in this study were described in **Table 1**.

Western blots

Cells were lysed in RIPA lysis buffer (Beyotime) and all the procedures were following the manufacturer's protocol. Subsequently the cell lysates were boiled in 5X SDS-PAGE loading buffer for 10 min and then resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used in this study: NRSN2 (1:1000, Proteintech), GAPDH (1:5000, Proteintech), p-Akt, Akt, p-mTOR, mTOR, p-GSK3β, GSK3β, β-catenin and the second antibodies were purchased from Cell Signaling Technology and with 1:1000 dilutions. The bound antibodies were visualized with the ECL kit (Thermo Scientific).

Construct stable cell lines

To generate stable silenced NRSN2 cell lines, Vectors containing shRNAs were purchased from Sigma-Aldrich. To over-express NRSN2, vectors containing the ORF of NRSN2 were obtained from Genecopoeia. 293T cells were transfected with those vectors by lipofectamine 2000 (Invitrogen), all the procedures were

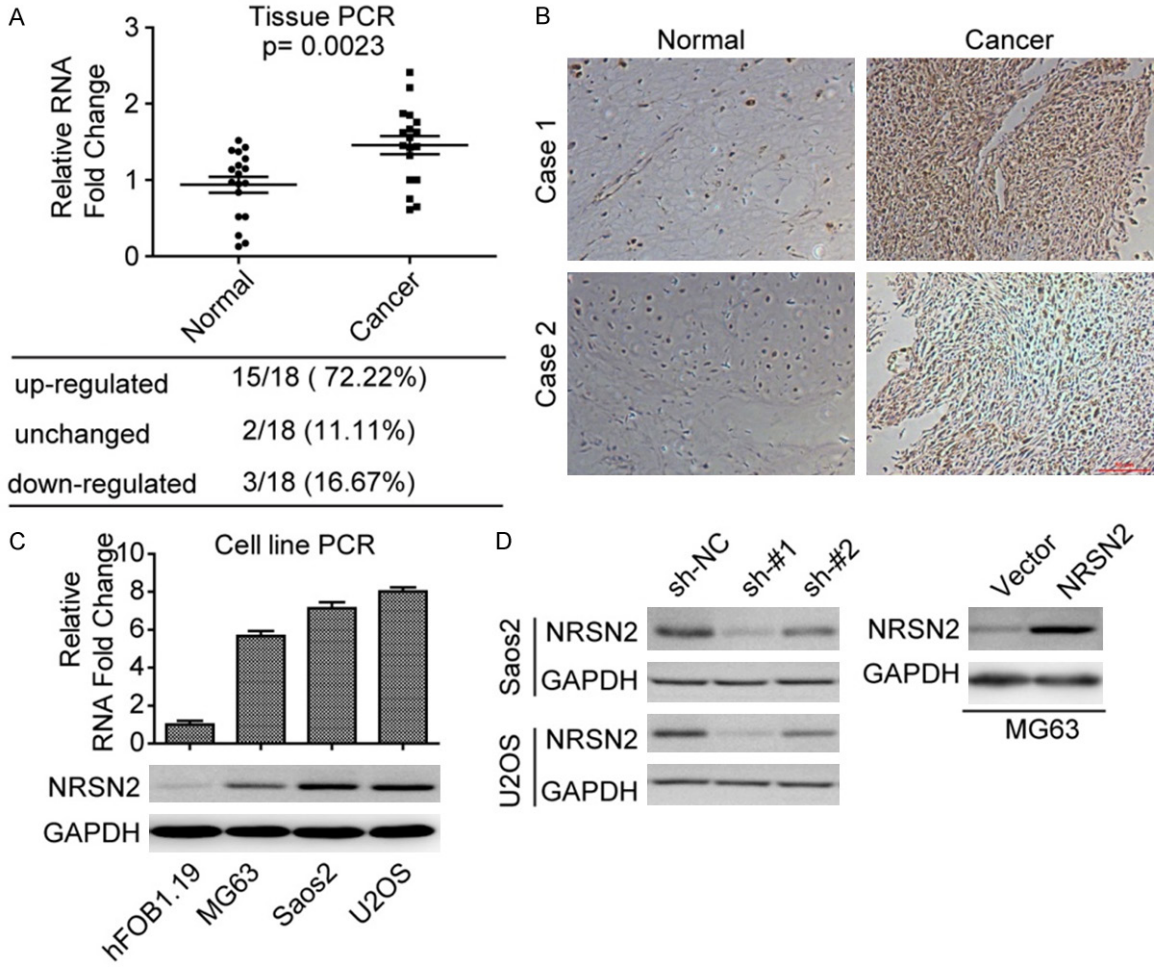

Figure 1. NRSN2 is significantly highly expressed in osteosarcoma tissues and cell lines. A. The mRNA level of NRSN2 is significantly highly expressed in 18 paired osteosarcoma tissues. B. Representative images of IHC show that NRSN2 is elevated in cancer tissues. C. The mRNA and protein level of NRSN2 is highly expressed in osteosarcoma cells when compared with immortalized normal osteoblast hFOB1.19. D. The protein level of NRSN2 is significantly altered in indicated cells.

according to the manufactures' protocols. The supernatant media containing virus was collected by centrifugation to remove cellular contaminant. The resulting viruses were used to infect indicated cells, and then integrated cells were selected by 2 μg/ml puromycin for 2 weeks. The alterations of NRSN2 in those cells were confirmed by western blots before further analysis. The sequence of shRNAs were described in **Table 1**.

CCK8 cell viability assays

Cells were seeded into a 96-well plate at 3×10^3 cells per well with 100 ul cultured medium and cultured at 37°C, 5% CO_2. The cell viability was quantified by addition 10 μl of cell counting kit (CCK-8, Dojindo). After 2 hours incubation, the plates were monitored by Power Wave XS microplate reader (BIO-TEK) at an absorbance 450 nm. For IWR-1-endo treated assay, IWR-1-endo was added to the medium in indicated concentration.

Colony formation assays

Colony formation in soft agar was tested to assay anchorage-independent growth. Stable transfected cell lines were suspended in the upper layer which consisting of culture medium with 1% FBS and 0.35% agar, which is above a basal layer of 0.6% agar in 6-well plates in a triplicate manner. The cell density was 1000 cells per well. Colonies were stained with 0.05% crystal violet, and all the visible colonies were counted by microscopy after 14-21 days.

