The Wnt/β-catenin pathway regulates self-renewal of cancer stem-like cells in human gastric cancer

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Abstract. Cancer stem cells (CSCs) possess the ability of self-renewal and tumor initiation. Targeting key signaling pathways that are active in CSC self-renewal is one approach to cancer therapy. Abnormal activation of the Wnt/β-catenin pathway has been described in a wide variety of human cancers and in CSCs; however, the role of this pathway in gastric CSCs has not been reported. In our study, we investigated whether the Wnt/β-catenin pathway plays an important role in gastric CSCs. First, we isolated cancer stem-like cells (CSLCs) from the human gastric cancer cell line MKN-45 using tumorsphere cultures. We tested whether tumorsphere cells were CSLCs using the following three criteria: i) We identified that the expression of the CSC marker CD44 was significantly greater in tumorsphere cells compared to adherent cells; ii) compared with adherent cells, the floating tumorsphere cells had greater self-renewing capacity; iii) *in vivo* xenograft studies showed that tumorsphere cells generate larger tumors than adherent cells at the same number. In addition, we studied the mechanism(s) by which the canonical Wnt signaling pathway acts in CSLCs. Western blotting and real-time PCR showed that the expression levels of β-catenin and c-myc, cyclin d1 and axin 2 were downregulated/upregulated with the inhibition/activation of the Wnt pathway. The pathway blocked by DKK-1 caused a higher reduction in the self-renewing capacity of MKN-45 tumorsphere cells and the pathway activated by lithium chloride improved the self-renewal of CSLCs. In conclusion, our data suggested that the Wnt/β-catenin pathway is essential for the self-renewal of CSLCs in human gastric cancer.

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Introduction

Tumor recurrence is one of the biggest problems in cancer therapy, and cancer stem cells (CSCs) are thought to be responsible for this. CSCs are a group of cells that have the ability of self-renewal and are tumor initiating. CSCs were first identified in human acute myeloid leukemia (1). Subsequent research has found that CSCs also exist in solid tumors, for instance in brain tumors (2), breast (3), colorectal (4) and liver cancer (5), carcinoma of cervix uteri (6), lung (7) and pancreatic cancer (8). Identification of molecular pathways essential for CSC self-renewal is critical for designing effective cancer therapeutics. The Wnt signaling pathway is an essential pathway that regulates non-neoplastic stem cells, including cell proliferation, differentiation and migration processes, which have been verified in various stem cells such as embryonic stem cells (9,10), neural progenitor cells (11,12), hematopoietic (13) and cardiovascular stem cells (14). The similarities between normal adult stem cells and CSCs suggest that the signaling pathways involved in somatic stem cell maintenance may also be involved in the regulation of CSCs. This assumption is verified in certain CSCs, such as colon (15), prostate (16) and cutaneous cancer (17), chronic myeloid leukemia (18), acute myelogenous leukemia (19) and hepatic carcinoma (20). The Wnt signaling pathway is a highly conserved signaling pathway.

In the canonical Wnt signaling pathway, Wnt ligands bind to Frizzled-low-density lipoprotein receptor-related protein-5/6 (LRP5/6) receptor complexes and activate the cytoplasmic scaffold protein Dishevelled (DVL), resulting in the inhibition of β-catenin phosphorylation and degradation. β-catenin accumulates in the cytoplasm and translocates into the nucleus, activating the target genes associated with T-cell factor/lymphoid enhancer factor (TCF/LEF). DKK1, a member of the dickkopf family of secreted proteins, acts by directly binding to the Wnt co-receptor LRP5/6 and disrupts Wnt-induced canonical Wnt/β-catenin pathway activation. Its inhibition effects on the Wnt signaling pathway, which regulates tumorigenesis, have been investigated in certain tumors: Zhu *et al* identified that DKK-1 secreted by mesenchymal stem cells was able to inhibit proliferation of human carcinoma cell lines through the canonical Wnt signaling pathway (21). Results of a previous study showed that DKK-1 acts as a tumor suppressor gene in colon cancer, with a higher secretion level following Wnt signaling pathway activation, which can be

Table I. Primers used for real-time PCR.

