Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;43:2133-2142
DOI: 10.1159/000484232
Published online: October 24, 2017
Accepted: June 26, 2017

Original Paper

A Novel Positive Feedback Loop Between NTSR1 and Wnt/β-Catenin Contributes to Tumor Growth of Glioblastoma

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Key Words
Neurotensin • NTSR1 • Wnt/β-Catenin • SR48692 • Glioblastoma

Abstract
Background/Aims: Neurotensin (NTS), an intestinal hormone, is profoundly implicated in cancer progression through binding its primary receptor NTSR1. The conserved Wnt/β-Catenin pathway regulates cell proliferation and differentiation via activation of the β-catenin/T-cell factor (TCF) complex and subsequent modulation of a set of target genes. In this study, we aimed to uncover the potential connection between NTS/NTSR1 signaling and Wnt/β-Catenin pathway. ***Methods:*** Genetic silencing, pharmacological inhibition and gain-of-function studies as well as bioinformatic analysis were performed to uncover the link between NTS/NTSR1 signaling and Wnt/β-Catenin pathway. Two inhibitors were used *in vivo* to evaluate the efficiency of targeting NTS/NTSR1 signaling or Wnt/β-Catenin pathway. ***Results:*** We found that NTS/NTSR1 induced the activation of mitogen-activated protein kinase (MAPK) and the NF-κB pathway, which further promoted the expression of Wnt proteins, including Wnt1, Wnt3a and Wnt5a. Meanwhile, the mRNA and protein expression levels of NTSR1 were increased by the Wnt pathway activator Wnt3a and decreased by the Wnt inhibitor iCRT3 in glioblastoma cells. Furthermore, pharmacological inhibition of NTS/NTSR1 or Wnt/β-Catenin signaling suppressed tumor growth *in vitro* and *in vivo*. ***Conclusion:*** These results reveal a positive feedback loop between NTS/NTSR1 and Wnt/β-Catenin signaling in glioblastoma cells that might be important for tumor development and provide potential therapeutic targets for glioblastoma.

Introduction

Neurotensin (NTS), a 13 endogenous amino acid peptide initially isolated from bovine hypothalamus, has been well studied as a neurotransmitter and hormone in the central nervous system [1]. Recently, emerging data suggest that NTS plays crucial roles in the

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numerous malignant phenotypes of cancers, such as proliferation, anti-apoptosis, invasion, metastasis and stem-like traits [2-5]. The effects of NTS are mainly mediated by a single class of cell-surface receptors, also known as Neurotensin receptors (NTSR), which is composed of three subtypes: NTSR1, NTSR2 and NTSR3. The NTSR1 and NTSR2 are G-protein coupled receptors (GPCR), while NTSR3, encoded by SORT1 gene, belongs in the sortilin receptor superfamily. Among these three receptors, NTSR1 exhibits highest affinity and mediates most biological functions of NTS in cancers [6]. Activation of NTSR1 can promote cell survival, proliferation, migration, and invasiveness in cancers through multiple signal transduction, including PKC, mitogen-activated protein kinase (MAPK), c-jun-NH-kinase, RhoGTPase, focal adhesion kinase, NF-κB and Wnt/β-catenin [7-10].

Wnt/β-catenin signaling is an important pathway regulates stem cell pluripotency and cell fate decisions during development [11]. Ample evidence have shown that dysregulation of Wnt/β-catenin signaling is associated with numerous disease pathologies, especially tumorigenesis [12, 13]. Activation of Wnt/β-catenin signaling leads to stabilization and nuclear translocation of β-catenin, which further activates T-cell factor/lymphoid enhancer-binding factor (TCF/LEF)-dependent transcription of downstream target genes. Recently, it has been demostrated that NTSR1 is a direct target of the Wnt/APC oncogenic pathways connected with the β-catenin/Tcf transcriptional complex [14]. And excitingly, NTS is also a direct target of the Wnt/β-catenin pathway and may be a mediator for neuroendocrine tumor cell growth [4].

Given these information above, we hypothesized that a positive feedback loop might be exist between NTS/NTSR1 and Wnt/β-catenin signaling. Previously, the oncogenic functions of NTS/NTSR1 and its mechanisms have been identified in glioblastoma. However, the potential relationship between NTS/NTSR1 and Wnt/β-catenin signaling has not been reported. In this study, we analyzed this hypothesis and aimed to find potential therapeutic targets for glioblastoma.

Materials and Methods

Cell culture and reagent

Glioblastoma cell lines A172, U87 and U251 were obtained from American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL streptomycin-penicillin in a humidified incubator at 37°C with 5% CO_2. NTS (1 mg/ml, Phoenix Pharmaceuticals, NY, USA) was dissolved in PBS as a stock. SR48692 and iCRT3 was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). TPCA-1 (S2824) and U0126 (S1102) and were purchased from Selleck (Shanghai, China). Wnt3a (R&D system, USA) was dissolved in PBS as a 10 μg/ml stock. For detection of cellular functions, glioblastoma cells were incubated with NTS, SR48692, TPCA-1, U0126, iCRT3, and Wnt3a at indicated concentrations in the absence of 10% FBS to avoid the effects of growth factors and hormones in FBS.