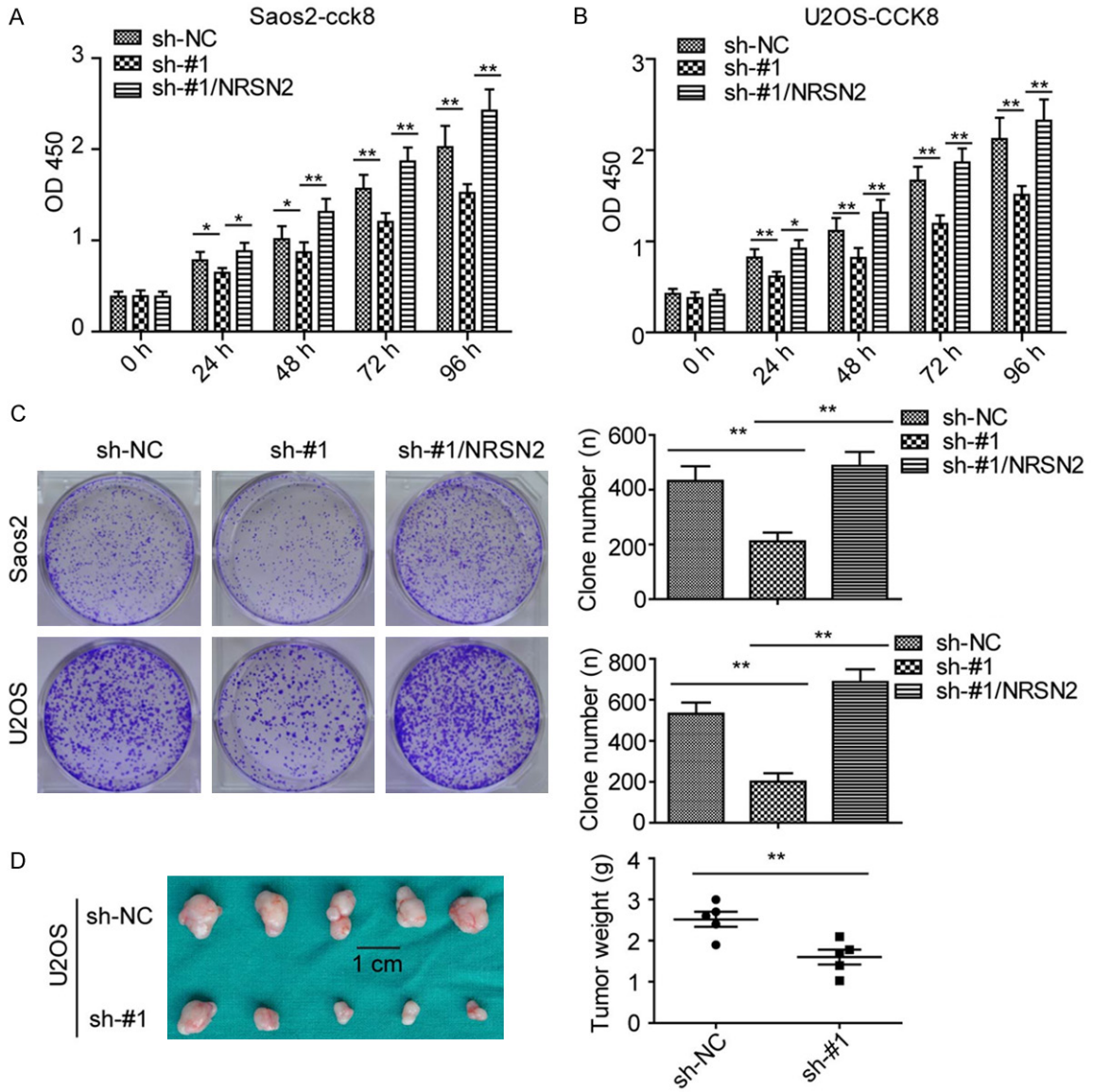

Figure 2. Knockdown NRSN2 inhibits osteosarcoma cell proliferation. (A, B) CCK-8 cell viability assays shown that knockdown of NRSN2 inhibits cell viabilities of Saos2 (A) and U2OS (B) cells and re-introducing NRSN2 elevates the cell viabilities in Saos2 and U2OS. (C) Silencing NRSN2 inhibits cell proliferation in Saos2 and U2OS, reintroducing NRSN2 rescue the cell inhibition effects in these two cell lines. (D) Knockdown NRSN2 inhibits the ability of subcutaneous tumor formation in U2OS. *p< 0.05, **p<0.01.

In vivo tumor formation assay

100×10^4 indicated stable cells were subcutaneously injected into right flank of 5 BALB/c (nu/nu) mice in each group. Tumor sizes were measured once a week and mice were sacrificed for the analysis of tumor burden after 4 weeks.

Luciferase reporter assays

Indicated cells were seeded in 96-well plates and transfected with TCF/β-catenin reporter plasmid (Wnt/β-catenin signaling) and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. After 48 hours incubation, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysis.

Statistics analysis

Data are expressed as the mean ± standard deviation. Student's ttest was used for comparisons between groups and p<0.05 was considered statistically significant difference.

