Biochemical and Biophysical Research Communications 392 (2010) 373–379

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Wnt/β-catenin signaling regulates cancer stem cells in lung cancer A549 cells

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ARTICLE INFO

Article history:
Received 30 December 2009
Available online 13 January 2010

Keywords:
Signal transduction
Lung neoplasms
OCT-4
Target therapy

ABSTRACT

Wnt/β-catenin signaling plays an important role not only in cancer, but also in cancer stem cells. In this study, we found that β-catenin and OCT-4 was highly expressed in cisplatin (DDP) selected A549 cells. Stimulating A549 cells with lithium chloride (LiCl) resulted in accumulation of β-catenin and up-regulation of a typical Wnt target gene cyclin D1. This stimulation also significantly enhanced proliferation, clone formation, migration and drug resistance abilities in A549 cells. Moreover, the up-regulation of OCT-4, a stem cell marker, was observed through real-time PCR and Western blotting. In a reverse approach, we inhibited Wnt signaling by knocking down the expression of β-catenin using RNA interference technology. This inhibition resulted in down-regulation of the Wnt target gene cyclin D1 as well as the proliferation, clone formation, migration and drug resistance abilities. Meanwhile, the expression of OCT-4 was reduced after the inhibition of Wnt/β-catenin signaling. Taken together, our study provides strong evidence that canonical Wnt signaling plays an important role in lung cancer stem cell properties, and it also regulates OCT-4, a lung cancer stem cell marker.

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Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer death in the world. Unfortunately, current therapy is still inadequate, and the 5-year survival rate for lung cancer remains poor [1]. In order to develop more effective therapies, it is important to obtain a better understanding of molecular biology of lung cancer. For this purpose, the identification and isolation of cancer stem cells (CSCs) may provide new insights into cancer therapies.

CSCs are a subpopulation of tumors that are responsible for tumor maintenance and spreading. These cells possess unlimited proliferation potential, ability to self-renewal and capacity to generate a progeny of differentiated cells that constitute the major tumor population [2]. Because of these properties, CSCs may be a promising target for cancer therapy. CSCs have many stem cell properties, but the mechanisms to maintain these properties remain unclear. Recently, many studies have demonstrated that the Wnt/β-catenin pathway is crucial in the maintenance of CSCs, such as leukemia [3–5], breast [6,7], melanoma [8], colon [9], liver [10] and cutaneous cancers [11].

In this study, we found that when the lung adenocarcinoma A549 cells were stimulated with LiCl, Wnt/β-catenin pathway was activated. Moreover, this stimulation significantly enhanced proliferation, clone formation and drug resistance abilities of the cells. More importantly, we observed an up-regulation of OCT-4, a stem cell marker after the stimuli. Then we used a reverse approach to inhibit Wnt signaling in A549 by knocking down the expression of β-catenin using RNA interference technology. And the opposite results were observed. Taken together, our study convincingly show that canonical Wnt signaling plays an important role in the regulation of OCT-4, which may be a novel marker of lung cancer stem cell and potentially a new target for lung cancer therapy.

Materials and methods

Cell culture. A549 lung adenocarcinoma cells were purchased from ShangHai Cell Biology Institute (China), and they were cultured in RPMI1640 (Hyclone) and 10% fetal bovine serum (FBS, FMG Biotech Co. Ltd) according to the supplier's instructions. The cells were passed every 2–3 days. Cultivated in serum-free condition for 24 h, the cells were stimulated with 10 mM LiCl (Amresco) for 24 h.

Drug select experiment. A549 cells were treated with cisplatin (5 μg/ml) for 2 days. A vast majority of the cells died and drug-resistant cells survived. After 7 days' culture, the living cells were collected for Western blot analysis.

Construction of β-catenin specific shRNA expression vector. The plasmid, PGCsilencer™U6/Neo/GFP/RNAi was used to express siRNAs in A549 cells. The coding regions corresponding to nucleotide positions 346–364, 1271–1289, and 2263–2281 of β-catenin sequence in GenBank (CTNNB1, NM001904) were selected to form siRNA target sequences. Then, three primer pairs were synthesized: one encoding nucleotide positions 346–364 (caGCAACA

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doi:10.1016/j.bbrc.2010.01.028

GTCTTACCTGGAC) followed by a 9 bp 'loop' (TTCAAGAGA) and an inverted repeat (CTNNB1-siRNA-1); the second encoding nucleotide positions 1271–1289 (aaACTACTGTGGACCACAAGC) also followed by the loop and the inverted repeat (CTNNB1-siRNA-2), and the third encoding nucleotide positions 2263–2289 (caAGCCACAaGATTACAAGAA) followed by the loop and the inverted repeat (CTNNB1-siRNA-3). Moreover, a negative control scrambled siRNA (CAAGGTCGGGCAGGAAGAG), which had no significant homology to human gene sequences, was taken as a negative control (NC). These sequences were synthesized and purchased from Genechem Company (China).

