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Differential effect of NF-κB activity on β-catenin/Tcf pathway in various cancer cells

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Abstract β-Catenin/Tcf and NF-κB pathways play an important role in biological functions. We determined the underlying mechanisms of differential interaction between two pathways in various human cancer cell lines. NF-κB positively regulated β-catenin/Tcf pathways in human glioblastoma, whereas it has an opposite effect on β-catenin/Tcf pathways in colon, liver, and breast cancer cells. Expression of lucine zipper tumor suppressor 2 (lzts2) was positively regulated by NF-κB activity in colon, liver, and breast cancer cells, whereas negatively regulated in glioma cells. Downregulation of lzts2 increased the β-catenin/Tcf promoter activity and inhibited NF-κB-induced modulation of the nuclear translocation of β-catenin. These data indicate that the differential crosstalk between β-catenin/Tcf and NF-κB pathway in various cancer cells is resulted from the differences in the regulation of NF-κB-induced lzts2 expression.
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1. Introduction

The Wnt family of secretory glycoproteins plays an important role in embryonic development, the induction of cell polarity, and in the determination of cell fate. Deregulation of Wnt signaling disrupts axis formation in embryos [1–3] and is associated with multiple human malignancies [4]. Wnt signaling also plays an important role in the proliferation and differentiation of stem cells including human mesenchymal stem cells (hMSCs) [5,6]. The NF-κB signaling pathway is another important signal transduction pathway that plays a critical role in activating the expression of genes involved in the immune and inflammatory response and in regulating cellular apoptosis [7–10]. NF-κB is an important mediator for the production of cytokine and chemokines in hMSCs [11].

Deng and colleagues [12] first suggested that β-catenin interacted with and inhibited NF-κB in human colon and breast cancer cells. They found that β-catenin could physically complex with NF-κB, resulting in a reduction of NF-κB DNA binding, transactivation activity, and target gene expression [12]. Subsequently, several studies have demonstrated that the activation of the β-catenin/Tcf pathway negatively regulates the NF-κB pathway in colon and breast cancer cells [12,13] and in response to bacterial stimulation in intestinal epithelial cells [14]. On the other hand, stimulation of the Wnt cascade through the upregulation of either Wnt or degradation-resistant β-catenin significantly enhances both baseline and TNF-α-induced NF-κB activity, which is mediated through the E3 ligase TrCP1, in vascular smooth muscles [15].

A recent study showed that lzts2 (lucine zipper tumor suppressor 2), a putative tumor suppressor [16], interacts with β-catenin, represses the transactivation of β-catenin, and affects the subcellular localization of β-catenin. We found that the activity of NF-κB regulated the expression of lzts2 in human adipose tissue-derived mesenchymal stem cells [17]. These findings highlight the complex interactions between the β-catenin/Tcf and NF-κB signaling pathways and further emphasize the importance of characterizing their interactive role in biological functions. In this study, we determined crosstalk between β-catenin/Tcf and NF-κB pathways in various human cancer cells.

2. Materials and methods

2.1. Cell culture

All cancer cell lines (human glioma cell line, U87MG and GBM-05; human colon cancer cell line, COLO201 and KM12C; and breast cancer cell line, SKBR3; human hepatocellular liver carcinoma cell line, HepG2 and Hep3B) were grown in the specified media (Dulbecco's modified Eagle's media containing 10% fetal bovine serum).

2.2. Reporter gene assay

All transient transfections were performed with Lipofectamine Plus Reagent (Invitrogen). The transient transfections were performed using pTOP-flash, pFOP-flash, pNF-κB-Luc, pCMV-β-Gal plasmid (Clontech Laboratories Inc., CA) and the p65 plasmid (pCMVp65 NF-κB). Reporter gene assay done as described [18].

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2.3. Nuclear and cytosolic fractionation and Western blot analysis
The cells were separated into cytoplasmic and nuclear fractions as described [19]. Monoclonal antibodies (β-catenin and β-actin; BD Biosciences Clontech, CA, NF-κBp65, Santa Cruz Biotechnology Inc., CA) were used for immunoblotting.