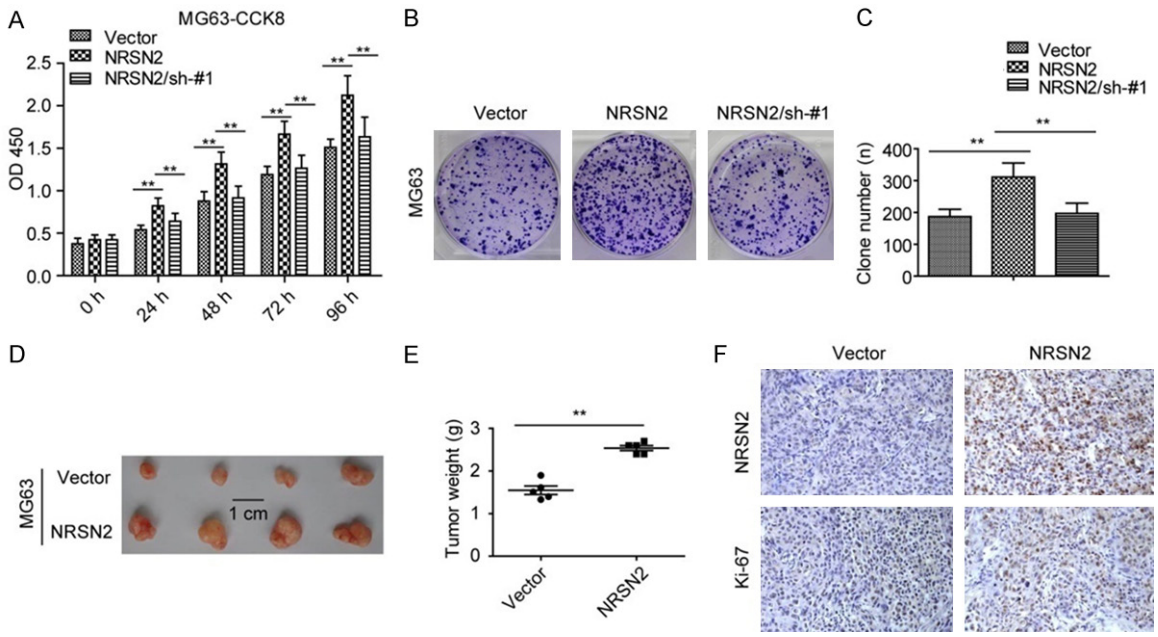

Figure 3. Overexpressing NRSN2 promotes osteosarcoma cell proliferation. A. Elevating the expression of NRSN2 promotes the cell viability and silencing NRSN2 rescues the elevated effects in MG63 cells. B, C. Overexpression of NRSN2 promotes cell proliferation and silencing NRSN2 rescues the heightened effects in MG63 cells. D. Overexpression of NRSN2 promotes the ability of subcutaneous tumor formation in MG63. E. Elevated level of NRSN2 in MG63 promoted the tumor weight. F. The level of KI-67 is positively correlated with NRSN2. *p<0.05, **p<0.01.

Results

The level of NRSN2 is elevated in osteosarcoma tissues and cell lines

Recently, NRSN2 has been reported plays contradictory roles in HCC and NSCLS. Wang et al. and Ma et al. reported that NRSN2 is down-regulated and plays a tumor suppressive role in HCC development. Zhang et al. found that NRSN2 is up-regulated in NSCLC tissues and plays oncogenic role in NSCLC progression [4, 6]. The contradictory effects of NRSN2 in different kinds of malignant tumors prompted us to investigate its role in osteosarcoma. Firstly, we determined the mRNA level of NRSN2 in 18 fresh malignant osteosarcoma tissues and matched adjacent tissues. Surprisingly, as illustrated in **Figure 1A**, we found that NRSN2 is more commonly highly determined in osteosarcoma malignant tissues. Moreover, we performed IHC staining assays revealed that NRSN2 I highly expressed in osteosarcoma tissues (**Figure 1B**). Furthermore, we found we also detected both mRNA and protein level of NRSN2 in immortalized osteoblast hFOB1.19 and osteosarcoma cell lines MG63, Saos2 and U2OS (**Figure 1C**). The results from these cell lines were consistent with fresh tissues; both the mRNA and protein levels of NRSN2 were highly expressed in these malignant cell lines compared with hFOB1.19, a normal osteoblast cell line. These results suggest that NRSN2 is highly expressed in the osteosarcoma and the highly expressed pattern of NRSN2 might play a role in the aggressive progression of osteosarcoma.

