δ-Catenin promotes tumorigenesis and metastasis of lung adenocarcinoma

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Abstract. δ-Catenin coded by gene CTNND2 has been found to be overexpressed in various types of cancers, including prostate, breast, lung and ovarian cancers. However, the function of δ-catenin in lung carcinoma remains largely unknown. In the present study, we revealed that δ-catenin acts as an oncogene promoting the malignancy of lung adenocarcinoma. When δ-catenin proteins of Lewis lung cells were depleted by knocking out *Ctnnd2* via CRISPR/Cas9 technology, the cells lost the tumorigenic and metastatic abilities *in vivo*. Consistently, overexpression of *Ctnnd2* enhances the subcutaneous tumorigenesis and distant metastasis of Lewis lung cells *in vivo*. However, δ-catenin promotes cell proliferation and cell cycle progression of Lewis lung cells. Mechanistically, δ-catenin enhances G1-S phase transition in cooperation with canonical Wnt signaling in Lewis lung cells. Moreover, δ-catenin promotes oncosphere formation of lung adenocarcinoma cells and is associated with the expression of cancer stem cell markers, which indicates δ-catenin enhances colonization and invasion via cancer stem cell maintenance. Taken together, our data suggest that δ-catenin may serve an important role in the malignancy of lung adenocarcinoma through activating canonical Wnt signaling and cancer stem cell maintenance. Our research indicates that δ-catenin can be a new potential target for the treatment of lung adenocarcinoma.

Introduction

Lung carcinoma is the leading cause of cancer-related death, for which the 5-year survival rate for lung cancer patients is less than 20%. Lung cancer can be divided into small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC comprises ~85% of all lung cancer cases (1). Epidermal growth factor receptor (EGFR) mutation has been found in lung cancer for years and its kinase inhibitors are used for clinical treatment. However, it is disappointing that the efficacy is limited (2). Therefore, research has focused on new biomarkers for lung cancer diagnosis and therapies are promising.

δ-Catenin coded by the gene CTNND2 is recognized to be primarily expressed in the brains of normal people (3,4). However, in recent years, various research has revealed that δ-catenin also can be a biomarker for cancers, since it has been found to be overexpressed in various types of cancers, including prostate, breast, lung and ovarian cancer (3,5-7). δ-Catenin can be expressed as different variants in different type of cancers (3). In some cell lines of ovarian, breast and esophageal cancer, a full length δ-catenin transcript is overexpressed. However, in other cancer types, lung cancer included, δ-catenin species with N- or C-terminal truncations are expressed. The distributions of different δ-catenin species are also different. N-terminus of δ-catenin is prominently associated with the cytoplasmic distribution, whereas the carboxyl-terminus of δ-catenin can be subject to significant translocation to the nuclei (3).

Overexpression of full-length or truncated δ-catenin is usually associated with malignancy and poor prognosis. In prostate cancer, overexpression of δ-catenin is due to -9 G>A mutation in 5'-UTR promoters (6). Moreover, truncated mutations of δ-catenin found in prostate cancer have been reported to promote cancer cell survival via metabolic reprogramming, hypoxia pathways and Wnt signaling response (8). It has been also reported that δ-catenin promotes the epithelial cell marker, E-cadherin processing to regulate malignancy in prostate cancer (9). In ovarian cancer, δ-catenin is overexpressed and associated with advanced stage. δ-catenin regulates the ovarian cancer cell proliferation, invasion and cell cycle (7). In non-small cell lung cancer, δ-catenin has been reported to be expressed much higher in malignant tissues than in benign tissues. Moreover, δ-catenin is expressed in the cytoplasm, correlated with high Dvl3, CD31 and VEGF expression, suggesting that δ-catenin may be related to angiogenesis and lymphangiogenesis. High expression of δ-catenin in lung cancer is associated with poor prognosis (10,11). However, Dai *et al* reported

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that δ-catenin may affect lung cancer prognosis through δ-catenin/Kaiso pathway (5), but the detailed mechanisms of how δ-catenin enhances the malignancy of lung cancer remain largely unknown.

