Knockdown of Slit2 promotes growth and motility in gastric cancer cells via activation of AKT/β-catenin

RONGLIANG SHI[1*], ZHEN YANG[1*], WEIYAN LIU[1], BINGYA LIU[2], ZIPING XU[1] and ZIPING ZHANG[1]

[1]Department of General Surgery, Central Hospital of Shanghai Minhang District, Shanghai 201100;
[2]Department of General Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital,
Shanghai Jiao Tong University, School of Medicine, Shanghai 200025, P.R. China

Received September 8, 2013; Accepted October 25, 2013

DOI: 10.3892/or.2013.2887

Abstract. We previously showed that Slit2 was highly expressed in gastric cancer tissues that exhibit less advanced clinicopathological features, suggesting a tumor suppressor role for Slit2. In the present study, we investigated the effects of Slit2 knockdown on gastric cancer cells. Slit2-specific shRNAs were used to generate Slit2-knockdown SGC-7901 gastric cancer cells. Cell proliferation assay, Annexin V/PI double staining and cell cycle analysis were used to investigate the role of Slit2 knockdown in cell growth. Wound-healing and *in vitro* migration/invasion assays were performed. Subcutaneous tumor formation and peritoneal spreading in nude mice were employed to examine the *in vivo* effects of Slit2 knockdown. Cell signaling changes induced by Slit2 knockdown were analyzed by immunoblotting. Slit2 knockdown increased gastric cancer cell growth in monolayer and soft agar/Matrigel 3D culture. Slit2 knockdown inhibited apoptosis but did not alter cell cycle progression. Slit2-knockdown cells formed larger tumors and produced more peritoneal metastatic nodules in nude mice. Slit2 knockdown increased AKT phosphorylation, activated anti-apoptotic signaling, suppressed GSK3β activity and induced β-catenin activation. Blocking the effects of PI3K/AKT using pharmacological inhibitors abolished the ability of Slit2 knockdown to induce apoptosis resistance and cell migration/invasion. These results indicate that Slit2 knockdown promotes gastric cancer growth and metastasis through activation of the AKT/β-catenin-mediated signaling pathway.

Introduction

Gastric cancer is the fourth most common cancer worldwide and is the second most common cause of cancer-related mortality. Gastric cancer has a poor prognosis (1) due to its high rate of metastasis. Dysregulation of oncogenic signaling pathways such as Wnt/β-catenin, Hedgehog and Notch signaling pathways play important roles. Crosstalk among these different signaling pathways has been documented (2-4). Slits are secreted proteins that regulate axon guidance, branching and neuronal migration during development of the central nervous system (CNS) (5). The Slit proteins control the migration of neurons and neural crest cells by enhancing the interaction between novel Slit-regulated Rho GTPase-activating proteins (srGAPs) and the Robo receptor (6). Slit2 inhibits *in vitro* migration of various types of mammalian cells outside the CNS, such as leukocytes (7), dendritic cells (8) and vascular smooth muscle cells (9). Recently, Slit2 was found to contribute to gut development (10). Slit2 protein is located in the outer gut mesenchyme in regions that partially overlap with the secretion of netrin-1. Functionally, vagal sensory axons are responsive to Slit proteins and are, thus, repelled by Slits secreted in the gut wall and prevented from reaching inappropriate targets (10).

Slit2 is involved in many classic signaling pathways that are critical for development and tumorigenesis; hence, it plays an important role in the regulation of many cellular functions such as the cell cycle, apoptosis and invasion (11). The role of Slit2 in human cancers is controversial. The antitumor role of Slit2 is supported by its role in suppressing tumor growth when activated (12). The supportive evidence is that the loss of Slit2 expression has been noted in many cancer types. The SLIT2 gene is frequently inactivated by hypermethylation in its promoter region or allele loss in lung, breast, liver and colorectal cancers and malignant gliomas (13-15). Slit2 can also inhibit cell migration through targeting the AKT-GSK3β signaling pathway (16,17). On the contrary, it was reported that ectopic expression of Slit2 induced E-cadherin degradation and, thus, caused epithelial-mesenchymal transition (EMT) in colorectal cancer (18). Slit2 signaling activates Rho GTPase family proteins such as Rac1 and Cdc42 (19). However, whether Slit2 inhibits tumorigenesis of gastric cancer has not been demonstrated.

Whether Slit2 promotes or inhibits gastric cancer development and progression is not clear. Hence, in the present study, we examined the role of Slit2 in the growth and motility of gastric cancer cells. We used a Slit2-knockdown cell model

Correspondence to: Dr Ziping Zhang or Professor Weiyan Liu, Department of General Surgery, Central Hospital of Shanghai Minhang District, 170 Xinsong Road, Shanghai 201100, P.R. China
E-mail: roundshi@gmail.com
E-mail: wyliu13@163.com

[*]Contributed equally

Key words: Slit2, gastric cancer, apoptosis, metastasis, AKT, β-catenin

to investigate the *in vitro* and *in vivo* roles of Slit2 in gastric cancer and the related mechanisms.

Materials and methods

Cell culture. Human gastric cancer cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), penicillin and streptomycin (Gibco-BRL, Gaithersburg, MD, USA). Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay. Soft agar colony formation assay was performed by using 0.3% agar in complete medium with cells as the feeder layer and 0.6% agar in complete medium as the bottom layer. 3D Matrigel culture was performed using Matrigel matrix (BD Biosciences, San Jose, CA, USA). Oxaliplatin was obtained from Sigma.