Gene	Forward primer	Reverse primer
Axin 2	GAGTGGACTTGTGCCGACTTCA	GGTGGCTGGTGCAAAGACATAG
c-myc	GCAGCTGCTTAGACGCGCTGGA	CGCAGTAGAAATACGGCTGCAC
Cyclin d1	ATGTTCGTGGCCTCTAAGATGA	CAGGTTCCACTTGAGCTTGTTC
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

explained with a negative feedback loop (22). Lithium chloride (LiCl) activates the Wnt signaling pathway by its inhibition effect on GSK-3β, which is part of the degradation complex.

In this study, we investigated whether the canonical Wnt signaling pathway activated or depressed by LiCl/DKK-1 produced any effect on the self-renewal of CSLs in the gastric cancer cell line MKN-45.

Materials and methods

Cell culture, formation of tumorspheres and sub-tumorspheres. The human gastric cancer cell line MKN-45 obtained from Central Experiment Labor of Xiangya was cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% antibiotic mixture. Cells were passaged every 2-3 days. Tumorspheres were derived by placing the MKN-45 cells into serum-free 1640 culture medium supplemented with human recombinant epidermal growth factor (EGF) (Peprotech, Rockville, NJ, USA) at a concentration of 20 ng/ml, human recombinant basic fibroblast growth factor (bFGF) (Peprotech) at a concentration of 10 ng/ml, 1% N-2 (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic mixture, and the MKN-45 cells were plated in 6-well ultra-low attachment cell plates (Costar, Cambridge, MA, USA) at 1,000 cells per well. Ten days later, plates were analyzed for tumorsphere formation and were quantified using an inverted microscope at a magnification of x40 and x100.

Tumorspheres were dissociated at a density of 1,000 cells per ml and 100 µl single cell suspension was seeded in each well of a 96-well plate in serum-free medium as described above. Each well was examined, and only the wells that contained a single cell were marked. Two weeks later, the wells were analyzed for sub-tumorsphere formation. Normal adherent cells were used as a control for their ability to form sub-tumorspheres. To test the effect of the Wnt/β-catenin pathway on the formation of sub-tumorspheres, dissociated cells were treated with LiCl 10 mM (Sigma, St. Louis, MO, USA), DKK-1 200 ng/ml (Peprotech) or control, phosphate-buffered saline (PBS), for 24 h and the formation of sub-tumorspheres was observed.

Western blot analysis. Tumorsphere cells or adherent cells were washed with ice-cold PBS and lysed directly in nuclear lysis buffer to collect whole cell extracts. The protein concentration of the total cell lysates was tested by an Enhanced BCA Protein assay kit (Beyotime, Institute zf Biotechnology, Haimen, China). Protein samples for western blotting were prepared by boiling following the addition of denaturing sample buffer. Proteins were then separated using SDS-PAGE on a 10% gel, and transferred onto a nitrocellulose membrane

by DYY-6C. Membranes were incubated at 4°C overnight with primary antibody, and subsequently incubated with secondary antibodies for 1 h at 37°C. Protein bands were scanned using Genegnome Bio Imaging System (Syngene, Frederick, MD, USA) following the application of BeyoECL plus (Beyotime). The following concentrations were used for primary antibodies: CD44 antibodies 1:500 (Epitomics, Burlingame, CA, USA), β-catenin antibody 1:2000 (Epitomics) and β-actin antibody 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Quantitative real-time PCR. Total RNA from the cell line was prepared with TRIzol (Takara) according to the manufacturer's instructions. The cDNA synthesis was carried out according to the protocol of the PrimeScript® RT Master Mix reagent kit (Takara DRR036S). The PCR reaction was performed with a Step OnePlus™ Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). The PCR primers were synthesized by BioSune Biotechnology (China), and the sequences (5'-3') are shown in Table I.

Xenograft model. An equal number (1x10^{6}) of freshly dissociated cells was suspended in 200 µl PBS, and the tumorsphere cells were injected into the right underarm of each male nude mouse (n=6 per group) and the same number of adherent cells was injected into the left underarm of each mouse. Thirty days later, mice were sacrificed by cervical dislocation and tumors were excised. Tumors were measured and then placed in 10% formalin for hematoxylin and eosin (H&E) staining. Tumor size was calculated every 3 days using the formula: 2 x larger diameter x (smaller diameter)[2].

Hematoxylin and eosin. The excised tumors from nude mice were fixed in 10% formalin, embedded in paraffin and processed by standard histological methods. Tissue sections were then stained with H&E to assess morphology.