Knockdown of NRSN2 inhibits osteosarcoma cell proliferation both in vitro and in vivo

As NRSN2 is highly expressed in osteosarcoma tissues and cell lines, we then selected Saos2 and U2OS, which were relative highly, expressed NRSN2 among these cell lines, to knockdown NRSN2 for biological functions investigation and the protein level was significantly changed (**Figure 1D**) Previous studies revealed that NRSN2 is involved in cancer cell proliferation and survival, and then we selected these stable cell lines to perform CCK-8 cell viability assays. As shown in **Figure 2A** and **2B**, knockdown NRSN2 significantly inhibits cell viability, both in Saos2 and U2OS cell lines. To confirm these results, we then re-introduced NRSN2 to these silencing cells and the cell viability inhibited effects were totally rescued in these two cell lines (**Figure 2A** and **2B**). Moreover, we also

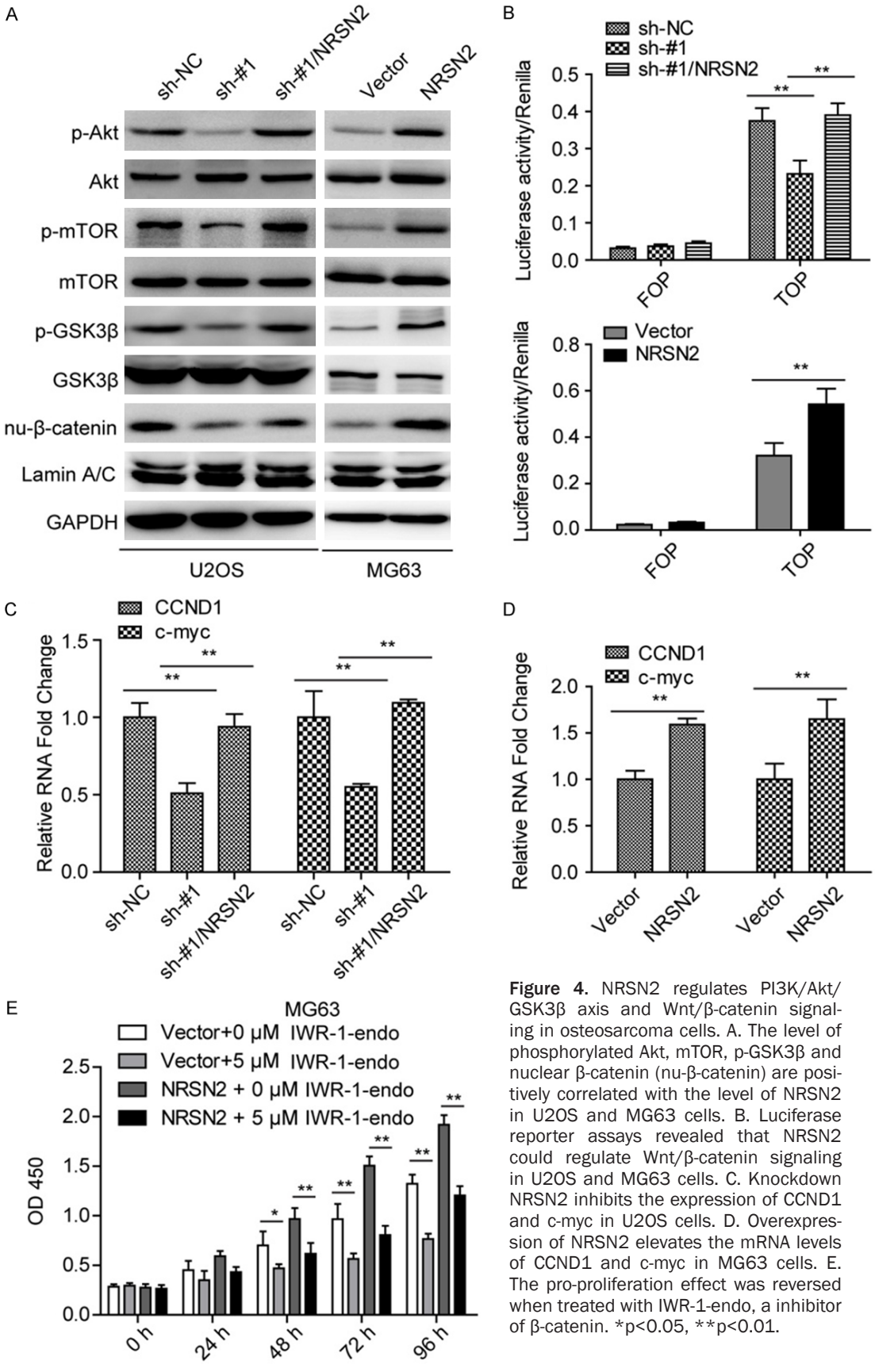

Figure 4. NRSN2 regulates PI3K/Akt/GSK3β axis and Wnt/β-catenin signaling in osteosarcoma cells. A. The level of phosphorylated Akt, mTOR, p-GSK3β and nuclear β-catenin (nu-β-catenin) are positively correlated with the level of NRSN2 in U2OS and MG63 cells. B. Luciferase reporter assays revealed that NRSN2 could regulate Wnt/β-catenin signaling in U2OS and MG63 cells. C. Knockdown NRSN2 inhibits the expression of CCND1 and c-myc in U2OS cells. D. Overexpression of NRSN2 elevates the mRNA levels of CCND1 and c-myc in MG63 cells. E. The pro-proliferation effect was reversed when treated with IWR-1-endo, a inhibitor of β-catenin. *p<0.05, **p<0.01.

performed soft agar cell clone formation assays in Saos2 and U2OS stable cell lines. As shown in **Figure 2C**, silencing NRSN2 remarkably inhibited the anchorage independent cell proliferation in these stable cell lines, and when reintroduced NRSN2 to these silencing cell lines, the inhibitions were totally reversed (**Figure 2C**). In addition, we also employed U2OS stable cell lines performed