Small interfering RNA transfection. The transfection was performed according to the instruction of the Lipofectamine2000 (Invitrogen). For stable transfection, A549 cells were transfected with CTNNB1-RNAi plasmid, the negative control vector. Then, the cells were selected with a standard medium containing 600 μg/ml G418 (Gibco) for 14 days, and G418-resistance colonies were pooled to establish stable A549 transfectants. The stable transfected cells were then used for subsequent studies.

Clone formation assay. For the assay, 100 cells were plated in RPMI1640/10% FBS on six-well plates per well, and were cultured for 14 days. The number of the clones ($\geqslant$50 cells) was assessed by counting under a microscope.

Cell proliferation experiment. Cells were plated in 96-well plates at 5×10^3 per well. After 2-day culture, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma) was added and cultivated at 37 °C for 4 h, then disposed the culture, added 150 μl dimethylsulfoxide (DMSO, Sigma) in each well to dissolve the formazam product. Absorbance (A) was measured at 490 nm.

Cell migration assay. The migratory capacity of cells was analyzed using Millicell chambers with 8 μm pore size (Millipore). Samples each containing 5×10^5 cells in 0.8 ml of serum-free medium were added to the upper compartments. The lower compartments were filled with 1 ml of RPMI1640 containing 10% FBS as a source of chemoattractants. The chambers were incubated for 24 h at 37 °C and 5% CO_2. After incubation, cells on the top surface of the filters were wiped off with cotton swabs. Cells that had migrated into the lower compartment and attached to the lower surface of the filter were counted after staining with Giemsa. The migration rate was expressed as numbers of migrated cells.

Chemotherapeutic treatment. First, 2500 cells were plated in 96-well plate, and after 24 h, DDP was added in different concentrations (2, 4, 6, 8 and 10 μg/ml), with phosphate buffered solution (PBS) as a control. A MTT assay was performed after 3-day culture.

Immunochemistry. To identify subcellular localization of β-catenin, A549 cells were plated onto slides, resulting in 80% confluence, and they were treated with 10 mM LiCl for 24 h. After paraformaldehyde fixation (4%) was performed for 30 min at 4 °C, blocking was carried out with goat serum for 1 h to minimize unspecific binding of the antibody. The β-catenin antibody (abcam, ab2982) was applied at a 1:100 dilution for 1 h. As a specificity control, PBS was used instead of the primary antibody to exclude unspecific binding of the secondary antibody. The procedure was performed according to the instruction of Histostain™-PluS Kits (ZSGB-BIO, SP9001).

Western blotting. Harvested cultured cells were lysed in SDS lysis buffer (Beyotime, P0013G). The concentration of proteins in cell lysates was quantified by the Enhanced BCA Protein Assay Kit (Beyotime, P0010S), and 90 μg protein was loaded in each lane. Samples were electrophoresed with SDS–PAGE (10%) and blotted for 1 h onto nitrocellulose filter (NC) membranes. The membranes were stained with Ponceau-S to check loading and transfer, and then blocked with 5% non-fat milk overnight at 4 °C. Blots were incubated with the primary antibodies for 2 h, and then with the secondary antibodies for 1.5 h at room temperature, before being visualized by ECL plus (Hyglo, E2100C). The integral optical density (IOD) of each sample was measured. The antibodies we used were as follows: OCT-4 antibodies (abcam, ab19857, 1:700); β-catenin antibody (abcam, ab2982, 1:400); β-actin antibody (CWBIO, CW0097, 1:1000); HRP-conjugated goat-anti-rabbit IgG (Jingmei Biotech, SB200, 1:5000).