Transfection

The small interfering RNA against NTSR1 (siNTSR1), versus the negative control (NC) were purchased from GenePharma (Shanghai, China). For transfection, A172 and U87 cells seeded in 6-well plate were transfected with siNTSR1 by using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) in accordance with the manufacturer's instructions. Briefly, the culture medium was replaced by 500 μl of transfection mixture containing 8 μl of Lipofectamine 2000 and 15 μl of 20 μM siNTSR1 or si-Ctrl in Opti-MEM; then transfected cells were cultured for 72 h and harvested for western blotting validation. The NTSR1 overexpressing plasmids as well as an empty vector were transfected with FuGENE transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Quantitative Real-time PCR

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Shanghai) according to the manufacturer's protocol. Then 1 μg RNA for each sample was reversely transcribed and the resulting cDNA

was amplified using the GoTaq® 2-Step RT-qPCR System (Promega, Madison, USA) in the ViiA 7 Real-Time PCR System (ABI). The relative mRNA expression was normalization with reference to the quantification of GADPH level. The following primers were used: MYC, forward, 5'-GTCAAGAGGCGAACACACAAC-3'; reverse, 5'-TTGGACGGACAGGATGTATGC-3'; CCND1, forward, 5'-CAATGACCCCGCACGATTTC-3'; reverse, 5'-CATGGAGGGCGGATTGGAA-3'; MMP7, forward, 5'-GAGTGAGCTACAGTGGGAACA-3'; reverse, 5'-CTATGACGCGGGAGTTTAACAT-3'; Wnt1, forward, 5'-CGATGGTGGGGTATTGTGAAC-3'; reverse, 5'-CCGGATTTTGGCGTATCAGAC-3'; Wnt3a, forward, 5'-CTCGCTGGCTACCCAATTTG-3'; reverse, 5'-AGGCTGTCATCTATGGTGGTG-3'; Wnt5a, forward, 5'-TCGACTATGGCTACCGCTTTG-3'; reverse, 5'-CACTCTCGTAGGAGCCCTTG-3'; NTSR1, forward, 5'-AGCAGTGGACTCCGTTCCT-3'; reverse, 5'-GTTGGCAGAGACGAGGTTGT-3'; GAPDH, forward, 5'-ACAACTTTGGTATCGTGGAAGG-3'; reverse, 5'-GCCATCACGCCACAGTTTC-3';

Western blot analysis

Protein extracts were isolated with 1× cell lysis buffer. Equal amounts of protein were resolved on SDS-PAGE gel and electrophoretically transferred to polyvinylidene difluoride membranes. After blocking with 5% defatted milk, membranes were incubated overnight with primary antibodies at 4°C, followed by a horseradish peroxidase–labeled secondary antibody for 1 hour. Primary antibodies used in this study: NTSR1 (1:1000, Abcam), NF-κB, p-NF-κB, ERK1/2 and p-ERK1/2 (diluted at 1:1000) were purchased from Cell Signaling Technology and antibody against GADPH (1:2000, Abcam). Immunoblots were developed using the enhanced chemiluminescence detection system (Millipore).

Luciferase reporter assay

Cells were plated at 4×10^5 cells/wells in 24-well plates and transiently transfected with the TopFlash (0.5 μg) and the Renilla reporter (0.05 μg) using Lipofectamine 2000 (Invitrogen, CA, USA). The NTS, Wnt3a, SR48692 and iCRT3 treatment were added to A172 or U87 cells for 24 h after plating. The cells were harvested and luciferase activity was measured two days after transfection. The luciferase activity was measured by using the Dual Luciferase Reporter Assay System (Promega, USA).

Cell proliferation and cell apoptosis assay

Cells were seeded into 96-well plates to a density of 5×10^3 cells per well and incubated in the culture medium with indicated treatment for an additional 48 h. Cell viability and cell apoptosis assays were carried out using a Cell Counting kit-8 (Dojindo, Tokyo, Japan) and a Caspase-Glo 3/7 assay kit (Promega, USA) according to the manufacturer's instructions, respectively.