Slit2 knockdown. For the knockdown of Slit2 expression, the Slit2 shRNA sequences were obtained as reported in a previous publication (20): Slit2 shRNA1#, 5'-CCGGCCTCACCTTA ATTCTTAGTTACTCGAGTAACTAAGAATTAAGGTGA GGTTTTTG-3' and Slit2 shRNA 2#, 5'-CCGGCCTGGA GCTTTCTCACCATATCTCGAGATATGGTGAGAAAG CTCCAGGTTTTTG-3'. The shRNAs were cloned into pGPU6/Neo (Shanghai GenePharma Co., Shanghai, China). Plasmids were transfected into gastric cancer cells using Lipofectamine 2000 (Invitrogen). G418 was used for stable clone selection.

Cell migration and invasion assays. Cell migration was analyzed by a Transwell chamber assay. Cell invasion assays were performed using BD BioCoat™ Matrigel™ Invasion chambers. FCS (10%) was used as the chemoattractant. Cells on the lower surface of the insert were fixed and stained followed by counting under a light microscope. Cells were visualized using an Olympus BX50 microscope (Olympus Opticol Co., Tokyo, Japan). Images were captured using Nikon Digital Sight DS-U2 (Nikon, Tokyo, Japan), and NIS-Elements F3.0 software was used (Nikon).

Wound-healing assays. Cells were cultured in 6-well plates and grown to confluency. The wound area was generated by scraping cells with a 200-μl pipette tip across the entire diameter of the well and extensively rinsed with the medium to remove all cellular debris. Low-serum DMEM with mitomycin (2 μg/ml) was then added to inhibit cell proliferation during the experiment, and the closing of the wound was observed at different time-points. The speed of closure reflected wound-healing ability. Cells were visualized using an Olympus IX71 microscope (Olympus Opticol Co.). Images were captured using Nikon Digital Sight DS-U1 and NIS-Elements F3.0 software was used.

Reverse transcription and quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the cultured cells using the RNeasy Mini kit (Qiagen), and cDNA was synthesized with oligo(dT) primers by using the SuperScript First-Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocols. Gene expression was assessed by qRT-PCR using an Applied Biosystems 7500 Fast Sequence Detection System (Life Technologies Corp., Carlsbad, CA, USA). The PCR reaction mixture consisted of QuantiTect SYBR-Green PCR Master Mix (2X QuantiTect SYBR-Green kit, contains Hot Start Taq® DNA polymerase, QuantiTect SYBR-Green PCR buffer, dNTP mix, SYGB I, Rox passive reference dye and 5 mM $MgCl_2$) (Qiagen), 0.5 μmol/l of each primer and cDNA. The transcription of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize expression data. The comparative Ct (threshold cycle) method was used to calculate the relative changes in gene expression.

The primers used for amplifying Slit2 were 5'-AACTGC CTTCGGGTAGATGC-3' (forward) and 5'-GAATGGCCCG AAGAGGTGAA-3' (reverse). The primers used for amplifying GAPDH were 5'-TTGGCATCGTTGAGGGTCT-3' (forward) and 5'-CAGTGGGAACACGGAAAGC-3' (reverse).

Western blotting. Whole cell lysates were harvested using RIPA cell lysis buffer supplemented with a protease inhibitor cocktail (Sigma). Antibodies against Slit2 (Abcam), Bcl-2, Bcl-XL, Bad, p-Histone H3-Ser6, GAPDH, c-Myc and cyclin D1 (Epitomics, Burlingame, CA, USA), GSK3β, p-GSK3β Ser9, β-catenin, p-β-catenin Ser675, pAKT, AKT, p-BAD Ser136, PARP, cleaved-PARP and Ncad (Cell Signaling Technology, Danvers, MA, USA) were used at a 1:1,000 dilution. The signals were detected using ECL chemiluminescent reagent (Pierce Chemical Co., Rockford, IL, USA).

In vivo tumorigenesis and metastasis. Male BALB/c nu/nu nude mice (Institute of Zoology, Chinese Academy of Sciences, Shanghai, China) were housed in a specific pathogen-free environment at the Animal Laboratory Unit, School of Medicine, Shanghai Jiao Tong University, China. The mice received humane care, and the study protocol complied with the institution's guideline and animal research laws. Cells ($1x10^6$) were subcutaneously injected into 4-week-old male BALB/c mice. Tumor length (L) and width (W) were measured, and the tumor volume was calculated using the equation: Volume = (W^2 x L)/2 (21). Mice were sacrificed 28 days after injection under anesthesia. Eight mice were used in each group.

In order to produce peritoneal spreading experimental metastasis, $2x10^6$ cells were injected into 5-week-old male BALB/c nude mice intraperitoneally. After 6 weeks, the mice were sacrificed under anesthesia. Ten mice were used in each group. The macrometastatic nodules were visualized under a microscope.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Values are represented as means ± standard deviation (SD) of samples measured in triplicate. Each experiment was repeated three times, unless otherwise indicated. The significance of differences between experimental groups was analyzed using the Student's t-test and two-tailed distribution.