In the present study, we investigated the roles of δ-catenin in the development of lung adenocarcinoma via Lewis lung cell tumorigenesis and metastasis models. We found that δ-catenin enhances Lewis lung cell subcutaneous tumorigenesis and metastasis *in vivo*. Our data reveal that δ-catenin enhances Lewis lung cell proliferation and cell cycle progression. δ-Catenin may contribute to G1-S phase transition in cooperation with canonical Wnt signaling in Lewis lung cells. Furthermore, δ-catenin promotes the oncosphere formation of Lewis lung cells. The expression of cancer stem cell marker, CD133 and *Aldh1*, may be modulated by δ-catenin. In the present study, novel mechanisms of how δ-catenin promotes lung adenocarcinoma malignancy are revealed. Our data suggest that δ-catenin may be a biomarker and a new therapy target for lung adenocarcinoma.

Materials and methods

Plasmids, antibodies and reagents. The mouse *Ctnnd2* sgRNAs were subcloned into pLVR-sgRNA-CMV-Cas9-GFP and pU6gR-MCS2-CMV-Cas9-SV40-mCherry plasmids, separately (GeneCopoeia, Guangzhou, China). The sequences of sgRNA1 were: forward, 5'-ATCCGCCGGGCGCCAGGG CGGCCC-3' and reverse, 5'-AAACGGGCCGCCCTGGCGC CCGGC-3'; sgRNA2 forward, 5'-ATCCGCGGCGGGTGCA TGTTCGCC-3' and reverse, 5'-AAACGGCGAACATGCAC CCGCCGC-3'. The human CTNND2 were subcloned into pEZ-M02 (EX-Z7373-M02-5; GeneCopoeia). Antibodies were used in the present study: monoclonal anti-δ-catenin (ab54578; Abcam, Cambridg, UK); anti-CD133 (11-1331-80; eBioscience, San Diego, CA, USA); anti-β-catenin (844602; BioLegend, San Diego, USA); control IgG (I5381; Sigma-Aldrich, St. Louis, MO, USA) and anti-β-actin (A1978; Sigma). Tris-HCl, NaCl and other chemicals were from Sigma.

CRISP/Cas9 system. The two sgRNAs were subcloned into pLVR-sgRNA-CMV-Cas9-GFP and pU6gR-MCS2-CMV-Cas9-SV40-mCherry plasmids, separately (GeneCopoeia). Transfection of plasmids into Lewis lung cells were performed with polyethylenimine (Polysciences, Inc., Warminster, PA, USA). G418 (20 μg/ml) (Sigma) was used to select cells in which pLVR-sgRNA-CMV-Cas9-GFP and pU6gR-MCS2-CMV-Cas9-SV40-mCherry plasmids were expressed. The Cas9 protein could break the genome of *Ctnnd2* at two different sites guided by the two different sgRNAs, resulting in an ~300 bp DNA deleted fragment. Monoclone cells were picked out to extract genome DNAs. Monoclone cells with the targeted genes knocked out were identified by PCR. The primer sequences were: forward, 5'-CGGGAGGAGCCTCGCTCT-3' and reverse, 5'-CGGGAGGAGCCTCGCTCT-3'.

Cells and transfection. Lewis lung and A549 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The ATCC number of Lewis lung cells was CRL-1642. The ATCC number of A549 cells was CCL-185. The cells were purchased April 5, 2014. Lewis lung cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA); A549 cells in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% FBS (HyClone). Transfection of DNA plasmids into Lewis lung and A549 cells were performed with polyethylenimine (Polysciences, Inc.), and the stable cell lines were selected with 20 μg/ml G418 (Sigma).

Mice. B6/C57 mice used in the present study were bred and maintained in a specific pathogen-free animal facility at Fujian Medical University. Mice were euthanized with carbon dioxide asphyxiation. All animal experiments were approved by the Animal Ethical Committee of Fujian Medical University.