Animal studies

A172 cells were used to establish a subcutaneous xenograft and to determine the anti-tumor effects of SR48692 and iCRT3. This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Third Military Medical University. All procedures were done according to protocols approved by the ethics committee of the Third Military Medical University. NOD-SCID BALB/c mice were inoculated subcutaneously in the right back with 2×10^6 A172 cells. The growth of the primary tumors was recorded every 4 days. SR48692 (10 mg/kg) and iCRT3 (5 mg/kg) was diluted in PBS i.p. triweekly when tumors grew to ~200 mm^3. The control mice were treated with blank PBS containing 5% (v/v) DMSO. Tumor volume was evaluated with the following formula: volume = tumor length × $width^2/2$. The mice were sacrificed 24 days after pharmaceutical treatment. The tumors were resected and embedded in paraffin, and the Ki67 staining was analyzed by immunohistochemistry.

Statistical analysis

Statistical analyses were performed by using SPSS 13.0 statistical package software (SPSS, Chicago, IL, USA) or GraphPad Prism (GraphPad Software Inc., San Diego, CA). The two-tailed unpaired Student's t test and one wayanalysis of variance (ANOVA) were used to determine the statistical significance of differences between two groups and among more than three groups, respectively. P value less than 0.05 was considered statistically significant.

Results

NTSR1 modulates the activity of Wnt/β-catenin signaling

To investigate the effects of NTS/NTSR1 on Wnt/β-Catenin pathway, loss of function of NTSR1 in two glioblastoma cell lines, A172 and U87, was performed. By Western blotting analysis, genetic silencing of NTSR1 resulted in pronounced decrease in NTSR1 protein level in both A172 and U87 cells (Fig. 1A). In the presence of 50 nM NTS, luciferase reporter assay showed that NTSR1 knockdown markedly reduced the activity of Wnt/β-Catenin pathway (Fig. 1B). To further confirm this finding, a selective pharmacological antagonist of