Results

Knockdown of Slit2 promotes gastric cancer cell growth in vitro. To investigate the role of Slit2 knockdown in gastric cancer cells, we generated the Slit2-knockdown

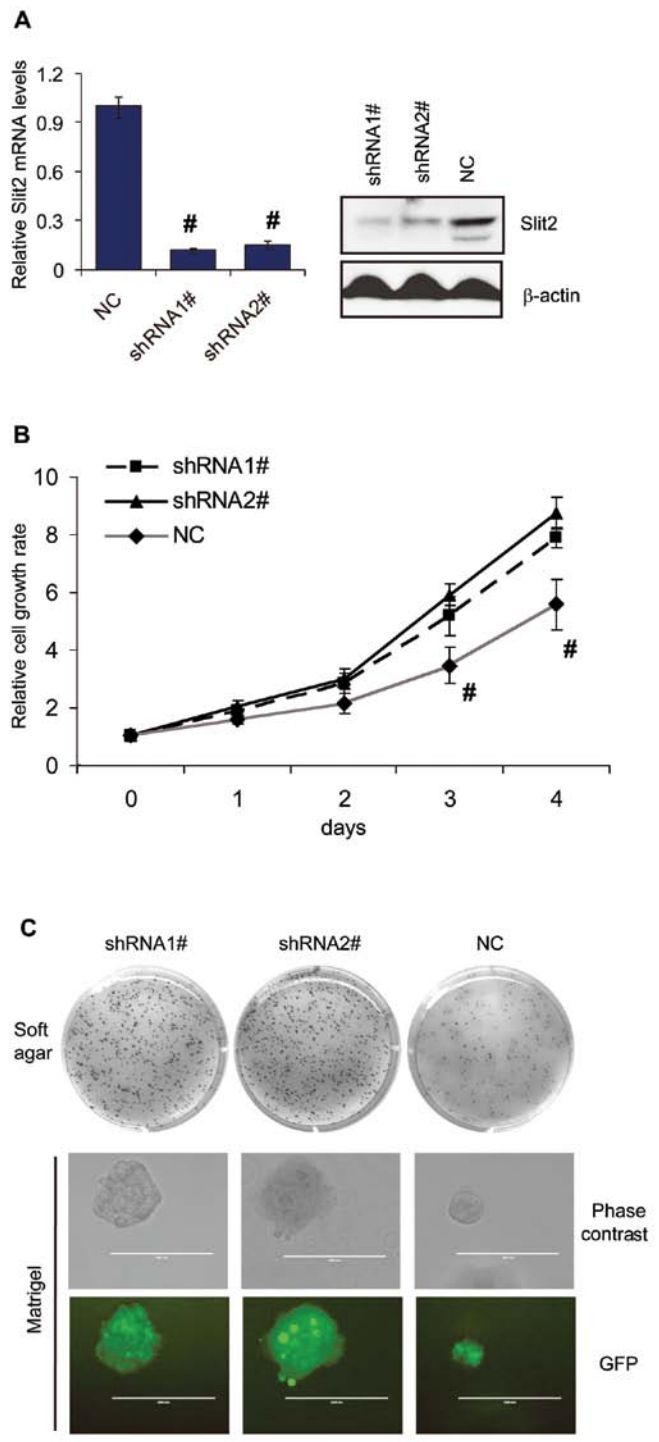

Figure 1. Knockdown of Slit2 promotes gastric cancer growth. (A) The efficacy of Slit2 knockdown was examined by qRT-PCR (left panel) and immunoblotting (right panel). (B) Cell proliferation assays were performed to show the effect of Slit2 knockdown on cell growth in a monolayer culture. #P<0.05. (C) The effects of Slit2 knockdown on cell growth in soft agar (upper panel) and Matrigel (lower panel) 3D culture.