2.4. Decoy oligodeoxynucleotide (ODN) technique
The NF-κB decoy technique was performed as described previously [20].

2.5. Small interfering RNA (siRNA) transfection
siRNA duplex oligo (on-TARGET plus SMART pool, Dharmacon Inc., CO) targeting lzts2 (leucine zipper, putative tumor suppressor 2) mRNA or non-targeting duplex oligo (on-TARGET plus siCONTROL, Dharmacon Inc., CO) as a negative control were transfected using DharmaFECT Transfection Reagent.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis
The total cellular RNA was isolated from the cells and reverse transcribed using the conventional protocols. The cDNAs were amplified by PCR with 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. PCR amplification was performed using the following primer sets: GAPDH 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′, lzts2, 5′-CTGTGTCCTGGAAGGAAAGC-3′, 5′-CTCCCACTTGGTCTCCTCAA-3′. The designed primers were used to amplify the PCR, and GAPDH was used as a control.

2.7. RT-PCR
The primer sequences used in the experiment were as follows: β-actin, 5′-CTGGTGCCT GGG GCG-3′, 5′-AGCCTCGCCTTTGCCGA-3′; lzts2, 5′-AGAAGCGGCAATTGCAGGAC-3′, 5′-CTCGCCTGATTTCTGGCACA-3′. Real-time quantitation done as described [21].

2.8. Statistical analysis
Comparisons between two groups were analyzed via a Student's *t*-test. ($P < 0.05$), and comparisons between three groups were analyzed by ANOVA with a Student–Newman–Keuls post hoc test ($P < 0.05$). Data are presented as means ± S.E.

3. Results

We determined the effect of inhibition of NF-κB signaling by treatment with SN50, an inhibitor of NF-κB nuclear translocation on β-catenin/Tcf signaling. In this experiment, we used two colon cancer cells (COLO-201 and KM12C), two glioma cells (U87MG and GBM-05 [22]), two hepatoma cells (HepG2 and Hep3B), and one breast cancer cells (SKBR3). The treatment with SN50 (50 or 100 μg/ml) inhibited the luciferase activity of the NF-κB promoter-luciferase construct in all of cells to be tested in the experiment (Fig. 1A). The treatment of SN50 inhibited the luciferase activity of the Top-Flash construct in glioma cell lines, U87MG and GBM-05, without affecting that of the Fop-Flash construct (Fig. 1B). In contrast, the treatment of SN50 (50 or 100 μg/ml) increased Tcf promoter activity in the COLO-201, KM12C, SKBR3, HepG2, and Hep3B cell lines (Fig. 1B).

To confirm the differential crosstalk between the NF-κB and β-catenin/Tcf signaling pathways in cancer cell lines, we trea-