RNA preparation and real-time PCR. Total RNA from cell lines was prepared with Trizol (Sangon, BS410) following the manufacturer's instruction. The cDNA synthesis was carried out according to the protocol of the PrimeScript ™RT reagent Kit (TaKaRa, DRR037S) with a starting amount of 400 ng RNA. The PCR reaction was performed with a Roche Lighter 2.0 DNA Amplification System (Roche, Switzerland). The SYBR Green PCR reaction mixture (TOYOBO, Japan) was used according to the manufacturer's instruction. The PCR primers were synthesized by BioSune Biotechnology (China), and their sequences were as follows (5′–3′):

CCND1F: TGATGCTGGGCACTTCATCTG;
CCND1R: TCCAATCATCCCGAATGAGAGTC;
CTNNB1F: CTCTGGTGGATATGGCCAGGA;
CTNNB1R: CAGATCTGGCAGCCCATCAA;
OCT-4F: GCAATTTGCCAAGCTCCTGAA;
OCT-4R: GCAGATGGTCGTTTGGCTGA;
GAPDHF: GCACCGTCAAGGCTGAGAAC;
GAPDHR: TGGTGAAGACGCCAGTGGA.

Statistical analysis. Statistical analysis was performed with SPSS 16.0. The difference was considered statistically significant at $P < 0.05(*)$ or $P < 0.01(**)$.

Results

OCT-4 and β-catenin are highly expressed in drug selected A549 cells

After being affected by DDP (5 μg/ml) for 2 days, the cells were collected to examine the OCT-4 and β-catenin expression. Surprisingly, the expression of OCT-4 was strikingly enhanced in DDP selected cells, and so was the expression of β-catenin (Fig. 1).

Activation and inhibition of the Wnt/β-catenin pathway in A549 cells

After A549 cells were stimulated with LiCl for 24 h, Western blot analysis was used to investigate whether this stimuli could up-regulate the expression of β-catenin at the protein level. We used β-actin as a loading control, and used β-catenin/β-actin to evaluate the relative expression of β-catenin. We found a dramatic increase of β-catenin after the stimulation (Fig. 2A). To investigate whether LiCl stimulation influences the subcellular localization of β-catenin, we performed immunocytochemistry. While untreated A549 cells (control) exhibited cytoplasmic staining for β-catenin, treatment with LiCl (10 mM for 24 h) displayed a clear nuclear staining of β-catenin (Fig. 2B), indicating that LiCl stimulation could lead to the accumulation and translocation of β-catenin from the cytoplasm into the nucleus. To evaluate whether LiCl-mediated accumulation of β-catenin would lead to the activation of typical Wnt target genes, the mRNA expression level of cyclin D1 was quantified by real-time PCR, and we observed a significant induction (Fig. 2C).

Taken together, these findings demonstrate that the stimulation with 10 mM LiCl for 24 h in A549 cells can up-regulate the expression of β-catenin at the protein level, its translocation into nucleus as well as the enhanced expression of Wnt target gene cyclin D1, all of which highlight the activation of the Wnt/β-catenin pathway.

We transfected A549 cells with β-catenin interfering plasmid to knockdown the expression of β-catenin. The cells transfected successfully showed green fluorescence, which could be an indicator for transfection efficiency. By Western blot analysis, we observed that the expression of β-catenin at the protein level was dramati-

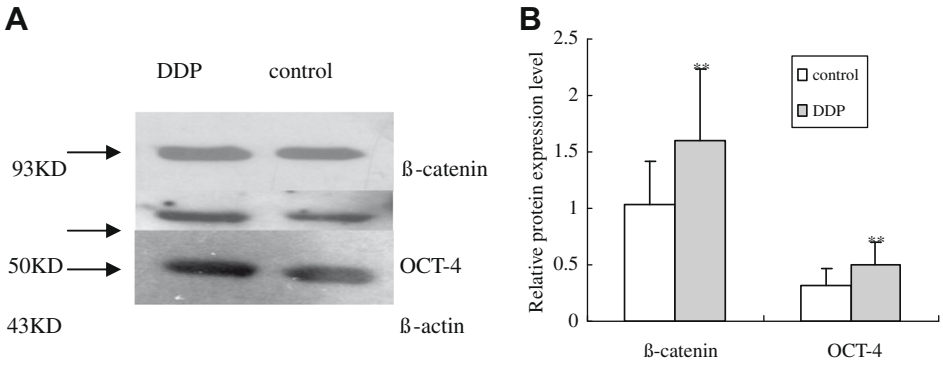

Fig. 1. The level of the expression of β-catenin and OCT-4 in DDP selected cells. (A) Western bolt detected an expression of β-catenin (about 93KD) and OCT-4 (about 50 KD). The expression of β-actin was used as a loading control. (B) The expression of β-catenin and OCT-4 was up-regulated in DDP selected cells. $^{**}P < 0.01$.

cally knocked down in the cells transfected with CTNNB1-RNAi-2 (Fig. 2D). So we chose the cells transfected with CTNNB1-RNAi-2 plasmid for subsequent experiments. The expression of β-catenin and cyclin D1 at the mRNA level were also down-regulated in the CTNNB1-RNAi-2 plasmid transfected A549 cells (Fig. 2D). So CTNNB1-RNAi-2 interfering led to down-regulation of β-catenin and cyclin D1, thereby further inhibiting the Wnt/β-catenin pathway.