Tumorigenesis and metastasis. Cells ($5x10^5$) of WT and *Ctnnd2* knock out LLCs were subcutaneously injected into the abdomen of each B6/C57 mouse to make the subcutaneous tumorigenesis mouse model. Tumor sizes were measured by tumor length and width using clippers and then tumor volume was calculated using the formula V = (L x W x W)/2, where V is tumor volume, W is tumor width, and L is tumor length. Cells ($1x10^6$) of LLCs were injected into the tail veins of B6/C57 mice to make the metastasis model. LLCs tend usually to transfer to the lungs and bones. Survival curves of mice were calculated by GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Hematoxylin and eosin stain. The tissues are embedded in paraffin to be cut into 3 μm tissue sections. Tissue sections were dewaxed with xylene. Then, sections were rehydrated with 100, 95 and 75% alcohol gradients. Hematoxylin was stained for 20 min, and then sections were differentiated with 1% hydrochloric acid for 30 sec. After 15 min of PBS blue staining, eosin was stained for 3 min. After rinsing, sections were dehydrated with a gradient of 95-100% alcohol. The sections were cleared with xylene two times. Then, sections were mounted with a neutral resin.

Flow cytometry. Flow cytometric analysis was performed using BD FACS C6 Flow Cytometer. To monitor the cell cycle, after cells were fixed with 70% ethanol overnight, 5 μg/ml propidium iodide was used for staining. Then, 20,000 cells were collected for analysis. To monitor cell surface expression of CD133, anti-CD133-FITC antibody and control IgG were used for staining, and 5,000 cells were collected for analysis. The percentage of each phase in cell cycle and the expression of CD133 were analyzed by FlowJo 7.6.1 software.

Quantitative real-time PCR. Total cell RNA was isolated with TRIzol (Invitrogen), and cDNA was synthesized with ReverTra Ace (Promega, Madison, USA). Real-time PCR was performed with an ABI QuantStudio 5 system. The expression level of genes was measured using the comparative Ct method. Expression values were normalized to GAPDH expression. The primer sequences were as follows. *Ctnnd2* (mouse): forward, 5'-CCTCCGAATAGACAATGACC-3' and reverse, 5'-GAGAAGCAGCCTTGACCAC-3'; CTNND2 (human): forward, 5'-GCTCCGAATAGACAATGACC-3' and reverse, 5'-GAGATGCAGCCTTGACCAC-3'; c-myc forward,

5'-ATGCCCCTCAACGTGAACTTC-3' and reverse, 5'-GTCGCAGATGAAATAGGGCTG-3'; p-21: forward, 5'-GTGATTGCGATGCGCTCATG-3' and reverse, 5'-TCTC TTGCAGAAGACCAATC-3'; Gapdh forward, 5'-CATGGCC TTCCGTGTTCCTA-3' and reverse, 5'-CCTGCTTCACCAC CTTCTTGAT-3'.

Immunoblotting and immunoprecipitation assay. Cells were lysed with TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, pH 7.5). For immunoblotting assay, cell lysates were mixed with 4X loading buffer (40 mM Tris-HCl, 200 mM DTT, 4% SDS, 40% glycerol, 0.032% bromophenol blue, pH 8.0). The samples were run with 4% stacking gel and 10% separating gels. Then, proteins on the gels were transferred to nitrocellulose filter membranes for incubation with antibodies. The membrane exposure was carried out with Thermo Pierce ECL and FluorChem E (Protein Sample). For immunoprecipitation assay, cell lysates were incubated with β-catenin antibody or control IgG overnight. Then, protein A agarose (Thermo Fisher Scientific, Inc., Waltham, MA, USA) beads were added to bind antibodies and targeted proteins for 2 h. Protein A agarose bead elution was collected for immunoblotting.

Cell proliferation assay. Equal amount of cells were placed in the 6-well plates, and then subjected to different treatments. Every day the cell numbers were counted.

Matrigel 3D cultures. Cells in 2D cultures were trypsinized and resuspended in media, and 20,000 cells were plated on each 100 μl Matrigel (BD BioSciences, New Jersey, NY, USA). Media containing 10% FBS was added for cell growth (12).

Cell migration and wound-healing assays. Cell migration and wound-healing assays were as previously described (13).

Statistical analysis. The Student's t-test, one-way ANOVA, Wilcoxon rank sum and log-rank test were used. $P<0.05$ were considered statistically significant.