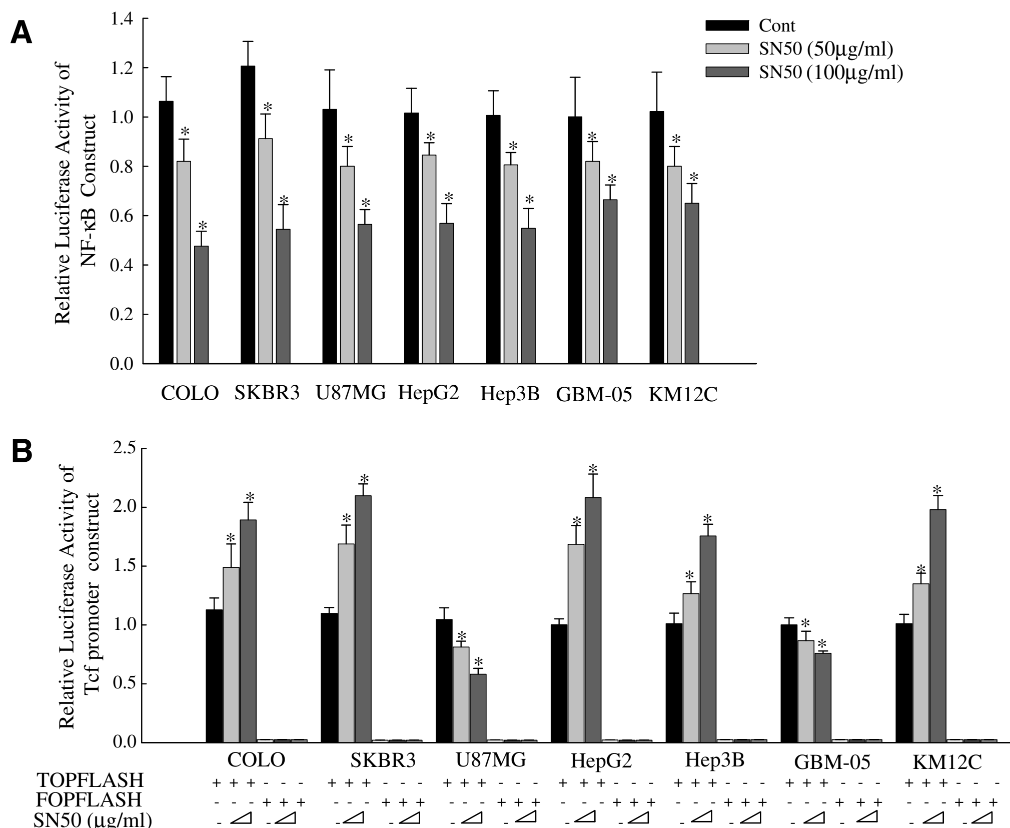

Fig. 1. Effect of NF-κB inhibitors on NF-κB and β-catenin/Tcf signaling pathways in cancer cells. (A) Cells were cotransfected with NF-κB reporter constructs and β-Gal vector. (B) Cells were cotransfected with either TOPFLASH or FOPFLASH reporter constructs and β-Gal vector. Transfected cells were treated without or with SN50 (50 and 100 μg/ml) for 48 h. Luciferase activity was normalized by β-galactosidase activity. Data represent means ± S.E.M. of four different experiments. $^{*}P < 0.05$ compared with data in the absence of SN50.

ted COLO-201, SKBR3, U87MG, HepG2, Hep3B, KM12C, and GBM-05 cancer cells with mutant and NF-κB decoy. The treatment with NF-κB decoy decreased the luciferase activity of NF-κB in comparison to treatment with the mutant decoy in all cancer cell lines (Fig. 2A). In Tcf promoter activity, treatment with NF-κB decoy increased activity of Tcf promoter in colon, hepatic, and breast cancer cells in contrast with glioma cells in which the treatment of NF-κB decoy decreased Tcf promoter activity (Fig. 2B). The overexpression of p65 by plasmid transfection increased the luciferase activity of both the Tcf and NF-κB promoters in U87MG and GBM-05, but decreased Tcf promoter activity in COLO-201, KM12C, HepG2, Hep3B, and SKBR cells (Fig. 2A and B).

To understand the mechanisms underlying the crosstalk between the NF-κB and β-catenin/Tcf signaling pathways in cancer cells, the β-catenin levels in the total, nuclear, and cytosolic fractions were determined by Western blot analysis. The SN50 treatment inhibited the nuclear translocation of p65 in COLO-201, SKBR3, and U87MG cells. Although SN50 (100 μg/ml) treatment did not affect the total β-catenin levels, it increased the β-catenin levels in the cytosolic fractions and decreased the β-catenin levels in the nuclear fractions of U87MG, indicating that SN50 inhibited nuclear translocation of β-catenin as well as NF-κB (Fig. 2C). In contrast, SN50 increased nuclear translocation of β-catenin in COLO-201 and SKBR3.