Proliferation, clone formation, migration, drug resistance abilities and the expression of OCT-4 are enhanced after the stimulation in A549 cells

To investigate whether the stimulation of LiCl influences the proliferation ability of A549 cells, we performed a MTT proliferation experiment. After stimulated with LiCl (10 mM for 24 h), we observed an increase in cell proliferation by 32.24% (Fig. 3A). In addition, this stimulation increased the ability of clone formation by 100% through clone formation assay (Fig. 3B). The migration experiment shown an enhanced migration ability of A549 cells treated with LiCl (27.250 ± 2.500 vs. 52.5 ± 4.041, $P < 0.01$) (Fig. 3C). Also in the chemotherapeutic treatment experiment, the cells treated with LiCl (10 mM for 24 h) showed an enhanced drug resistance ability in all DDP concentrations we examined (Fig. 3D).

Since the Wnt/β-catenin pathway significantly affected the maintenance of stem cells, we next asked whether the activated Wnt/β-catenin pathway by 10 mM LiCl observed in A549 cells could affect the expression of OCT-4, a stem cell marker of embryonic stem cells and a biological marker of lung CSCs. Surprisingly, we found an up-regulation of OCT-4 at the protein level by Western blot (Fig. 3E). Moreover, the expression of OCT-4 was increased at the mRNA level by real-time PCR (Fig. 3F).

Knocking down the Wnt/β-catenin pathway reduces the proliferation, clone formation, migration, drug resistance abilities and the expression of OCT-4 in transfected A549 cells

Since LiCl application resulted in an increase of stem cell properties, we were interested in whether inhibition of Wnt signaling accomplished by knocking down β-catenin would lead to the opposite behaviors of A549 cells. We performed a MTT cell proliferation experiment and found that knocking down β-catenin resulted in a significant reduction of cell proliferation (Fig. 4A). Moreover, we also examined the clone formation ability, revealing a high reduction of clone formation ability in A549 cells (Fig. 4B). The migration abilities was also inhibited (33 ± 2.739 vs. 16 ± 3.808, $P < 0.01$) (Fig. 4C). The chemotherapeutic treatment experiment also showed the decreased drug resistance ability (Fig. 4D).

These results strongly suggested that inhibition of Wnt signaling in A549 cells by knocking down β-catenin not only down-regulated typical Wnt target genes but also decreased cell proliferation, clone formation, migration and drug resistance abilities.

In view of A549 cells with an increased expression of OCT-4 upon the activation by LiCl, we analyzed whether inhibition of this pathway by knocking down β-catenin would affect OCT-4 in an opposite way. We collected the transfected cells and performed Western blotting and real-time PCR analysis. As expected, there was a prominent decline of OCT-4 at the protein level (Fig. 4E), and there was a decrease at the mRNA level (Fig. 4F).

Discussion

Cancer is most likely a disease of stem cells. More and more studies suggest that CSCs play a role in the formation and progression of the tumor, such as chemoradiation resistance, metastasis, and recurrence [12,13]. Therefore, CSCs may be a new target in cancer therapy.

CSCs have been isolated from various kinds of tumors, such as leukemia [14], breast [15], colon [16], brain [17] and prostate carcinomas [18]. Although research about CSCs has been in bulk, many difficulties still remain in the CSC therapy. The dominate one is lacking of an accepted and universal cancer stem cell marker. OCT-4, also known as POU5F1, is a key regulator of self-renewal and differentiation in embryonic stem cells [19]. Recently, OCT-4 has been detected in many tumors [20–24], and it is regarded as a CSC marker. Levings et al. [25] found that OCT-4 identifies tumor-initiating cells in osteosarcoma. Chiou et al. [26] suggested that OCT-4 plays an important role in maintaining the self-renewal property of CSCs in oral squamous cell carcinoma. More recently, Chen et al. [27] found that knocking down the expression of OCT-4 in CD133(+) lung cancer cells can significantly inhibit the abilities of tumor invasion and colony formation as well as the chemoradio resistance. Therefore, OCT-4 appears to be a key player in maintaining the stem cell properties of CD133(+) lung cancer cells. A new study showed that CD133 alone cannot be used as a cancer stem cell marker for A549 cells. CD133(−) cells contain similar cancer stem cells as well as CD133(+) cells [28]. So we deduced OCT-4 should be a more significative marker for A549 lung cancer stem cells. Vera [29]found that drug selected cancer cells have all the properties of CSCs, such as high clonogenic efficiency, enrichment in SP phenotype, expressing markers associated with CSCs and low expression of epithelial differentiation markers (cytokeratins 8/18). In our study, after treatment with DDP (5 μg/ml) for 2 days, the expression of OCT-4 in living A549 cells was notably up-regulated. So we deduced OCT-4 should be a more significative marker for A549 lung cancer stem cells.