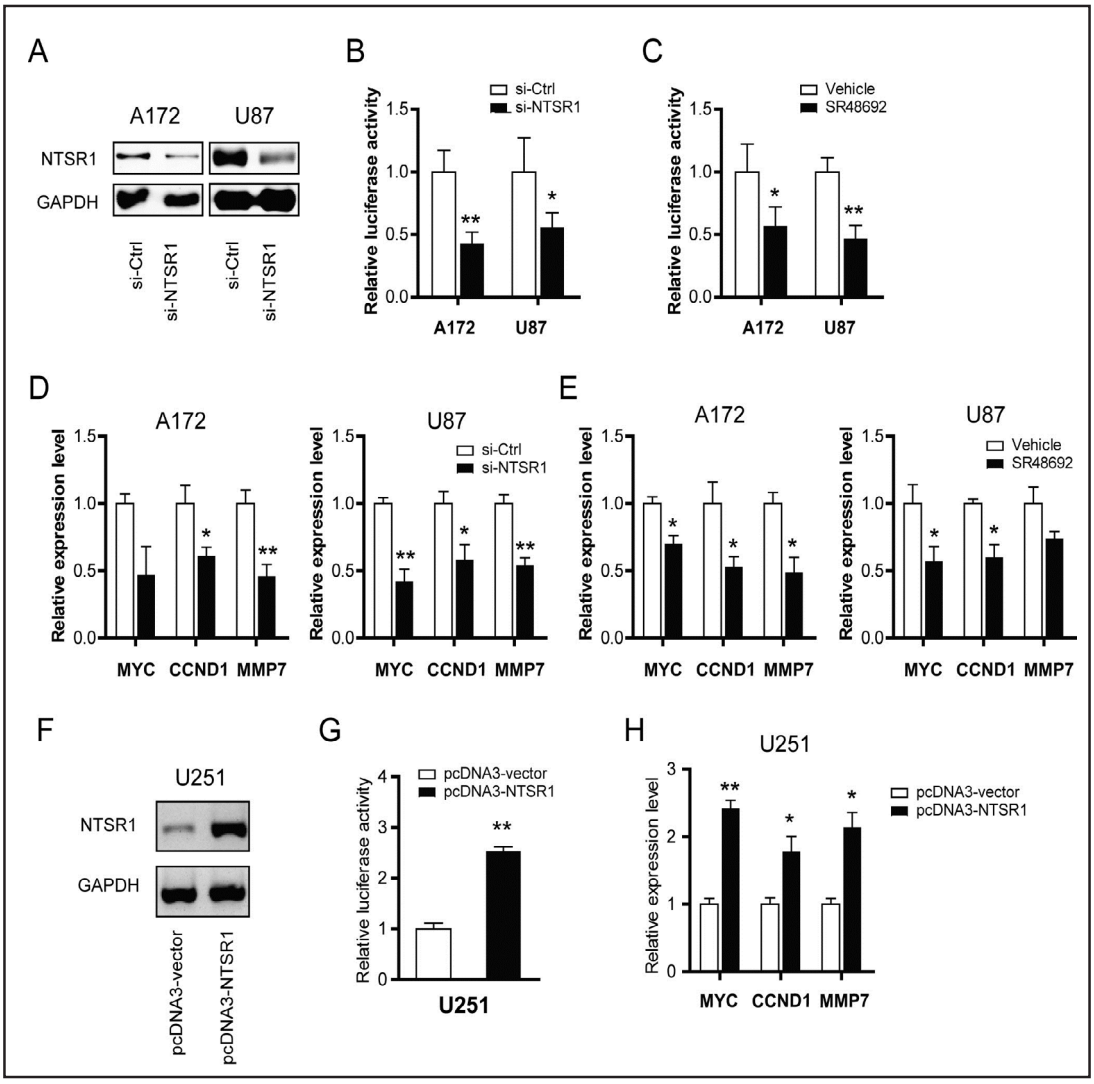

Fig. 1. NTSR1 modulates activity of Wnt/β-catenin signaling. (A) The protein level of NTSR1 was measured by western blotting in si-NTSR1 and si-Ctrl A172 and U87 cells. GAPDH was used as a loading control. Quantification of luciferase activity of Wnt/β-catenin signaling in A172 and U87 cells with NTSR1 knockdown (B) and inhibition (C) in the presence of NTS stimulation. Downstream targets (MYC, CCND1 and MMP7) of Wnt/β-catenin signaling in A172 and U87 cells with NTSR1 knockdown (D) and inhibition (E) in the presence of NTS stimulation was detected by RT-qPCR. (F) The overexpression efficiency of NTSR1 in U251 cells was measured by western blotting. (G) Quantification of luciferase activity of Wnt/β-catenin signaling in U251cells with NTSR1 overexpression. (H) The effects of NTSR1 overexpression on the expression of MYC, CCND1 and MMP7. A-E, *p<0.05 and **p<0.01 (vs. si-Ctrl or Vehicle); G and H, *p<0.05 and **p<0.01 (vs. pcDNA3-vector).

NTSR1, SR48692, was used. As shown in Fig. 1C, A172 and U87 cell lines treated with 5 μM SR48692 exhibited a significant reduction of TOP Flash activity when compared with the DMSO control group. Consistently, in the presence of 50 nM NTS, either NTSR1 knockdown or pharmacological inhibition decreased the expression of the downstream targets of Wnt/β-Catenin pathway, including MYC, CCND1 and MMP7 (Fig. 1D and 1E). Moreover, overexpression of NTSR1 in U251 cells by transfection of pcDNA3.1-NTSR1 plasmid markedly increased the protein level of NTSR1 (Fig. 1F). And overexpression of NTSR1 in U251 cells significantly enhanced the activity of Wnt/β-Catenin pathway and the expression level of its downstream targets (Fig. 1G and 1H).

Activation of NTSR1 promotes Wnt expression through NF-κB and MAPK signaling

It is well known that activation of NTSR1 induces the formation of Ras-GTP, which further activates Raf and the downstream MAPK cascade. And NTS can also induce NF-κB signaling through calcium-dependent PKC activation. We therefore tested whether the regulatory role of NTS/NTSR1 on Wnt/β-Catenin pathway is mediated by NF-κB and MAPK signaling or not. As shown in Fig. 2A, inhibition of NTSR1 with SR48692 or NTSR1 knockdown resulted in a significant decrease in the phosphorylation level of NF-κB and MAPK, suggesting the downstream cascades of NTS/NTSR1 signaling. Previously, it has been reported that exogenous NTS stimulation can upregulate the expression of Wnt proteins in liver cancer [9]. To test this hypothesis in glioblastoma, we detected the effects of NTS on the expression of Wnt1, Wnt3a and Wnt5a upon NF-κB and MAPK inhibition. Expectedly, all the Wnts detected were significantly elevated by NTS and largely compromised by NF-κB and MAPK inhibitors, TPCA-1 and U0126 (Fig. 2B). Collectively, these data above suggest that NTS/NTSR1 can enhance Wnt/β-Catenin pathway by upregulating the expression of Wnts through NF-κB and MAPK signaling.