A recent study showed that lzts2 is involved in the nuclear translocation of β-catenin [16]. Therefore, we determined whether differential regulation of lzts2 expression by NF-κB according to cell types is responsible for opposite responses of β-catenin/Tcf pathway by NF-κB activity. RT-PCR and real-time PCR analysis showed that SN50 (100 μg/ml) and NF-κB decoy increased lzts2 expression in U87MG and decreased it in COLO-201, SKBR3, HepG2, Hep3B, and KM12C cells. In contrast, p65 overexpression by plasmid transfection decreased lzts2 expression in U87MG and increased it in COLO-201, SKBR3, HepG2, Hep3B, and KM12C cells (Fig. 3A and B). To further confirm the role of lzts2 on the interaction between β-catenin/Tcf and NF-κB pathways, siRNA duplex oligonucleotides were transfected for the downregulation of lzts2 expression. lzts2 siRNA-trans-

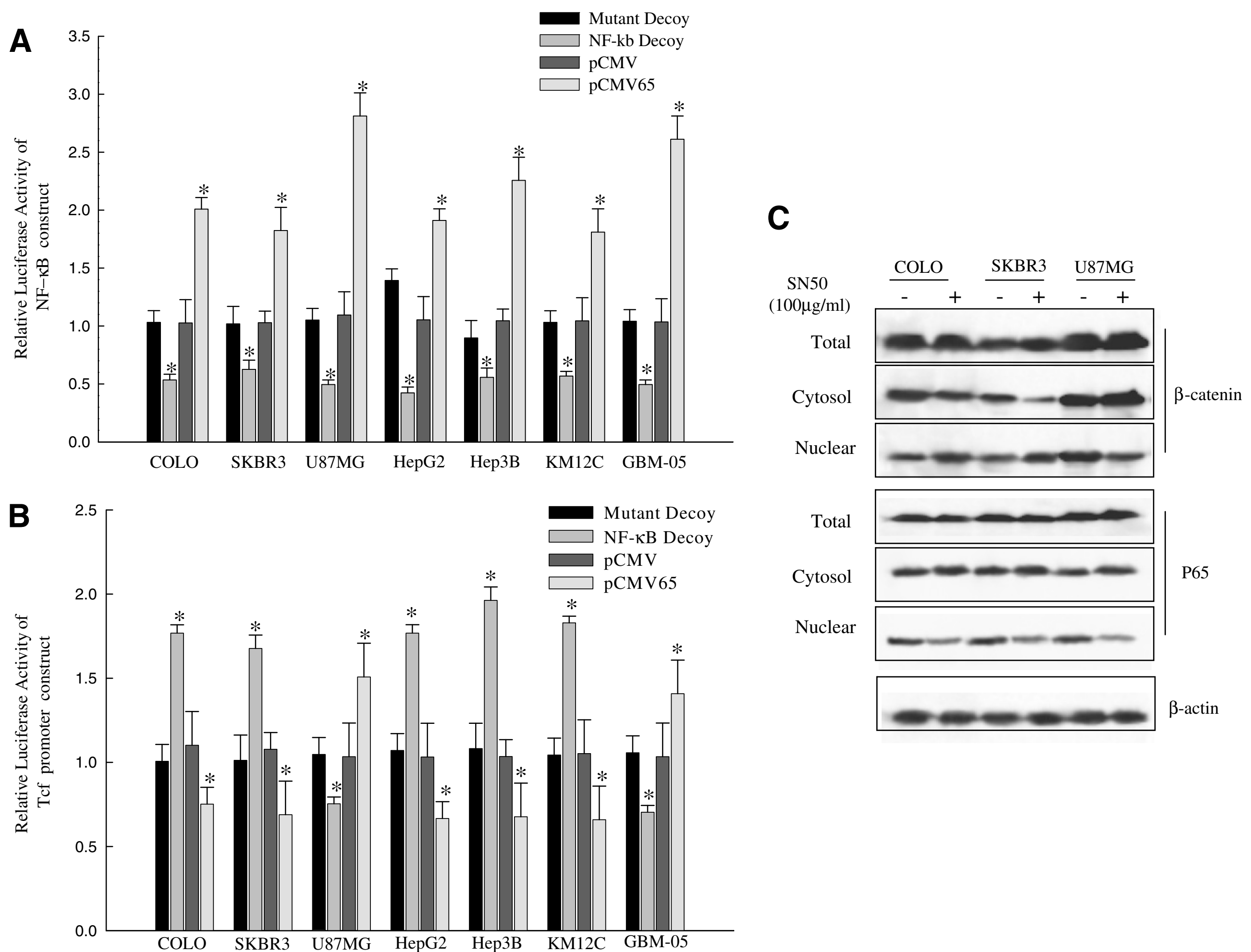

Fig. 2. Effect of NF-κB activity on β-catenin/Tcf pathway in cancer cells. (A,B) Cells were cotransfected with either NF-κB(A) or Tcf (B) reporter constructs vector in combination with β-Gal vector, and incubated with either mutant or NF-κB decoy for 48 h. To determine the effect of p65 overexpression, cells were cotransfected with either pCMV65 or pCMV empty vector in combination with reporter constructs. Luciferase activity was determined at 48 h after transfection and normalized by β-galactosidase activity. Data represent means ± S.E.M. of four different experiments. $^{*}P < 0.05$ compared with the data of mutant decoy or pCMV vector-transfected cells. (C) Effect of SN50 on nuclear translocation analysis of NF-κB and β-catenin in cancer cells. Cells was treated without or with SN50 (100 μg/ml) for 48 h and then total, cytosolic and nuclear proteins were immunoblotted with antibodies against β-catenin, p65 and β-actin. One representative experiment from three independent experiments was shown.