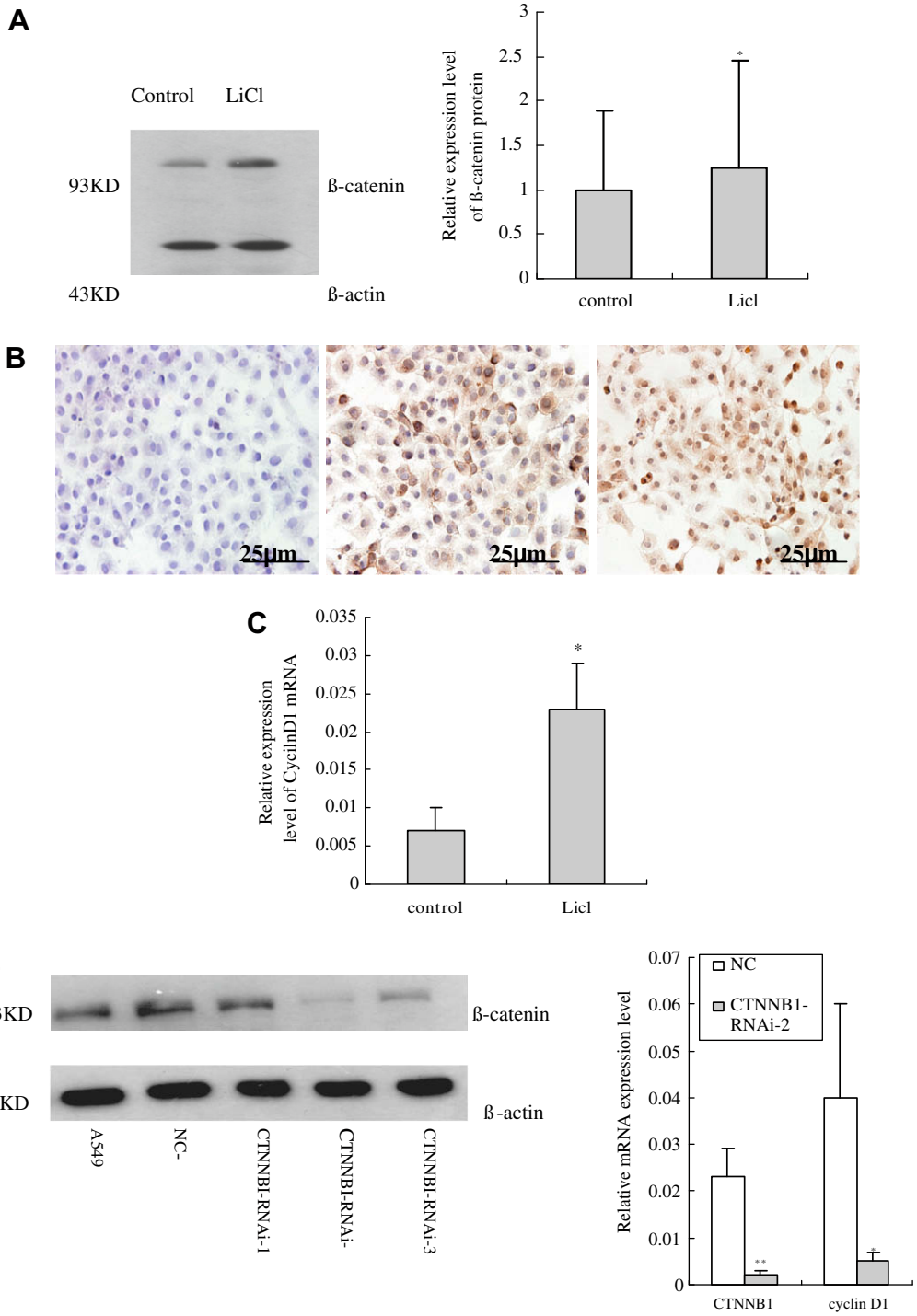

Fig. 2. Activation and inhibition of Wnt/β-catenin pathway. (A) Western blot detected an increased expression of β-catenin (about 93 KD) in A549 cells after stimulation of LiCl (10 mM for 24 h), and β-actin was used as a loading control, he expression of β-catenin was enhanced.*$P < 0.05$. (B) With immunocytochemistry, there was no nonspecific binding of the antibody in negative control with PBS, staining for β-catenin was mainly in the cytoplasm and cell membranes of control A549 cells. And in A549 cells stimulated with LiCl (10 mM and 24 h), β-catenin was accumulated in the cytoplasm and translocated to the nucleus. (C) The expression of cyclin D1 mRNA, regarded as a target gene of Wnt/β-catenin pathway, was increased as a result of LiCl treatment. *$P < 0.05$. (D) After transfection, especially in CTNNB1-RNAi-2 transfected cells Western blot showed the down-regulated protein expression of β-catenin, as well as the expression of β-catenin and cyclin D1 at the mRNA level. *$P < 0.05$, **$P < 0.01$.

The Wnt/β-catenin pathway is one of the most important signal transduction pathways in tumor genesis and progression. Recently, this pathway has been implicated in the maintenance of stem and progenitor cells in adult tissues of skin, blood, gut, prostate, muscle and the nervous systems [30]. CSCs have many common characteristics with tissue stem cells, so many studies are carried out to test whether this pathway is also involved in the maintenance of CSCs. Recent studies have demonstrated that the Wnt/β-catenin pathway influences the maintenance of CSCs in many tumors [3–11].

In our study, an increased expression of β-catenin was detected in DDP selected A549 cells, suggesting that the Wnt/β-catenin pathway plays an important role in CSCs. Then we used LiCl, a GSK-3β inhibitor, as an activator of the Wnt signaling pathway.