Statistical analysis. The experiments were repeated three times. Quantitative data were presented as the means ± SD. The data were tested whether they were normally distributed or not. If data obeyed normal distribution, we analyzed the results using the Student's t-test; if data did not obey normal distribution, we analyzed the results using the Mann-Whitney U test with SPSS 18.0 statistical software. P<0.05 was considered to indicate a statistically significant difference.

Results

Tumorsphere cells possessed the characteristics of cancer stem-like cells (CSLCs). MKN-45 adherent cells were grown
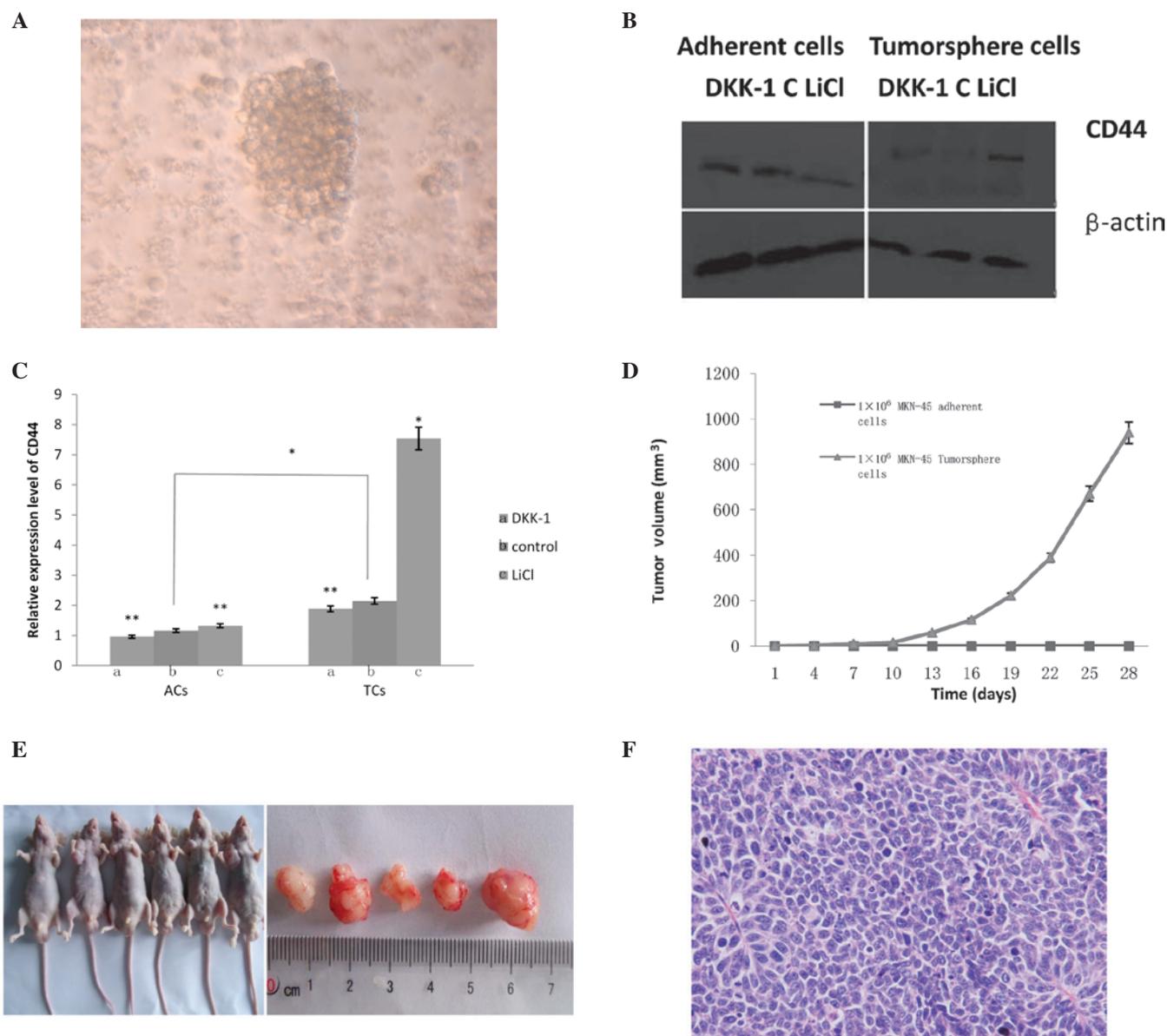
Figure 1. Tumorsphere cells possessed the characteristics of CSLCs. (A) Tumorspheres after 10 days of incubation in serum-free medium (original magnification, x600). (B) Western blot analysis of CD44 on adherent and tumorsphere cells following different treatments. (C) Relative expression level of CD44, regulated by β-actin. *P<0.05, **P>0.05. (D) Subcutaneous tumor volume changes over time. (E) Subcutaneous tumor growth in nude mice following injection of the same number (1x10⁶) of MKN-45 ACs to the left underarm and TCs to the right underarm. (F) H&E staining analysis of xenografts derived from MKN-45 tumorsphere cells (original magnification, x400). ACs, adherent cells; TCs, tumorsphere cells; C, control; CSCLs, cancer stem-like cells; H&E, hematoxylin and eosin; LiCl, lithium chloride.