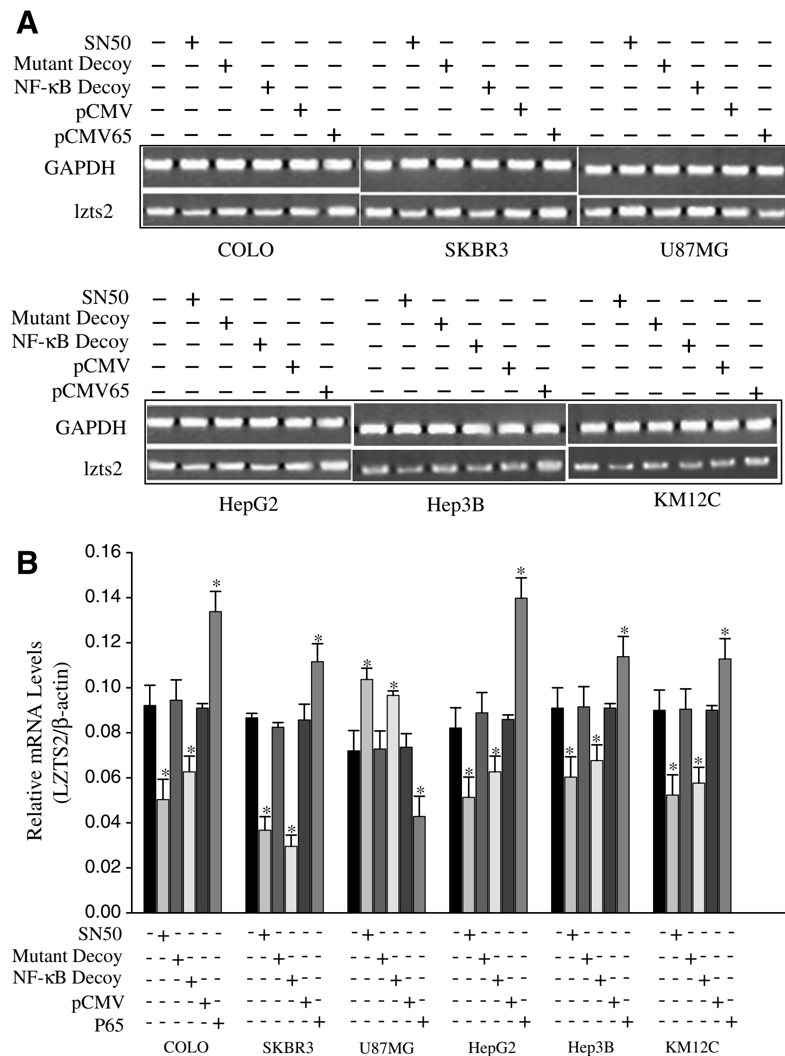

Fig. 3. Effect of NF-κB signal on lzts2 expression in cancer cells. (A) Cells was transfected with pCMV and pCMV65 plasmid or treated with mutant decoy, NF-κB decoy and SN50 (100 μg/ml). Total RNAs were isolated at 48 h after transfection or treatment, and lzts2 mRNA level in cancer cells was determined by RT-PCR (A) and real-time PCR (B). Data represent means ± S.E.M. of three different experiments. $^{*}P < 0.05$ compared with the data of control cells.

fected cells showed significant downregulation of the lzts2 gene in the cancer cells (by 70–80%) (Fig. 4A and B). We then determined Tcf and NF-κB promoter activity in lzts2 siRNA-transfected cells. The downregulation of lzts2 increased Tcf promoter activity in COLO-201, SKBR3, U87MG, HepG2, Hep3B, and KM12C cells (Fig. 4C). The transfection of lzts2 siRNA increased NF-κB promoter activity in U87MG, whereas it inhibited NF-κB promoter activity in COLO-201, SKBR3 HepG2, Hep3B, and KM12C cells (Fig. 4D). To confirm these findings, Western blot analysis was used to determine the levels of β-catenin in the cancer cell lines. The downregulation of lzts2 expression increased the total and nuclear β-catenin levels, but decreased the cytosolic β-catenin levels in the cancer cells (Fig. 4E).

To further determine the role of lzts2 on β-catenin/Tcf pathway, we measured the effect of lzts2 downregulation on nuclear translocation of β-catenin. In COLO-201, SKBR3, and HepG2 cells, the treatment of SN50 (100 μg/ml) increased nuclear translocation of β-catenin, but the overexpression of p65 decreased it. In U87MG and GBM-05 cells, the treatment of SN50 decreased nuclear translocation of β-catenin, but the overexpression of p65 increased it. In lzts2 downregulated cells, the alterations of the ratio of nuclear/cytosolic β-catenin levels induced by p65 overexpression or SN50 treatment were significantly decreased (Fig. 4F).

4. Discussion

β-Catenin inhibited the activity of NF-κB in colon and breast cancer cells [12], but induced the activation of NF-κB in vascular smooth muscle cells [15] and human embryonic kidney cells [23]. Studies concerning the crosstalk between NF-κB and the β-catenin/TCf pathways have revealed that the interaction between the two pathways occurs at multiple cellular levels. The data from studies in colon and breast cancer cell lines showed that β-catenin is able to bind to NF-κB and reduce the amount of NF-κB DNA binding [12]. The