xenograft subcutaneous tumor formation assay to validate the results form in vitro. As shown in **Figure 2D**, stable knockdown NRSN2 significantly inhibited tumor growth *in vivo*, both the tumor size and weight formed by silenced cells is significantly smaller and lighter than control group. Taken together, these results suggest that knockdown NESN2 could inhibit cell proliferation both *in vitro* and *in vivo*.

Overexpression of NRSN2 promotes osteosarcoma cell proliferation in vitro and in vivo

We then selected MG63, which NRSN2 is relative lower in these cell lines, to overexpress NRSN2 for biological functions confirmation assays and the protein level in significantly elevated (**Figure 1D**). Firstly, we also performed CCK-8 cell viability assays. Confirmedly, we found that when overexpression of NRSN2 in MG63 cells, the cell viability was significantly elevated, furthermore, we also found knockdown the level of NRSN2 in the Overexpressing cells, the cell viability was suppressed (**Figure 3A**). Moreover, we soft agar cell clone formation assays shown that overexpression of NRSN2 could significantly elevate the ability of cell clone formation in MG63 cells. And knockdown the NRSN2 in the overexpression cells could reverse the promoted effects (**Figure 3B** and **3C**). In addition, we also used the Overexpression stable cell lines performed subcutaneous tumor formation assays in nude mice. As illustrated in **Figure 3D** and **3E**, we found that elevated the level of NRSN2 in MG63, the tumor size and weight were significantly larger and heavier than control group. Furthermore, we also performed IHC staining in these tumors. As shown in **Figure 3F**, we found that the level of KI-67, a proliferative cell marker, is positively correlated with NRSN2. In conclusion, the gain-function assays in MG63 cells validated the results that highly expressed NRSN2 could promote osteosarcoma cell proliferation and tumor growth both in vitro and in vivo.