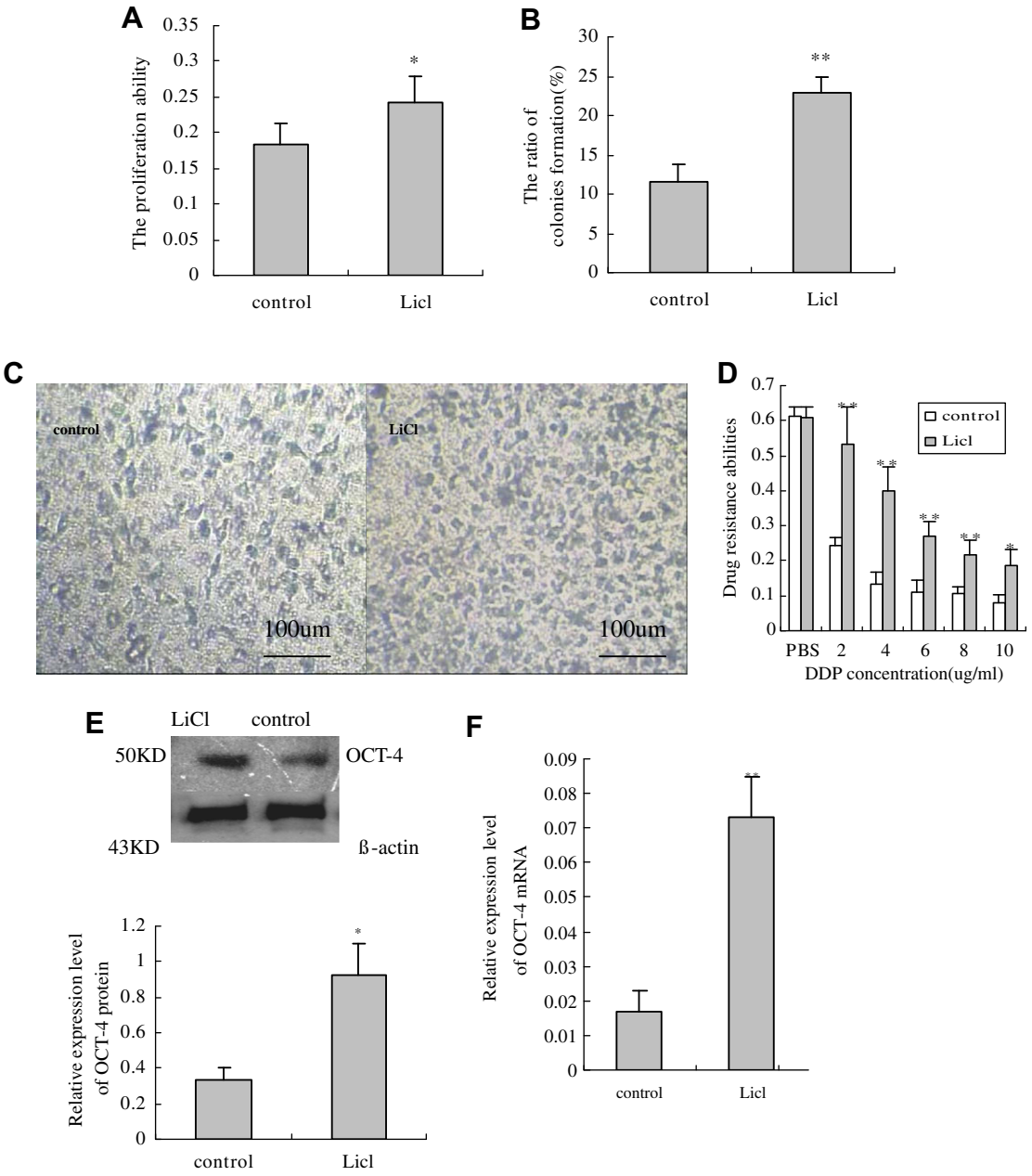

Fig. 3. Activation of Wnt/β-catenin pathway leads to up-regulation of stem cell properties and stem cell marker OCT-4. (A) The proliferation ability was up-regulated. $^{*}P < 0.05$. (B) The clone formation ability was increased. $^{**}P < 0.01$. (C) The migration ability was enhanced. $^{**}P < 0.01$. (D) The drug resistance ability was enhanced in all concentrations we examined. $^{*}P < 0.05$, $^{**}P < 0.01$. (E) By Western-blot, we detected an increased expression of OCT-4 after LiCl stimulation.$^{*}P < 0.05$. (F) The expression of OCT-4 was increased at the mRNA level through real-time PCR. $^{**}P < 0.01$.

After incubation of A549 cells with LiCl (10 mM for 24 h), we detected an enhanced expression of β-catenin by Western blotting analysis, as well as a clear accumulation of β-catenin in the cytoplasm and a translocation to the nucleus by immunocytochemistry. But this up-regulation was not observed at the mRNA level (data not shown), suggesting that the underlying mechanism is not transcriptional activation but the reduced degradation of β-catenin via the destruction complex [31]. The accumulation of β-catenin resulted in a significant induction of typical Wnt target gene cyclin D1 (about three times). So we conclude that the stimulation with LiCl (10 mM for 24 h) can activate the Wnt/β-catenin pathway efficiently.

When the pathway was activated, we studied the CSC properties such as the proliferation, clone formation, migration and drug resistance abilities. Strikingly, we observed a dramatic increase in proliferation, migration and clone formation abilities, which was also founded in the DDP resistance experiment. To our surprise, an up-regulation of OCT-4 was detected at both mRNA and protein levels. Conversely, when the pathway was blocked by knocking down the key molecule β-catenin, we found an obvious decrease in proliferation, clone formation, migration and drug resistance abilities, as well as a decline in the expression of OCT-4 at the protein level and the mRNA level. In a previous study, Wen et al. [10] found that the Wnt/β-catenin signaling pathway plays an important role in the maintenance of the stem cell properties of $OV6^{+}$ liver CSCs. Ilaria et al. [11] found that the Wnt/β-catenin signaling pathway contributes to the maintenance of CD34 cutaneous CSCs.