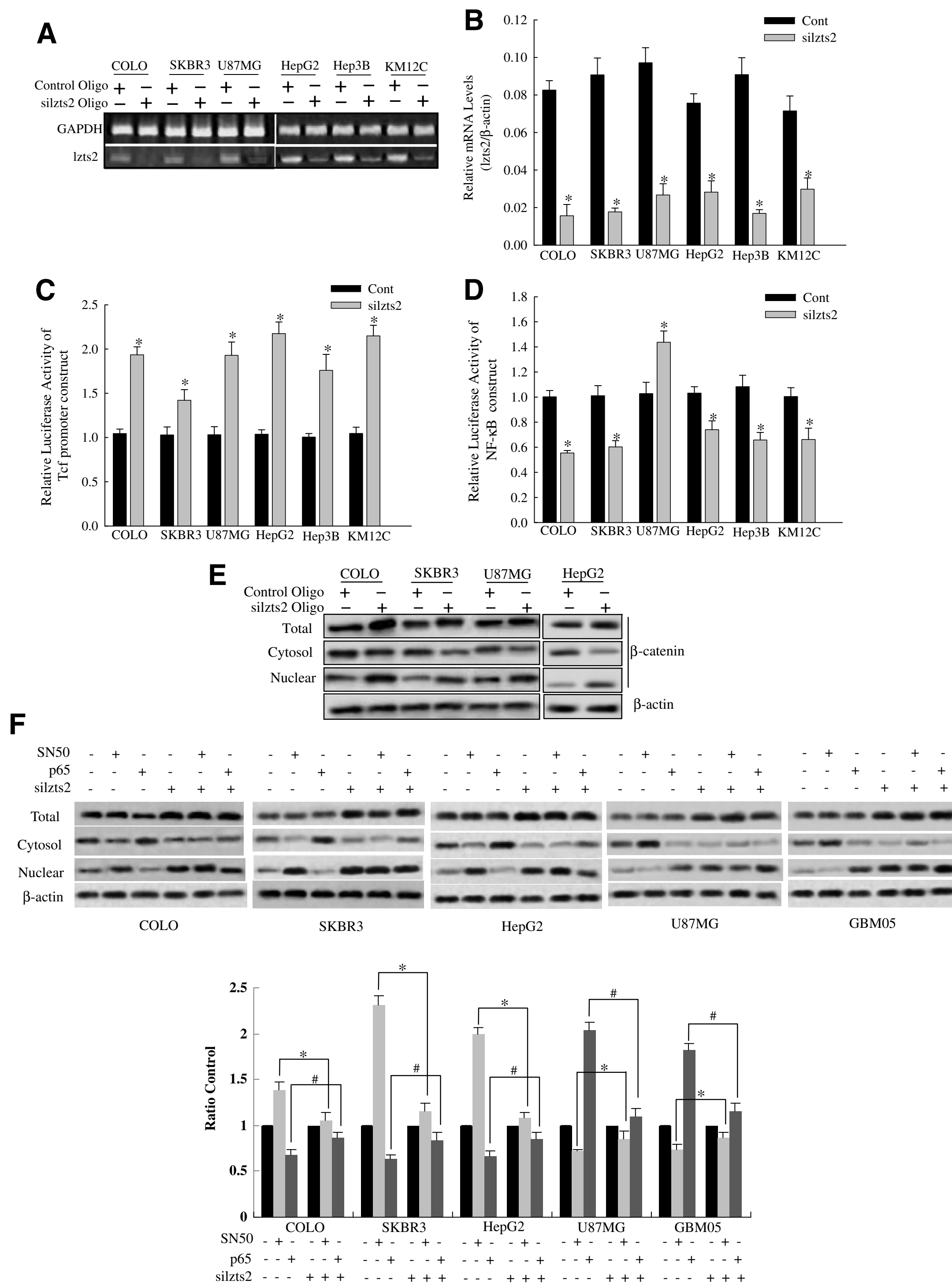

Fig. 4. Effect of lzts2 down regulation on NF-κB and β-catenin/Tcf signals in cancer cells. (A,B) lzts2 knock-down in cancer cells. lzts2 expression in lzts2 siRNA or non-target siRNA-transfected cells were determined by RT-PCR (A) and real-time PCR (B). (C,D) lzts2 siRNA or non-target siRNA-transfected cells were transfected with Tcf (C) and NF-κB (D) reporter constructs vector. Luciferase activity was determined at 48 h after transfection and normalized by β-galactosidase activity. Data represent means ± S.E.M. of four different experiments. $^{*}P < 0.05$ compared with control oligo-transfected cells. (E) Western blot analysis of total, cytosolic and nuclear proteins extracts obtained from lzts2 siRNA or non-target siRNA-transfected cells. One representative experiment from three independent experiments was shown. (F) Effect of lzts2 downregulation on the ratio of nuclear/cytosolic β-catenin levels induced by p65 overexpression or SN50 treatment. Cells were transfected with control or lzts2 siRNA oligonucleotides. Two days after oligonucleotide transfection empty vector or p65 plasmid were transfected. SN50 was treated just after transfection of plasmids. Quantitation of β-catenin level was done by an Image analyzer and the ratio of nuclear to cytosolic β-catenin level was calculated. Data represent the ratio to the control data in control oligo or lzts2 siRNA oligo transfected group (means ± S.E.M. of three different experiments). $^{*}P < 0.05$ compared with SN50-treated control oligo and vector-transfected cells. $^{*}P < 0.05$, compared with p65 and control oligo-transfected cells.