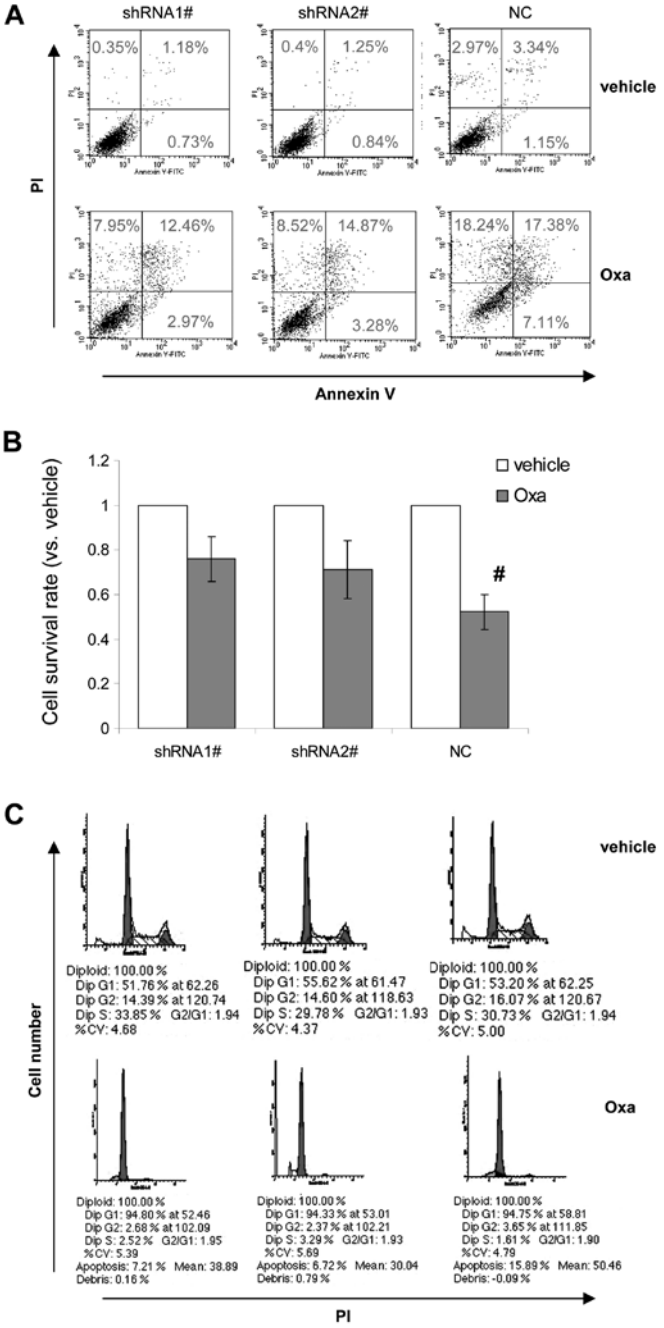

Figure 2. Knockdown of Slit2 promotes gastric cancer survival. (A) Effects of Slit2 knockdown on apoptosis under a normal condition (upper panel) and following oxaliplatin stimulus (lower panel). Cells were treated with oxaliplatin for 24 h and then labeled with Annexin V/PI followed by FACS analysis. (B) Cell proliferation of Slit2-knockdown and vector-control cells following oxaliplatin exposure. MTT assays were performed 24 h after oxaliplatin treatment. #P<0.05. (C) Effects of Slit2 knockdown on cell cycle progression in normal culture (upper panel) following oxaliplatin exposure (lower panel). Cells were treated with oxaliplatin for 24 h and then labeled with PI for cell cycle analysis.

(shRNA1# and shRNA2#) gastric cell model via transfection of Slit2-shRNAs and control-shRNA (NC) into SGC-7901 gastric cells (Fig. 1A). We first examined the effects of Slit2 knockdown on cell growth. Slit2 knockdown significantly increased SGC-7901 cell growth in monolayer (Fig. 1B) and 3D culture. Slit2-knockdown cells formed more and larger spheres in soft agar (Fig. 1C, upper panel) and Matrigel (Fig. 1C, lower panel) than the control-shRNA transfected cells. Transfection of Slit2-shRNAs into another gastric cancer cell line MKN45 showed similar effects (data not shown). These data suggest that Slit2 knockdown promotes gastric cancer cell growth.

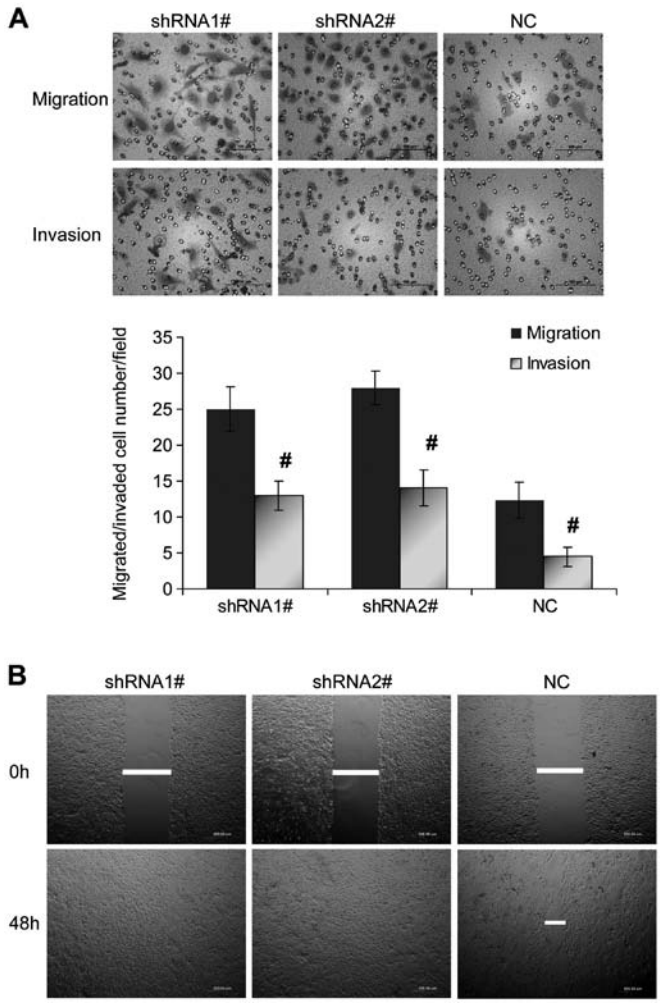

Figure 3. Effect of Slit2 knockdown on the migration and invasion of gastric cancer cells. (A) Slit2 knockdown promoted the migration and invasion of SGC-7901 cells. The plot shows the migrated/invaded cell numbers. #P<0.05. (B) Slit2 knockdown promoted the wound healing ability of SGC-7901 cells. White bars show the distance between the two wound edges.