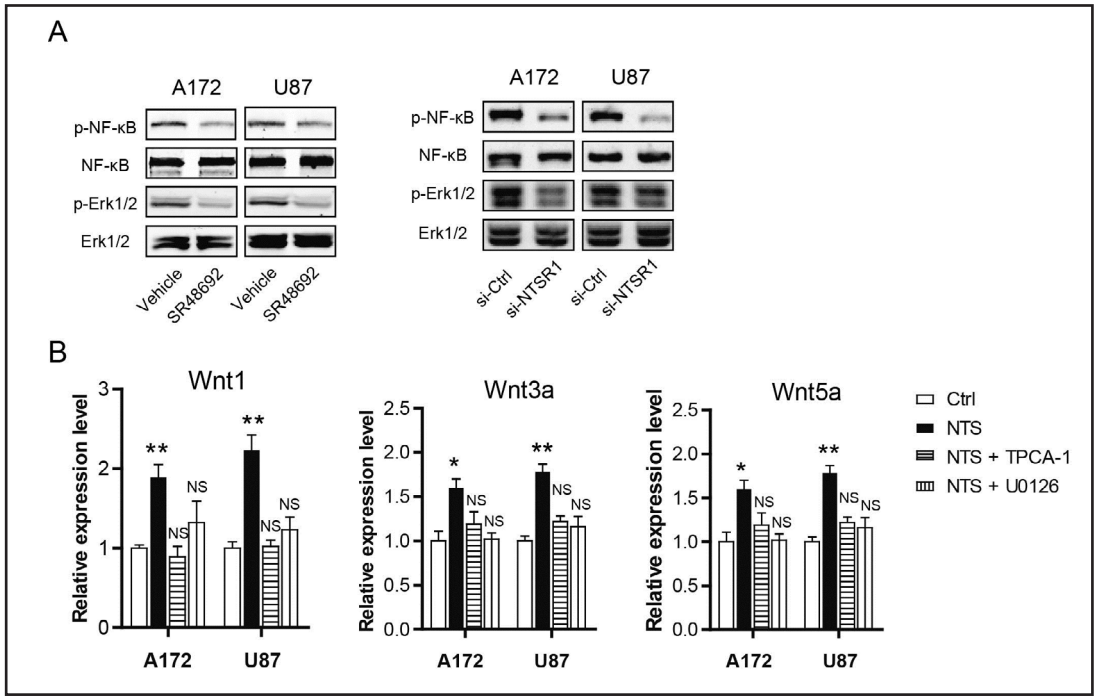

Fig. 2. Activation of NTSR1 promotes Wnt expression through NF-κB and MAPK signaling. (A) The protein level of p-NF-κB, NF-κB, p-ERK1/2 and ERK1/2 upon NTSR1 inhibition (SR48692) or knockdown was measured by western blotting. GAPDH was used as a loading control. (B) The mRNA levels of Wnt (Wnt1, Wnt3a and Wnt5a) in the presence of NTS stimulation and/or NF-κB (TPCA-1) and MAPK (U0126) inhibition were detected by RT-qPCR. *p<0.05 and **p<0.01 (vs. Ctrl).

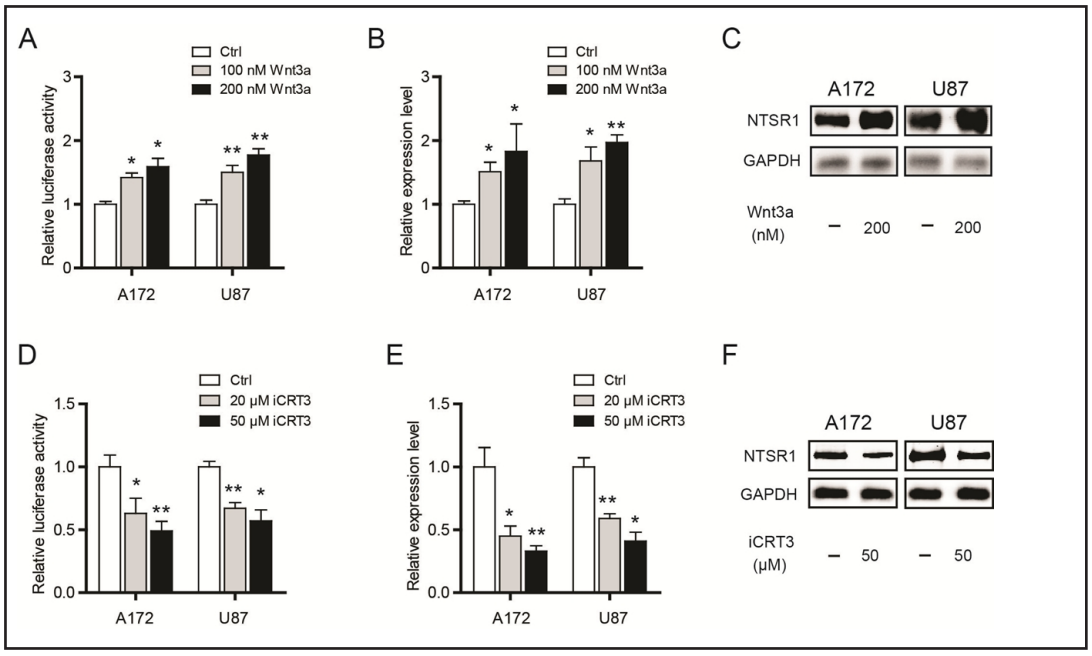

Fig. 3. Regulation of NTSR1 expression by Wnt/β-catenin signaling in glioblastoma cells. Quantification of luciferase activity of Wnt/β-catenin signaling with Wnt3a stimulation (A) or iCRT3 inhibition (D). The mRNA levels of NTSR1 with different concentrations of Wnt3a stimulation (B) or iCRT3 inhibition (E) were detected by RT-qPCR. (C) The protein levels of NTSR1 with 200nM Wnt3a stimulation or iCRT3 inhibition (F) were detected by western blotting. *p<0.05 and **p<0.01 (vs. Ctrl).