activity of NF-κB was enhanced by glycogen synthase kinase-3β (GSK-3β), which contributes to the degradation of β-catenin and represses the β-catenin/Tcf signaling pathway [4,24]. The transactivation of Tcf/Lef has been known to lead to the upregulation of the E3 ligase β-TrCP1 [15]. β-TrCP1 binds identical phosphorylated motifs on β-catenin and IκB, resulting in the tethering of the ubiquitin ligase machinery and subsequent degradation of IκB by the 26S proteasome [23,25,26], which can provide an explanation for the β-catenin-induced activation of NF-κB. However, these studies were focused on the β-catenin-mediated modulation of NF-κB, and the NF-κB-mediated modulation of the β-catenin/Tcf signaling pathways has not been studied well.

The results of our study showed that the modulation of the NF-κB signaling pathway differentially affects the β-catenin/Tcf signaling pathways according to the type of cancer cells. The modulation of NF-κB by the inhibition of NF-κB nuclear translocation, decoy oligonucleotides, and the overexpression of NF-κB directly affects the β-catenin/Tcf pathway in glioma cells, but inversely affects this pathway in colon, hepatic, and breast cancer cells. The mechanisms mentioned above do not provide a sufficient explanation for the NF-κB-induced modulation of β-catenin/Tcf pathways.

A recent study showed that lzts2 [16] interacts with β-catenin, represses the transactivation of β-catenin, and affects the subcellular localization of β-catenin. In this study, we demonstrated that NF-κB increased the expression of lzts2 in colon, breast, and hepatic cancer cells, but inhibited lzts2 expression in glioma cancer cells. These findings provide a good explanation for the differential effects of NF-κB activity on the β-catenin/Tcf pathways. That is, the increased nuclear translocation of β-catenin in colon, hepatic, and breast cancer cells by SN50 could be explained by a concomitant decrease in lzts2 expression. Conversely, the decreased nuclear translocation of β-catenin by SN50 in glioma cells could be related to the increase in lzts2 expression. The findings that the downregulation of lzts2 by RNA interference upregulated the levels of β-catenin, increased the translocation of β-catenin into the nucleus, increased the activity of the Tcf promoter, differentially regulated the activity of NF-κB according to cancer cell types and inhibited NF-κB-induced modulation of β-catenin translocation provide further support that lzts2 plays a central role in the interaction between the NF-κB and β-catenin/Tcf pathways. A schematic diagram in Fig. 5 illustrates our current understanding of the reciprocal interaction between the Wnt and NF-κB signaling pathways in cancer cells. Activation of NF-κB decreases lzts2 expression in colon, but increases it in. The changes in lzts2 level result in differential modulation of nuclear translocation of β-catenin and transactivation of Tcf/Lef.

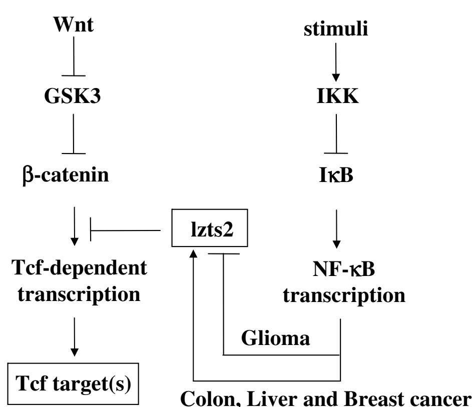

Fig. 5. lzts2 mediates crosstalk between NF-κB and β-catenin/Tcf pathways in cancer cells.

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