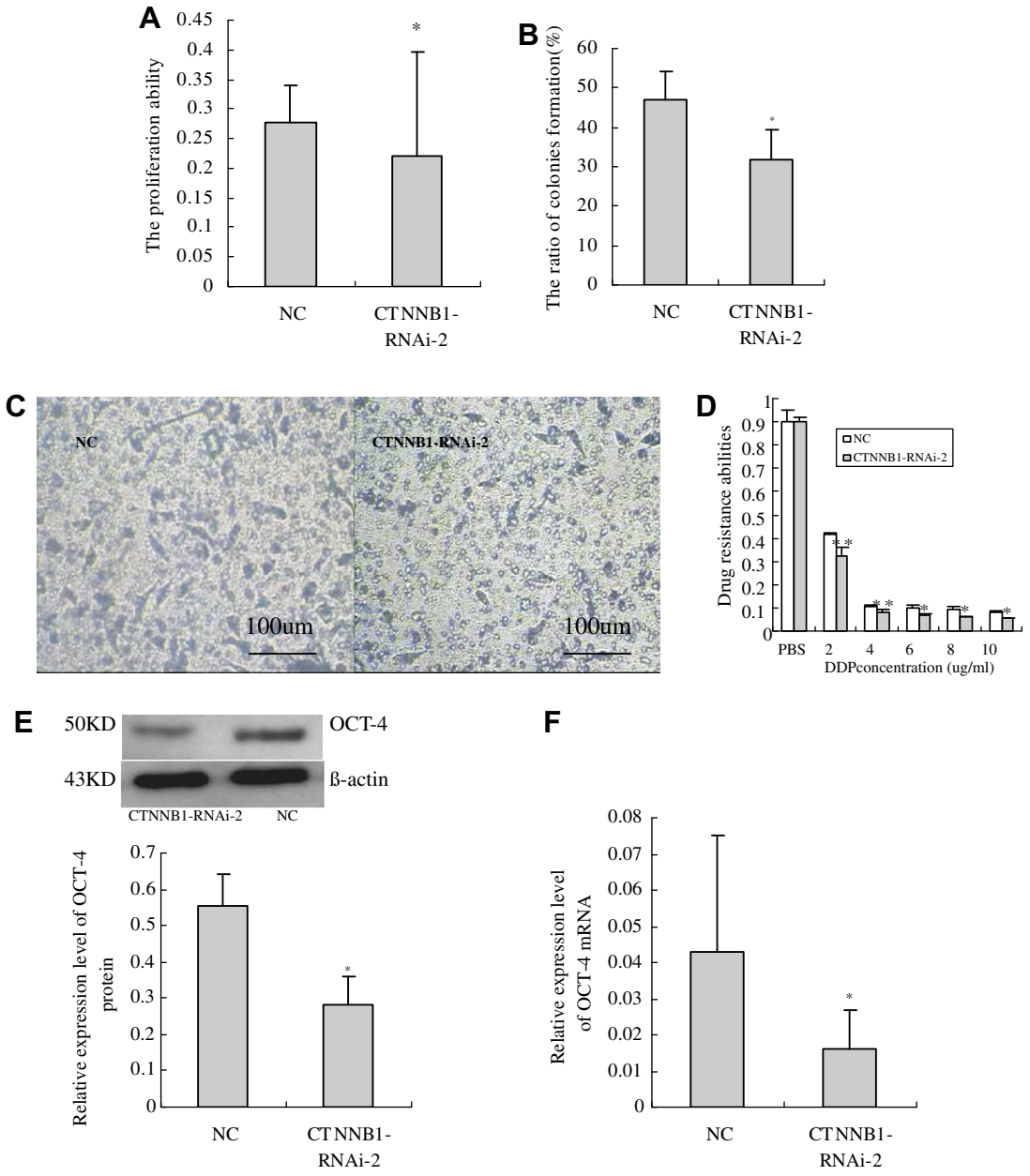

Fig. 4. Inhibition of Wnt/β-catenin pathway leads to downregulation of stem cell properties and stem cell marker OCT-4. (A) The proliferation ability was inhibited. $^{**}P < 0.01$. (B) The clone formation ability was inhibited. $^{*}P < 0.05$. (C) The migration ability was inhibited. $^{**}P < 0.01$. (D) The drug resistance ability was inhibited. $^{*}P < 0.05$, $^{**}P < 0.01$. (E) The decreased expression of OCT-4 at protein level was detected by Western blot analysis. $^{*}P < 0.05$. (F) There was also a decline of OCT-4 at the mRNA level. $^{*}P < 0.05$.

From our study, we conclude that Wnt/β-catenin may contribute to the regulation of OCT-4 lung CSCs. But the mechanism is still unclear and required more efforts in future.

For the first time, our study showed that OCT-4, a stem cell marker, was up-regulated in drug selected A549 cells, and it also may contribute to the maintenance of the CSCs. The expression of OCT-4 at both mRNA and protein levels was regulated by the Wnt/β-catenin pathway. We also found that the Wnt/β-catenin signaling pathway regulated the cancer stem cell properties of the A549 cells, such as proliferation, clone formation, migration and drug resistance abilities. We conclude that this regulation may partially result from the regulation of OCT-4 CSCs. Therefore, blocking the Wnt signal transduction pathway by applying the pathway antagonists or other methods may be an efficient therapy method for lung adenocarcinoma, and the targets is likely on the pathway itself as well as in the OCT-4 CSCs.

Acknowledgment

This work is supported by funds from 2009 Shandong provincial scientific and technological development projects (Grant No. 2009GG20002044).

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