in serum-free medium as described in Materials and methods. After 10 days, tumorspheres consisting of approximately 50-100 cells were observed (Fig. 1A).

We examined the expression of CD44 in dissociated tumorsphere cells and adherent cells using western blotting. The results showed that the expression of CD44 was significantly greater in tumorsphere cells than in adherent cells (Fig. 1B and C).

The self-renewing capacity of these tumorspheres was assessed by dissociating them into single cells and growing them at a density of 1,000 cells per ml. After 2 weeks, single cells derived from MKN-45 tumorsphere cells generated sub-tumorspheres at a rate of 31.95% compared with 15.63% of adherent cells (Fig. 3A). This suggested that the self-renewing

capacity of tumorsphere cells was significantly higher than that of adherent cells.

For xenograft studies, an equal number (1x10⁶) of freshly dissociated tumorsphere cells or control adherent cells was injected into each mouse (n=6 per group). The results showed that 83.3% (5/6) of the tumorsphere cells generated subcutaneous tumors, while no subcutaneous tumors were observed in the adherent cells whose activity was tested by trypan blue (Fig. 1D and E). This suggests that spheroid cells have a stronger tumorigenic capacity in nude mice than adherent cells.

The H&E examination of xenografts derived from MKN-45 tumorsphere cells showed significant atypia and pleomorphism, and these tumors closely resembled the original human tumor, a poorly differentiated adenocarcinoma (Fig. 1F).

These data suggest that tumorsphere cells represented CSLCs that had tumorigenic capacity.

β-catenin was more highly expressed in tumorsphere cells than in adherent cells, while no significant difference was detected in the pathway target genes. β-catenin is a key component of the Wnt/β-catenin signaling pathway, and the level of β-catenin target genes represents the active state of the pathway. Therefore, we examined the expression levels of β-catenin by western blotting, and c-myc, cyclin d1 and axin 2 by quantitative real-time PCR.

Western blotting showed that the expression levels of β-catenin were higher in tumorsphere cells than in adherent cells (Fig. 2A). We used β-actin as a loading control, and the relative expression of β-catenin was found to be significantly higher in tumorsphere cells than in adherent cells (Fig. 2B), whereas the expression level of c-myc, cyclin d1 and axin 2 showed no significant difference in CSLCs and adherent cells, as shown by real-time PCR (Fig. 2C).

LiCl activates the Wnt/β-catenin pathway, and activation of the pathway promotes the self-renewing capacity of MKN-45 tumorsphere cells. LiCl activates Wnt signaling by inhibiting GSK3β, leading to β-catenin stabilization and translocation into the nucleus. Western blot analysis was used to investigate whether LiCl treatment upregulated the expression of β-catenin in MKN-45 cells. We found a marked increase of β-catenin in MKN-45 tumorsphere cells after stimulation with LiCl (Fig. 2A and B). To evaluate whether LiCl-mediated accumulation of β-catenin would lead to the activation of typical Wnt target genes, the mRNA expression level of c-myc, cyclin d1 and axin 2 was quantified by real-time PCR. Our results showed that the mRNA level of c-myc and cyclin d1 were significantly higher in MKN-45 tumorsphere cells which were treated with LiCl than those not treated with LiCl (Fig. 3B). Although the axin 2 mean expression level in tumorsphere cells treated with LiCl was higher than that in control cells, no statistical significance was observed (Fig. 3B). We also found an upregulation of the stem cell surface marker CD44 at the protein level by western blotting (Fig. 1B and C). LiCl treatment led to a significant enhancement in the capacity for the formation of sub-tumorspheres in MKN-45 tumorsphere cells (Fig. 3A), while no significant difference was observed in adherent cells with or without stimulation by LiCl (Figs. 1B and C, 2A and B, 3A and C).