Regulation of NTSR1 expression by Wnt/β-catenin signaling in glioblastoma cells

To uncover whether Wnt/β-catenin signaling is involved in the regulation of NTSR1 expression, Wnt activator and inhibitor were used to test their effects on NTSR1 expression. As shown in Fig. 3A, Wnt3a, an effective Wnt activator, enhanced the activity of Wnt/β-Catenin signaling in a dose-dependent manner. Concomitantly, Wnt3a increased the mRNA (Fig. 3B) and protein (Fig. 3C) level of NTSR1 in both A172 and U87 cells. Then, one of the most selective inhibitors of Wnt, iCRT3 [15], significantly decreased TOP Flash activity and reduced the level of NTSR1 (Fig. 3D-3F). These data indicate that NTSR1 is regulated by Wnt/β-catenin signaling in glioblastoma and suggest a positive feedback loop between NTS/NTSR1 and Wnt/β-catenin signaling.

Pharmacological inhibition of the NTSR1/Wnt/β-catenin loop attenuates tumor growth of glioblastoma in vitro and in vivo

Next, we evaluated the therapeutic potential of targeting NTSR1/Wnt/β-catenin loop. Stimulation of A172 and U87 cells with NTS or Wnt3a significantly increased the TOP Flash activity, which was blocked by either SR48692 or iCRT3 (Fig. 4A). And expectedly, the proliferation rates of A172 and U87 cells were markedly upregulated by NTS or Wnt3a; and combined treatment with SR48692 or iCRT3 largely compromised the prolific effects of NTS and Wnt3a (Fig. 4B). Similarly, under serum starvation condition, cell apoptosis of A172 and U87 cells was reduced by NTS or Wnt3a treatment; and the anti-apoptotic effects of NTS and Wnt3a can be largely abrogated by SR48692 or iCRT3 (Fig. 4C). Finally, to determine the anti-tumor effects of SR48692 and iCRT3 *in vivo*, a subcutaneous xenograft model was used. As depicted in Fig. 4D, the tumor growth rates were markedly retarded by SR48692 or iCRT3 treatment. Consistently, the tumor-suppressive role of SR48692 and iCRT3 was accompanied with a reduction in Ki67 index, a proliferation marker (Fig. 4E). Taken together, these findings suggest that targeting the NTSR1/Wnt/β-catenin loop may provide promising therapies in the treatment of glioblastoma (Fig. 5).