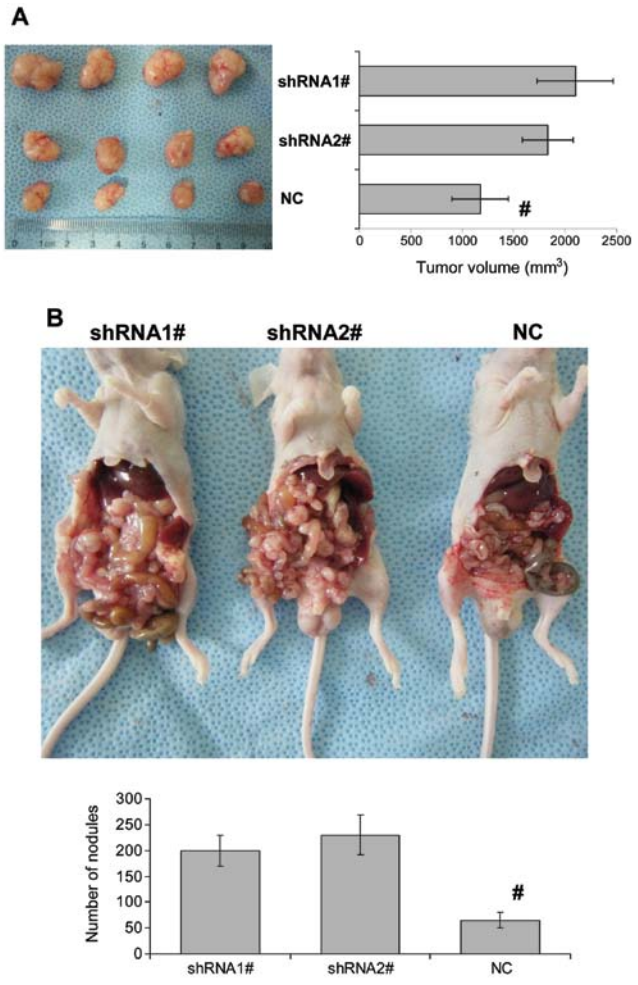

Figure 4. Effect of Slit2 knockdown on the *in vivo* growth and metastasis of gastric cancer cells. (A) Slit2 knockdown promoted the subcutaneous growth of SGC-7901 cells. The plot shows the tumor volume on the day of sacrifice. (B) Slit2 knockdown promoted the growth of metastatic nodules. The plot shows the number of metastatic nodules in the different groups. #P<0.05.

Knockdown of Slit2 inhibits gastric cancer cell apoptosis in vitro. We then determined whether Slit2 has an effect on protecting gastric cancer cells from apoptosis. We first examined the percenage of apoptosis in a normal culture. Slit2 knockdown decreased apoptotic cells when compared with the vector-control (Fig. 2A, upper panel). Next, we examined the effect of Slit2 knockdown on protecting cells from apoptotic stimuli. We treated cells with oxaliplatin, a chemotherapeutic drug which induces apoptosis and is commonly used in gastric cancer treatment. Cells were treated with oxaliplatin at 20 μM for 48 h, and analysis of apoptosis was performed using Annexin V/PI double staining. The percentage of apoptotic cells in the Slit2-knockdown groups was lower than that in the vector-control group (Fig. 2A, lower panel). Cell viability assessed by CCK-8 revealed that the percentage of surviving cells in the Slit2-knockdown cells treated with oxaliplatin was higher than that in the vector-control cells (Fig. 2B). Cell cycle distribution showed no difference between Slit2-knockdown and vector-control cells with (lower panel) or without (upper panel) oxaliplatin treatment (Fig. 2C). These results suggest that Slit2 knockdown promotes gastric cancer cell growth through inhibition of apoptosis.

Knockdown of Slit2 promotes gastric cancer cell metastasis in vitro. We next determined whether Slit2 knockdown regulates cell migration and invasion. Towards this aim, Boyden chambers coated without and with Matrigel were used for migration and invasion assays, respectively. Slit2 knockdown significantly increased the number of migrated and invaded cells (Fig. 3A). Wound healing assays were conducted to examine the speed of wound closure. Slit2-knockdown cells showed full closure of the wound at 48 h after scraping, while for the vector-control cells a visible distance between wound edges was still visible (Fig. 3B). These data suggest that Slit2 knockdown promotes gastric cancer cell migration and invasion.

Knockdown of Slit2 promotes gastric tumorigenesis and metastasis in vivo. We then used *in vivo* mouse models to further validate the *in vitro* observations. Slit2 knockdown or vector-control SGC-7901 cells were subcutaneously injected into 4-week-old nude mice. Mice injected with Slit2-knockdown

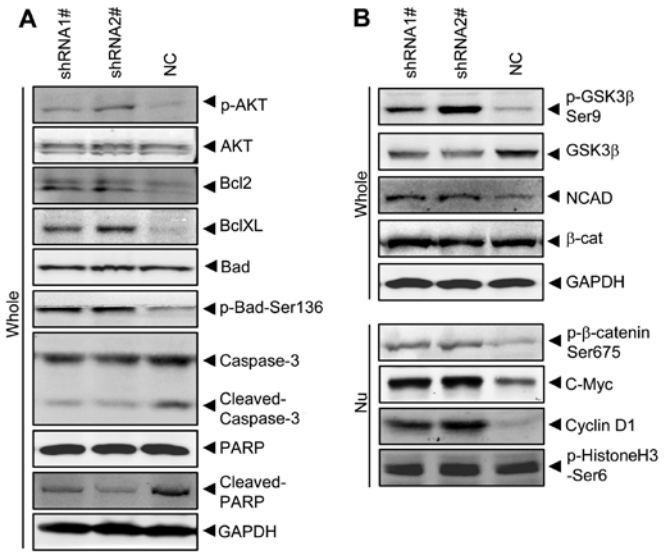

Figure 5. Knockdown of Slit2 activates AKT/β-catenin. (A) Immunoblot analysis of AKT activity and apoptosis-related proteins. (B) Immunoblot analysis of GSK3β and β-catenin activity. GAPDH and p-Histone H3-Ser6 were used as loading control.