Taken together, these findings demonstrate that stimulation with LiCl may increase the self-renewal ability of CSLCs through activation of the Wnt/β-catenin pathway. Additionally, it appears that only CSCs that have the ability of self-renewal and tumor initiation through abnormal activation of the Wnt/β-catenin pathway; thus, no changes in adherent cells following treatment with the same pathway activator were observed.

DKK-1 blocks the Wnt/β-catenin pathway and reduces the self-renewing capacity of MKN-45 tumorsphere cells. DKK-1 blocks the Wnt/β-catenin pathway by binding to LRP5/6, preventing formation of the Frizzled-Wnt-LRP5/6 complex. Western blot analysis was used to investigate whether DKK-1 treatment was able to downregulate the expression
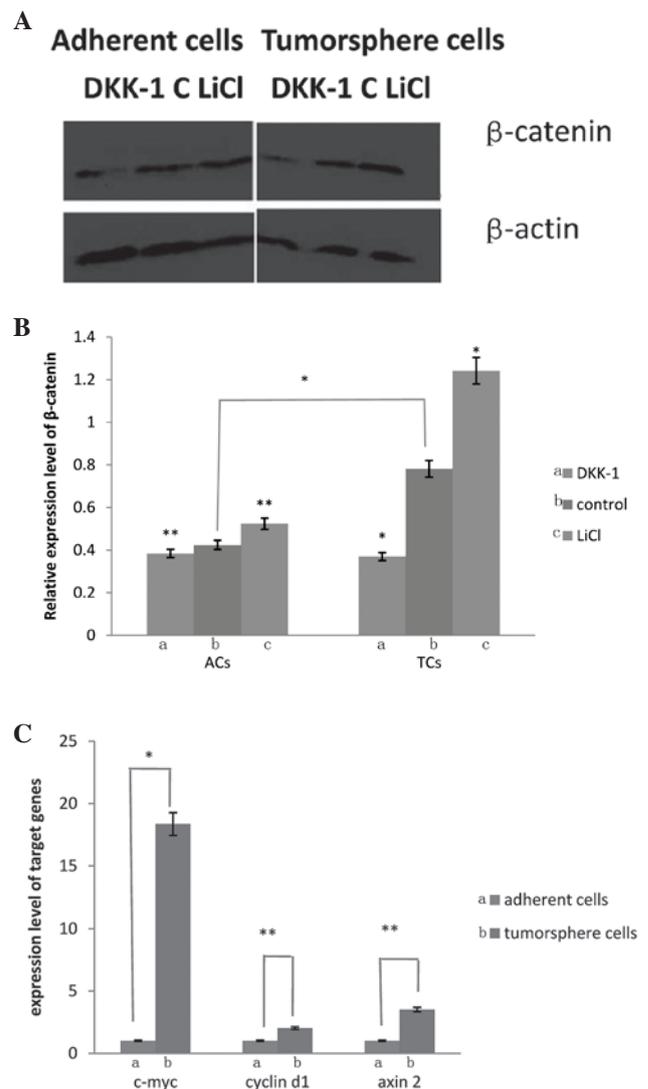
Figure 2. Wnt signaling in adherent and tumorsphere cells. (A) Western blot analysis of β-catenin on adherent and tumorsphere cells after different treatments. (B) Relative expression level of β-catenin, regulated by actin. *P<0.05, **P>0.05. (C) Expression levels of Wnt signaling target genes in adherent and tumorsphere cells. ACs, adherent cells; TCs, tumorsphere cells; C, control; LiCl, lithium chloride.

of β-catenin. We found a marked decrease of β-catenin in MKN-45 tumorsphere cells following the treatment (Fig. 2A and B). To evaluate whether DKK-1-mediated degradation of β-catenin would lead to the inhibition of typical Wnt target genes, the mRNA expression level of c-myc, cyclin d1 and axin 2 was quantified by real-time PCR. The results showed that the mRNA level of these target genes was significantly lower in MKN-45 tumorsphere cells that were treated with DKK-1 than those without DKK-1 treatment (Fig. 3B). CD44 was not downregulated as expected (Fig. 1B and C). The reason for this may be that the cell surface marker was not specific enough. DKK-1 treatment led to a significant reduction in capacity for the formation of sub-tumorspheres in MKN-45 tumorsphere cells (Fig. 3A). However, no significant differences were observed in adherent cells with or without treatment with DKK-1 (Figs. 1B and C, 2A and B, 3A and C).