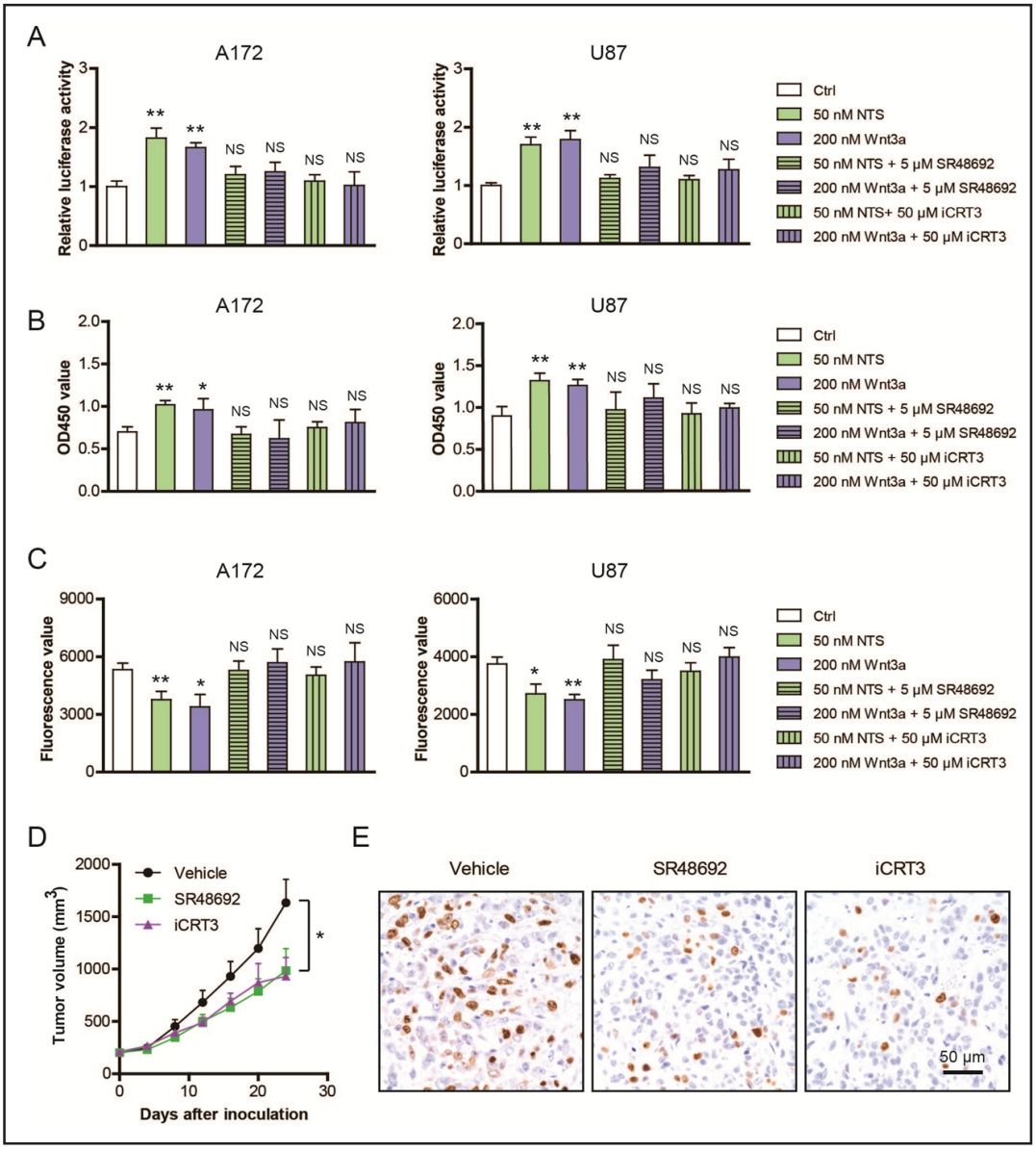

Fig. 4. Pharmacological inhibition of the NTSR1/Wnt/β-catenin loop attenuates tumor growth of glioblastoma in vitro and in vivo. (A) Quantification of luciferase activity of Wnt/β-catenin signaling in the presence of NTS, Wnt3a stimulation, and SR48692, iCRT3 inhibition. (B, C) The cell viability (B) and caspase-3/7 activity (C) of A172 and U87 cells upon indicated treatment. (D) Growth curves of subcutaneous xenograft mouse models upon SR48692 and iCRT3 treatment. (D) The Ki67 staining among Vehicle, SR48692 and iCRT3 group was analyzed by immunohistochemistry. *p<0.05 and **p<0.01 (vs. si-Ctrl or Vehicle).

Discussion

In the current study, we investigated the mutual regulation between NTS/NTSR1 signaling and Wnt/β-catenin pathway. First, NTS/NTSR1 signaling activated Wnt/β-catenin pathway through inducing the production of Wnt proteins. Second, Wnt/β-catenin signaling controlled the expression of NTSR1 in glimoblastoma cells. Moreover, pharmacological inhibition of NTS/NTSR1 signaling or Wnt/β-catenin pathway suppressed glimoblastoma cell growth *in vitro* and *in vivo*.