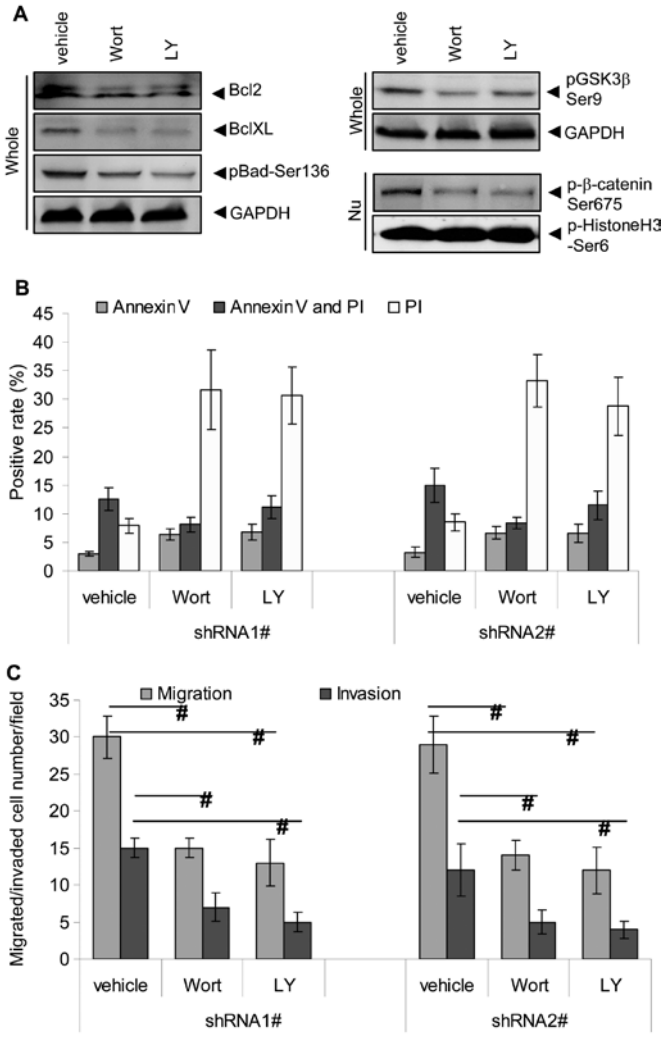

Figure 6. AKT inhibition attenuates the effect of Slit2 knockdown. (A) Wortmannin (Wort) and LY294002 (LY) attenuated the alterations in apoptosis-related protein and GSK3β/β-catenin activity. GAPDH and p-Histone H3-Ser6 were used as loading controls. (B) The effects of Wortmannin and LY294002 on oxaliplatin-induced apoptosis in Slit2-knockdown cells. Cells were treated with oxaliplatin for 24 h and then labeled with Annexin V/PI for analysis of apoptosis. (C) Effects of Wortmannin and LY294002 on migration and invasion in Slit2-knockdown cells. The plot shows the migrated/invaded cell numbers. #P<0.05.

cells formed larger tumors than those injected with vector-control cells after 28 days (Fig. 4A). As peritoneal spreading and metastasis are common in gastric cancer and are pivotal factors for its poor prognosis, we used a nude mouse model to investigate the influence of Slit2 knockdown on peritoneal metastasis. Consistent with the *in vitro* observations, we found that Slit2-knockdown cells formed more metastatic nodules than the number in the vector-control cells (Fig. 4B). The average number of metastatic nodules in mice injected with Slit2-knockdown cells was higher than that in mice injected with vector-control cells (Fig. 4B, plot). These results indicate that Slit2 knockdown promotes growth and invasion of gastric cancer cells in mice.

Knockdown of Slit2 activates AKT-mediated survival signaling. As Slit2 depletion was previously reported to activate AKT signaling (16), we examined whether the Slit2 knockdown-induced anti-apoptosis effect was through AKT activation as well. To better examine the apoptosis-related protein alterations, we stimulated cells with oxaliplatin for 24 h and collected protein for immunoblot analysis. Consistent with a previous finding, we found that AKT activity (shown as p-AKT) was increased in Slit2-knockdown cells (Fig. 5A). Since AKT induced a strong anti-apoptotic signal, we focused on the molecules of AKT-induced survival signaling. Activated AKT consecutively phosphorylates pro-apoptotic Bad protein to inhibit mitochondrial death cascade thereby promoting cell survival (22). Consistently, we observed high levels of p-Bad Ser136 (phosphorylated by AKT) in Slit2-knockdown cells. The expression of anti-apoptotic Bcl-2 and Bcl-XL was elevated in Slit2-knockdown cells (Fig. 5A). On the other hand, cleaved-caspase 3 and cleaved-PARP, two key molecules mediating apoptosis, were decreased in Slit2-knockdown cells, suggesting that the anti-apoptotic signal was significantly augmented in Slit2-knockdown cells.

Knockdown of Slit2 activates β-catenin signaling through AKT. Our previous study revealed a correlation between loss of Slit2 expression and nuclear β-catenin in gastric cancer tissues (23). AKT, on the other hand, is one of the major kinases that phosphorylated and inactivated GSK3β and subsequently released and activated β-catenin signaling. We speculated that β-catenin was activated in Slit2-knockdown cells by highly activated AKT. As expected, we found low GSK3β activity (shown as higher levels of its inactivated form p-GSK3β Ser9) and the highly activated β-catenin form in the nucleus (shown as higher activated form p-β-catenin Ser675) in Slit2-knockdown cells (Fig. 5B). We also revealed that c-Myc and cyclin D1, two downstream oncogenic targets of β-catenin signaling, were markedly increased in Slit2-knockdown cells (Fig. 5B). The level of N-cadherin (NCAD), a marker of EMT, was also found to be increased in Slit2-knockdown cells

(Fig. 5B). Together, these results suggest that Slit2 knockdown activates nuclear β-catenin signaling.

AKT inhibition abolishes the effects of Slit2 knockdown. To further validate the role of AKT in mediating the cellular functions and signaling alterations in Slit2-knockdown cells, PI3K/AKT inhibitors Wortmannin and LY294002 were used. As expected, the inhibition of AKT activity caused decreased Bcl-2, Bcl-XL, pBad Ser136, p-GSK3β Ser9 and p-β-catenin Ser675 levels (Fig. 6A). Application of Wortmannin (0.5 μM) or LY294002 (10 μM) (24) induced increased apoptosis in Slit2-knockdown cells treated with oxaliplatin (Fig. 6B). Cell migratory and invasive abilities were also inhibited by either Wortmannin or LY294002 (Fig. 6C). Similar to chemical inhibitors, AKT siRNAs abolished the anti-apoptotic and motility-inducing effects of Slit2 knockdown (data not shown). These data suggest that Slit2 knockdown activates AKT-mediated survival and β-catenin signaling.