Taken together, these findings demonstrate that stimulation with DKK-1 could decrease the self-renewal ability of CSLCs
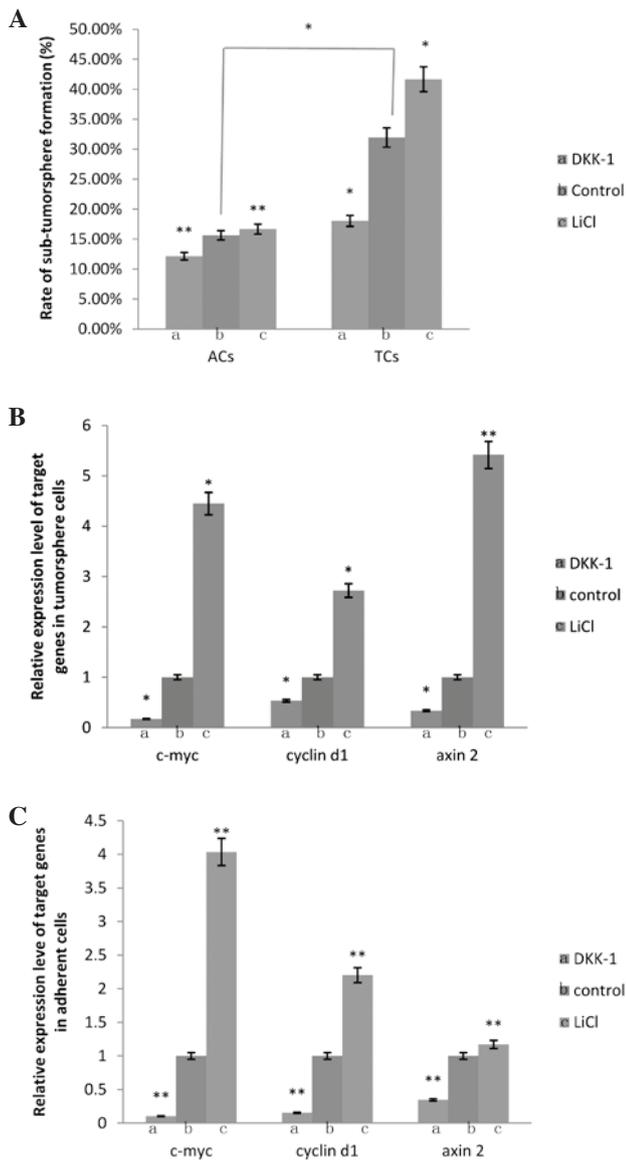
Figure 3. The effect of LiCl/DKK-1 on ACs and TCs. (A) The sub-tumorsphere formation ability of ACs and TCs under different conditions. (B) Expression level of Wnt signaling target genes in tumorsphere cells. (C) Expression level of Wnt signaling target genes in adherent cells. *P<0.05, **P>0.05. ACs, adherent cells; TCs, tumorsphere cells; LiCl, lithium chloride.

through inhibition of the Wnt/β-catenin pathway. Moreover, it appears that only CSCs have the ability of self-renewal and can initiate tumors through abnormal regulation of the Wnt/β-catenin pathway, which may explain the reason for the changes in adherent cells after treatment with the same pathway inhibitor not being detected.

Discussion

Despite the development of modern cancer therapy, cancer remains one of the major causes of mortality. CSCs are thought to be responsible for chemoresistance, metastasis and cancer recurrence. The elimination of CSCs may result in efficient cancer treatments. Signaling pathways, which regulate self-renewal of CSCs have been studied in a number of tumors, including the Wnt signaling pathway (17,20), Hedgehog

signaling pathway (23-25) and Notch signaling pathway (26). As for gastric cancer, for which the etiology is relatively clear, there is no research on the canonical Wnt signaling pathway regulating the self-renewal of its CSCs. Therefore, we performed a series of experiments to investigate whether or not this pathway is capable of regulating the self-renewal of gastric CSCs. The results showed that investigations should be conducted on how to effectively eliminate CSCs.