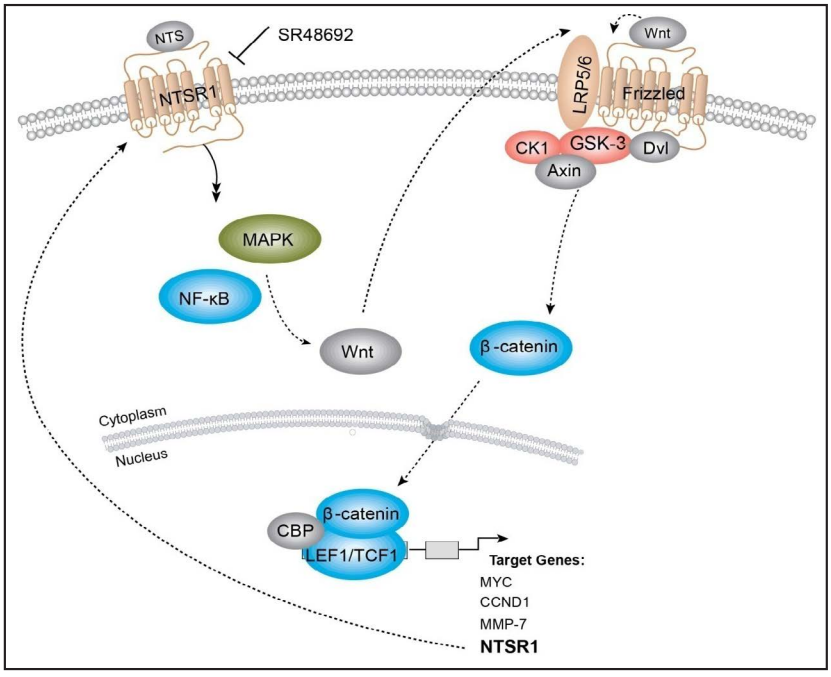

Fig. 5. A hypothesis of the positive feedback loop between NTS/NTSR1 and Wnt/β-catenin signaling in glioblastoma. Activation of NTSR1 leads to the activation of downstream Ras-dependent mitogen-activated protein kinase (MAPK) and the NF-κB pathway, which subsequent increase the expression of Wnt proteins. Activation of Wnt signaling triggers displacement of the multifunctional kinase GSK-3β from a regulatory APC/Axin/GSK-3β-complex that may involve substrate trapping and/or endosome sequestration (off-state). Stablized β-catenin is translocated to the nucleus and binds to LEF/TCF transcription factors to initiate gene expression (especially NTSR1). The element was obtained from Cell Signaling Technology (http://www.cellsignal.com/).

NTS/NTSR1 signaling plays versatile roles in the oncogenic signatures of glioblastoma. For example, NTS/NTSR1 signaling can stimulate glioblastoma cell proliferation by upregulating c-Myc and downregulating miR-29b-1 and miR-129-3p, and regulate stem-like traits of glioblastoma stem cells through activation of IL-8/CXCR1/STAT3 pathway [5, 16]. In this study, we found that NTS/NTSR1 signaling can promote glioblastoma tumor growth by activating Wnt/β-catenin pathway. Activation of NTSR1 resulted in downstream phosphorylation of NF-κB and Erk1/2, which ultimately increased the Wnt proteins. Blocking the NTSR1 signaling by the specific antagonist SR48692 inhibited the activity of Wnt/β-catenin signaling. Consistent with our observation, Ye et al. found that NTS-induced epithelial-to-mesenchymal transition (EMT) was correlated with the remarkable increase in Wnt1, Wnt3, Wnt5 expression and p-GSK3β level [9]. Furthermore, NTSR1 expression in hepatocellular carcinoma (HCC) was also positively correlated with the alteration of the Wnt/β-catenin pathway [17]. Therefore, we proposed that NTSR1 signaling-induced Wnt activated the canonical Wnt/β-catenin pathway.

Alterations in the Wnt/β-catenin pathway, resulting in TCF/LEF-dependent transcriptional gene activation, are commonly detected in human cancers. Notably, many receptors and their receptors have been identified as target genes of Wnt/β-catenin signaling [12]. For instance, β-catenin and Tcf mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/Ephrin-B [18]. In human colonic adenomas, NTSR1 gene activation was perfectly correlated with nuclear or cytoplasmic beta-catenin localization [14]. Recently, Kim et al. demonstrated that NTS itself was a direct target for Wnt/β-catenin signaling in neuroendocrine tumor cells in addition to NTSR1. In this study, we also revealed that NTSR1 was a target gene of Wnt/β-catenin signaling in glioblastoma. Thus, the Wnt/β-catenin pathway exhibited a positive regulatory effect on NTS/NTSR1 signaling at the level of the ligand and its receptor in glioblastoma. However, the precise model underlying the modulation of Wnt/β-catenin pathway on NTS/NTSR1 signaling warrants further investigation.

From a therapeutic point of view, we tested the potential anti-tumor effects by interference of the feedback loop between NTS/NTSR1 signaling and Wnt/β-catenin pathway. We demonstrated that activation of the NTS/NTSR1 signaling or Wnt/β-catenin pathway promoted tumor growth in glioblastoma, and pharmacological inhibition against NTSR1 signaling can largely suppress this effect *in vitro* and *in vivo*. The selective inhibitor of NTSR1, SR48692, has been demonstrated to show potent anti-tumor efficiency in the treatment of advanced colorectal cancer and small cell lung cancer. Meanwhile, SR48692 has been shown to be effective in inhibiting the prolific signaling in various cancers, such as colon cancer [19], pancreatic cancer [20, 21], and prostate cancer cells [22, 23]. Because NTSR1 is overexpressed in glioma and predicts a poor prognosis [24], targeted therapy against NTS/NTSR1 signaling may be a promising therapeutic strategy for treatment of glioblastoma. Besides, many inhibitors for Wnt/β-catenin pathway, such as PRI-724 and CWP232291, have been clinically well characterized and are also available for potential therapeutic use [25, 26].

In conclusion, our study reveals the positive feedback between NTS/NTSR1 signaling and Wnt/β-catenin pathway, which is critical for the tumor growth of glioblastoma. Therefore, we propose that targeting components of the feedback loop may represent candidate targets for therapeutic intervention of glioblastoma.

Acknowledgements

None.

Disclosure Statement

The authors confirm that there are no conflicts of interest.

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