Discussion

Slit2 is a large secreted protein with multi-functions during neural development and elongation. The roles of Slit2 in cancer have been recently studied. However, different studies have shown both a tumor-suppressor and a tumor-promoter role of Slit2. We found that knockdown of Slit2 in gastric cancer cells promotes cell growth and metastasis *in vitro* and *in vivo*. Knockdown of Slit2 activates AKT, enhances anti-apoptotic ability and activates β-catenin oncogenic signaling. Our findings suggest a tumor-suppressor role of Slit2 in gastric cancer.

Slit2 is considered to function as a tumor suppressor in certain types of human cancers. In breast cancer (25) and medulloblastoma (26) cells, Slit2 inhibits migration and invasion *in vitro*. The opposite findings have also been reported which suggest a tumor promoter role of Slit2 in other cancer types. Slit2 expression is increased in high-grade prostatic intraepithelial neoplasia, primary prostatic adenocarcinoma and metastatic prostatic adenocarcinoma but not expressed in normal prostate and benign prostatic hyperplasia (27). It has been reported that Slit2 expression is significantly associated with an increased metastatic risk and poorer overall survival in colorectal carcinoma patients (18). We previously discovered a negative correlation between Slit2 levels and malignant clinicopathological features in gastric cancer tissues which support a tumor-suppressor role of Slit2 in gastric cancer (23). In the present study, we further discovered the role of Slit2 in gastric cancer cells using a Slit2-knockdown model. Consistently, we showed that Slit2 knockdown promoted cell growth by inhibiting apoptosis, suggesting a tumor-suppressor role of Slit2 in gastric cancer.

Slit2 has been reported to inhibit the AKT signaling pathway (16,17). AKT plays a critical role in cell survival by providing protection from apoptosis (28). We revealed a strong anti-apoptotic effect of Slit2 knockdown with intact cell cycle progression, suggesting that cell cycle alteration was not involved in the regulation of Slit2 knockdown on growth in gastric cancer cells. Furthermore, we discovered high levels of anti-apoptotic and low levels of pro-apoptotic molecules in Slit2-knockdown cells. The fact that inhibition of AKT attenuated the expression of anti-apoptosis-related proteins further confirmed their correlation to AKT activity in Slit2-knockdown cells. Notably, the inactivation of GSK3β through AKT-mediated phosphorylation may also lead to downregulation of its pro-apoptotic activity and inhibition of the induction of cell death (29). Our findings suggest that loss of Slit2 expression facilitates gastric cancer cell growth and survival through AKT activation.

Slit2 was found to promote gastric cancer migration and invasion through its interaction with Robo1 in gastric cancer cells (30). The opposite findings were observed in glioma cells which indicated that Slit2 attenuated Cdc42 activation while N-cadherin and β-catenin remained intact (31,32). However, our previous study discovered that a high Slit2 level was related to cell membrane β-catenin and a low Slit2 level was related to nuclear β-catenin (23). As discovered, AKT phosphorylates GSK3β at Ser 9 to downregulate GSK3β activity (29). Hence, the inactivation of GSK3β can further induce the activation of β-catenin signaling. In the present study, we first showed that the activated nuclear β-catenin signaling was correlated with pAKT levels and secondly we showed that the inhibition of AKT activity attenuated nuclear β-catenin activity. Hence, we verified our previous findings and discovered a possible mechanism of AKT-mediated activation of β-catenin in Slit2-knockdown gastric cancer cells. Different from another report which showed no activation of β-catenin or N-cadherin mediated signaling (32), we showed a pronounced activation of β-catenin and increased N-cadherin expression following Slit2 knockdown. This discrepancy may be due to the different cell lines used in the studies.

In summary, Slit2 knockdown increased cell growth, decreased apoptosis and promoted cell migration and invasion. We further showed that Slit2 knockdown activated AKT and consequent β-catenin signaling. The present study suggests that Slit2 may function as a tumor suppressor in gastric cancer.

Acknowledgements

The present study was supported by a major program of the Minhang District Research Fund and by Shanghai City Health Bureau (Project No. 20134406).

References

1. Brenner H, Rothenbacher D and Arndt V: Epidemiology of stomach cancer. Methods Mol Biol 472: 467-477, 2009.
2. Katoh Y and Katoh M: Integrative genomic analyses on GLI1: positive regulation of GLI1 by Hedgehog-GLI, TGFβ-Smads, and RTK-PI3K-AKT signals, and negative regulation of GLI1 by Notch-CSL-HES/HEY, and GPCR-Gs-PKA signals. Int J Oncol 35: 187-192, 2009.
3. Fuxe J, Vincent T and Garcia de Herreros A: Transcriptional crosstalk between TGFβ and stem cell pathways in tumor cell invasion: role of EMT promoting Smad complexes. Cell Cycle 9: 2363-2374, 2010.
4. Wu WK, Cho CH, Lee CW, *et al*: Dysregulation of cellular signaling in gastric cancer. Cancer Lett 295: 144-153, 2010.
5. Yuan W, Zhou L, Chen JH, Wu JY, Rao Y and Ornitz DM: The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. Dev Biol 212: 290-306, 1999.
6. Wu W, Wong K, Chen J, *et al*: Directional guidance of neuronal migration in the olfactory system by the protein Slit. Nature 400: 331-336, 1999.
7. Wu JY, Feng L, Park HT, *et al*: The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors. Nature 410: 948-952, 2001.