Spheroid colony formation *in vitro*, cell surface markers and xenotransplanted tumors *in vivo* are three approaches that have been used to identify CSCs. We used the tumorsphere culture method to isolate CSCs (27,28). This is not only a way to obtain CSLCs, but also allows identification of the CSC characteristics of these cells. Using cell surface markers would be ideal for the isolation and identification of CSCs, if highly specific markers were identified. At present, markers that are not specific have been identified in various tumors (29-32). As for gastric cancer, Takaishi *et al* (27) found that CD44^{+} cells of gastric cancer behaved in the same manner as CSCs, thus CD44 may be regarded as a marker of gastric cancer stem cells. Our data have shown that tumorsphere cells express CD44 at significantly higher levels compared to adherent cells, and this expression identified CSC characteristics of the tumorsphere at the same time. We also used a tumorigenic assay, which showed that tumorsphere cells are more tumorigenic than adherent cells.

C-myc, axin 2 and cyclin d1 are β-catenin target genes. The Wnt signaling pathway is involved in the regulation of certain types of CSCs (15-20). Additionally, the canonical Wnt signaling is activated in mammary mesenchymal subpopulation cells, but not in the parental cells, in an autocrine manner (33). Therefore, target genes should be elevated in CSCs compared to non-CSCs. Our data showed no significant difference in the expression level of Wnt signaling target genes between CSLs and adherent cells. This may be explained as follows: First, CSLCs are not equal to CSCs, and tumorspheres contained both CSCs and a small amount of non-CSCs, which explains the term CSLs. Similarly, there is also an extremely small number of CSCs present in adherent cells. Secondly, CSCs are quiescent and slow-cycling cells, and serum-free conditions are not ideal for CSC self-renewal. By contrast, the small number of CSCs in adherent cells exhibit renewal following culture in serum medium. Our data support this explanation. The Wnt signaling pathway is not maintained in non-CSCs, the number of CSCs in adherent cells is extremely small and this small group of CSCs is already in an active self-renewal situation. Therefore, our data showed that activation/inhibition of the Wnt signaling pathway had no significant effect on adherent cells. However, since Wnt signaling is maintained in the regulation of CSCs in the MKN-45 cell line and CSCs are enriched in the tumorsphere cells, our data have shown that activation of the Wnt pathway increases the expression level of target genes, with the exception of axin 2, and improves the ability of self-renewal in MKN-45 tumorsphere cells. By contrast, inhibition of the pathway decreases the expression level of target genes significantly and the self-renewal ability is reduced. Our data are in agreement with a former experiment: Yang *et al* (20) showed that following treatment with GSK-3 inhibitor, tumorigenic liver progenitor cells were increased 2-fold to 11-fold relative to the control group. Inhibition of the Wnt/β-catenin pathway reduced the breast cancer stem cell

population *in vitro* and *in vivo* (34). Axin 2, a direct target of the Wnt pathway, whose mRNA is expressed in a restricted pattern, is a negative regulator of the Wnt/β-catenin pathway (35). Activation of the Wnt signaling pathway was expected to increase the expression level of target genes. However, no increase was found in the expression level of axin 2 following signal activation. The reason for this may be that axin 2 acts as a negative regulator of the Wnt/β-catenin pathway, and a negative feedback loop inhibits the expression of axin 2 during the active course of the pathway. Our explanation has been tested previously: Axin 2 mRNA was not detected, whereas other Wnt/β-catenin target genes were reactivated (36). Rough surface topography increases Wnt/β-catenin target genes but decreases axin 2 expression without affecting β-catenin mRNA levels (37).

We found that the Wnt signaling pathway is important in the regulation of self-renewal of gastric CSCs, while it has no significant effects on non-CSCs. The combination of a powerful Wnt signaling inhibitor and chemotherapy generally used at present is likely to effectively control cancer. A number of small-molecule inhibitors or biological inhibitors (38) have been studied in the Wnt signaling pathway, but none are able to eliminate CSCs effectively. The combined blocking of multiple targets and pathways, which have evolved in the self-renewal of CSCs, is required.

Acknowledgements

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