NRSN2 regulates PI3K/Akt/GSK3β axis and Wnt/β-catenin signaling in osteosarcoma

Previous studies gave the clues that NRSN2 could regulate PI3K/Akt signaling. In current study, we confirmed previous studies in osteosarcoma cells, as shown in **Figure 4A**, we found that the phosphorylated Akt (p-Akt) and mTOR (p-mTOR) were positively correlated with the level of NRSN2 both in U2OS and MG63. As we known, GSK3β is a co-intermediator of PI3K/Akt signaling, and Wnt canonical signaling. P-AKT could activate the phosphorylation of GSK3β, and phosphorylated GSK3β (p-GSK3β) could promote β-catenin degradation [16]. Considering these clues, we then determined the p-GSK3β and nuclear β-catenin in stable U2OS and MG63 stable cell lines. Surprisingly, we found that when knockdown NRSN2, the level of p-GSK3β was increased, consistently, the level of nuclear β-catenin was decreased. While, in the MG63 overexpression cell lines, the level of p-GSK3β is remarkably increased and nuclear β-catenin is heightened (**Figure 4A**). These findings indicate that NRSN2 might also regulate Wnt canonical signaling, which plays a pivotal role in the cell proliferation. We then performed luciferase reporter assays to investigate whether NRSN2 regulates Wnt/β-catenin signaling. Confirmedly, the results from luciferase reporter assays suggested that the level of NRSN2 is positively correlated with the activity of Wnt/β-catenin signaling. In addition, we also examined the levels of CCND1 and c-myc, renowned targets of Wnt/β-catenin [17], in these stable cell lines. And the mRNA levels of CCND1 and c-myc were also consistent with the level of NRSN2 (**Figure 4C** and **4D**). In addition, we also used β-catenin inhibitor IWR-1-endo treated stable MG63 cell lines, as showed in **Figure 4E**, when treated with IWR-1-endo, the pro-proliferation effects of NRSN2 were inhibited. In conclusion, these results suggest that NRSN2 could in some way regulate PI3K/Akt pathway and Wnt/β-catenin signaling in osteosarcoma.

Discussion

In current study, we report a poorly studied gene Neurensin 2 (NRSN2), which has been reported plays complex role in different kinds of cancer, plays an oncogenic role in osteosarcoma development for it could promote osteo-

sarcoma cell proliferation both in vitro and in vivo.

As we known, the alterations of the genomic including oncogenes activation like c-myc and tumor suppressive genes inactivation, for instance TP53, contribute to the tumorigenesis and cancer development including osteosarcoma [18, 19]. In current study, we found that the level of NRSN2 is more commonly elevated in malignant osteosarcoma tissues. And we further illustrated the highly expressed pattern could promote osteosarcoma cell proliferation and growth both in vitro and in vivo. These results indicate that NRSN2 is an oncogene in osteosarcoma tumorigenesis and development. Considering that the contradictory role of NRSN2 in HCC and NSCLC [4, 6], our findings enlarge our knowledge that NRSN2 as a tumor promoter in the field of tumor biology. However, the detailed mechanisms underlying the contradictory expression pattern and roles in osteosarcoma, NSCLC and HCC remain to be further investigated.

We further explored by which signaling pathways NRSN2 promotes osteosarcoma cell proliferation. Considering previous reports, we first examined the changes of PI3K/Akt pathways in NRSN2 stable osteosarcoma cell lines. We found that NRSN2 also could regulate PI3K/Akt in osteosarcoma for the level of p-Akt and p-mTOR is positively correlated with the level of NRSN2 in these stable cell lines. Furthermore, we also found that NRSN2 could through PI3K/Akt/GSK3β axis to activate Wnt/β-catenin signaling in osteosarcoma. As we known, PI3K/Akt/mTOR pathway is deregulated in numerous malignant tissues for its activation could promote cancer cell proliferation and metastasis [20]. In current study, NRSN2 activates PI3K/Akt/mTOR pathway in osteosarcoma further validates that NRSN2 plays an oncogenic role in osteosarcoma, however, how NRSN2 regulates this pathway remains to be illustrated in further study. In addition, Wnt canonical signaling has been reported plays an essential role in the early stage of cancer development, for it activates c-myc and CCND1, which robustly promotes cell proliferation [21, 22], in current study, we firstly report that NRSN2 could through PI3K/Akt/GSK3β axis to activate Wnt/β-catenin in osteosarcoma.

In conclusion, in current study, we found NRSN2 through PI3K/Akt/mTOR and Wnt/β-catenin signaling to promote osteosarcoma cell proliferation in vitro and in vivo. And as a membrane protein, NRSN2 obtains the potential to be a target for osteosarcoma treatment.

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Disclosure of conflict of interest

None.

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