8. Guan H, Zu G, Xie Y, *et al*: Neuronal repellent Slit2 inhibits dendritic cell migration and the development of immune responses. J Immunol 171: 6519-6526, 2003.
9. Liu D, Hou J, Hu X, *et al*: Neuronal chemorepellent Slit2 inhibits vascular smooth muscle cell migration by suppressing small GTPase Rac1 activation. Circ Res 98: 480-489, 2006.
10. Goldberg D, Borojevic R, Anderson M, Chen JJ, Gershon MD and Ratcliffe EM: Slit/Robo-mediated chemorepulsion of vagal sensory axons in the fetal gut. Dev Dyn 242: 9-15, 2013.
11. Dickinson RE and Duncan WC: The SLIT-ROBO pathway: a regulator of cell function with implications for the reproductive system. Reproduction 139: 697-704, 2010.
12. Qiu H, Zhu J, Yu J, Pu H and Dong R: SLIT2 is epigenetically silenced in ovarian cancers and suppresses growth when activated. Asian Pac J Cancer Prev 12: 791-795, 2011.
13. Dallol A, Da Silva NF, Viacava P, *et al*: SLIT2, a human homologue of the *Drosophila* Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. Cancer Res 62: 5874-5880, 2002.
14. Dallol A, Krex D, Hesson L, Eng C, Maher ER and Latif F: Frequent epigenetic inactivation of the SLIT2 gene in gliomas. Oncogene 22: 4611-4616, 2003.
15. Jin J, You H, Yu B, *et al*: Epigenetic inactivation of SLIT2 in human hepatocellular carcinomas. Biochem Biophys Res Commun 379: 86-91, 2009.
16. Chen WF, Gao WD, Li QL, Zhou PH, Xu MD and Yao LQ: SLIT2 inhibits cell migration in colorectal cancer through the AKT-GSK3β signaling pathway. Int J Colorectal Dis 28: 933-940, 2013.
17. Chang PH, Hwang-Verslues WW, Chang YC, *et al*: Activation of Robo1 signaling of breast cancer cells by Slit2 from stromal fibroblast restrains tumorigenesis via blocking PI3K/Akt/β-catenin pathway. Cancer Res 72: 4652-4661, 2012.
18. Zhou WJ, Geng ZH, Chi S, *et al*: Slit-Robo signaling induces malignant transformation through Hakai-mediated E-cadherin degradation during colorectal epithelial cell carcinogenesis. Cell Res 21: 609-626, 2011.
19. Wong K, Ren XR, Huang YZ, *et al*: Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. Cell 107: 209-221, 2001.
20. Huang P, Kishida S, Cao D, *et al*: The neuronal differentiation factor NeuroD1 downregulates the neuronal repellent factor Slit2 expression and promotes cell motility and tumor formation of neuroblastoma. Cancer Res 71: 2938-2948, 2011.
21. Safina A, Vandette E and Bakin AV: ALK5 promotes tumor angiogenesis by upregulating matrix metalloproteinase-9 in tumor cells. Oncogene 26: 2407-2422, 2007.
22. Kuo CT, Hsu MJ, Chen BC, *et al*: Denbinobin induces apoptosis in human lung adenocarcinoma cells via Akt inactivation, Bad activation, and mitochondrial dysfunction. Toxicol Lett 177: 48-58, 2008.
23. Shi R, Liu W, Liu B, Xu Z, Chen L and Zhang Z: Slit2 expression and its correlation with subcellular localization of β-catenin in gastric cancer. Oncol Rep 30: 1883-1889, 2013.
24. Pan G, Zhu Z, Huang J, *et al*: Semaphorin 5A promotes gastric cancer invasion/metastasis via urokinase-type plasminogen activator/phosphoinositide 3-kinase/protein kinase B. Dig Dis Sci 58: 2197-2204, 2013.
25. Alvarez C, Tapia T, Cornejo V, *et al*: Silencing of tumor suppressor genes RASSF1A, SLIT2, and WIF1 by promoter hypermethylation in hereditary breast cancer. Mol Carcinog 52: 475-487, 2012.
26. Werbowetski-Ogilvie TE, Seyed Sadr M, Jabado N, *et al*: Inhibition of medulloblastoma cell invasion by Slit. Oncogene 25: 5103-5112, 2006.
27. Bartholow TL, Becich MJ, Chandran UR and Parwani AV: Immunohistochemical staining of slit2 in primary and metastatic prostatic adenocarcinoma. Transl Oncol 4: 314-320, 2011.
28. Nogueira V, Park Y, Chen CC, *et al*: Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. Cancer Cell 14: 458-470, 2008.
29. Seo SB, Hur JG, Kim MJ, *et al*: TRAIL sensitize MDR cells to MDR-related drugs by down-regulation of P-glycoprotein through inhibition of DNA-PKcs/Akt/GSK-3β pathway and activation of caspases. Mol Cancer 9: 199, 2010.
30. Tie J, Pan Y, Zhao L, *et al*: MiR-218 inhibits invasion and metastasis of gastric cancer by targeting the Robo1 receptor. PLoS Genet 6: e1000879, 2010.
31. Xu Y, Li WL, Fu L, Gu F and Ma YJ: Slit2/Robo1 signaling in glioma migration and invasion. Neurosci Bull 26: 474-478, 2010.
32. Yiin JJ, Hu B, Jarzynka MJ, *et al*: Slit2 inhibits glioma cell invasion in the brain by suppression of Cdc42 activity. Neuro Oncol 11: 779-789, 2009.