Results

δ-Catenin enhances Lewis lung cell tumorigenesis and metastasis in vivo. Lewis lung carcinoma originate from a spontaneous lung tumor of a B6/C57 mouse (14,15). In the present study, to study the function of δ-catenin in lung cancer, we established *Ctnnd2* knockout Lewis lung cells (LLCs) via the CRISPR/Cas9 system (16,17). SgRNAs were designed in exon1 of *Ctnnd2* genome and ~300 bps DNA fragment may be deleted. According to our results, all four monoclones of Lewis lung cells contained *Ctnnd2* genome DNA fragment deletion (Fig. 1A). However, only KO2 and KO3 cell lines were chosen for the following research, since compared with KO1 and KO4 cell lines, mRNA levels of KO2 and KO3 cell lines were reduced (data not shown). Furthermore, proteins of δ-catenin were abolished in KO2 and KO3 cell lines (Fig. 1B). Next, WT and *Ctnnd2* knockout LLCs were subcutaneously injected into B6/C57 mice to examine their tumor formation ability. WT LLCs formed tumors with average volumes to ~1 cm^3 within 40 days, but *Ctnnd2* knockout LLCs could not form tumors, suggesting significant roles of δ-catenin in tumorigenesis (Fig. 1C). Furthermore, we injected WT LLCs into tail veins of B6/C57 mice, leading to lung and bone metastasis. However, *Ctnnd2* knockout LLCs did not have metastatic ability (Fig. 1D). Lung and bone metastasis were confirmed by tissue anatomy and histology analyses (Fig. 1D and E). Moreover, lung and bone metastasis of WT LLCs caused mice to die in <20 days but mice injected with *Ctnnd2* knockout LLCs survived for a long time (Fig. 1F). Results above indicated that loss of δ-catenin inhibited the tumorigenesis and metastasis of Lewis lung cells.

Consistently, overexpression of *Ctnnd2* enhances subcutaneous tumorigenesis and metastasis of LLCs. We established a stable LLC cell line with *Ctnnd2* overexpressed driven by CMV promoter (Fig. 1G). We used the human CTNND2 gene cDNA to transfect mouse lung tumor cells, since the mouse *Ctnnd2* cDNA and human CTNND2 cDNA are highly conserved, particularly the key regions. Human δ-catenin and mouse δ-catenin have alike functions. *Ctnnd2* overexpressed LLCs could form larger tumors subcutaneously than empty vector expressed LLCs at the same time point (Fig. 1H). Tail vein injection of either empty vector expressed LLCs or *Ctnnd2* overexpressed LLCs led to lung or bone metastasis. However, the metastasis of *Ctnnd2* overexpressed LLCs was much faster, resulting in shorter survival time of mice (Fig. 1I). Taken together, our data suggested that *Ctnnd2* coded δ-catenin promoted tumorigenesis and metastasis of Lewis lung cells *in vivo*.

δ-Catenin enhances Lewis lung cell proliferation. According to the results above, δ-catenin promotes tumor growth of Lewis lung cells *in vivo*. Therefore, we sought to exam whether δ-catenin enhanced the proliferation or cell cycle progression of Lewis lung cells. Our results showed that *Ctnnd2* knockout could make the cell cycle of Lewis lung cells arrest in G1 phase but *Ctnnd2* overexpression could enhance the growth of Lewis lung cells (Fig. 2A and B). Furthermore, c-myc, which promotes cell proliferation and G1-S phase transition (18-20), was downregulated when *Ctnnd2* was knocked out while it was upregulated with *Ctnnd2* overexpressed (Fig. 2C and D). However, the CDK inhibitor, p21 (21-23), was upregulated when *Ctnnd2* was knocked out (Fig. 2E). Taken together, δ-catenin promoted cell cycle progression and proliferation via c-myc and p21 in Lewis lung cells.

δ-Catenin promotes G1-S phase transition of Lewis lung cells in cooperation with canonical Wnt signaling. δ-Catenin was reported to promote prostate cancer malignancy by enhancing Wnt signaling pathway (8). Wnt signaling enhances cell proliferation by promoting cell cycle progression (24). Thus, we sought to analyze whether δ-catenin regulated the cell cycle of Lewis lung cells in cooperation with Wnt3a signaling. We found that *Ctnnd2* knockout attenuated G1-S phase transition induced by Wnt3a CM (Fig. 3A). Consistently, overexpression of *Ctnnd2* facilitated Wnt3a signaling to promote cell cycle progression (Fig. 3B). In our results, sub-G1 and sub-G2 phase were deducted, thus, the sum of G1, S and G2 was <100%. The activation of Wnt3a signaling results in the nuclear accumulation of β-catenin, which regulates the expression of downstream

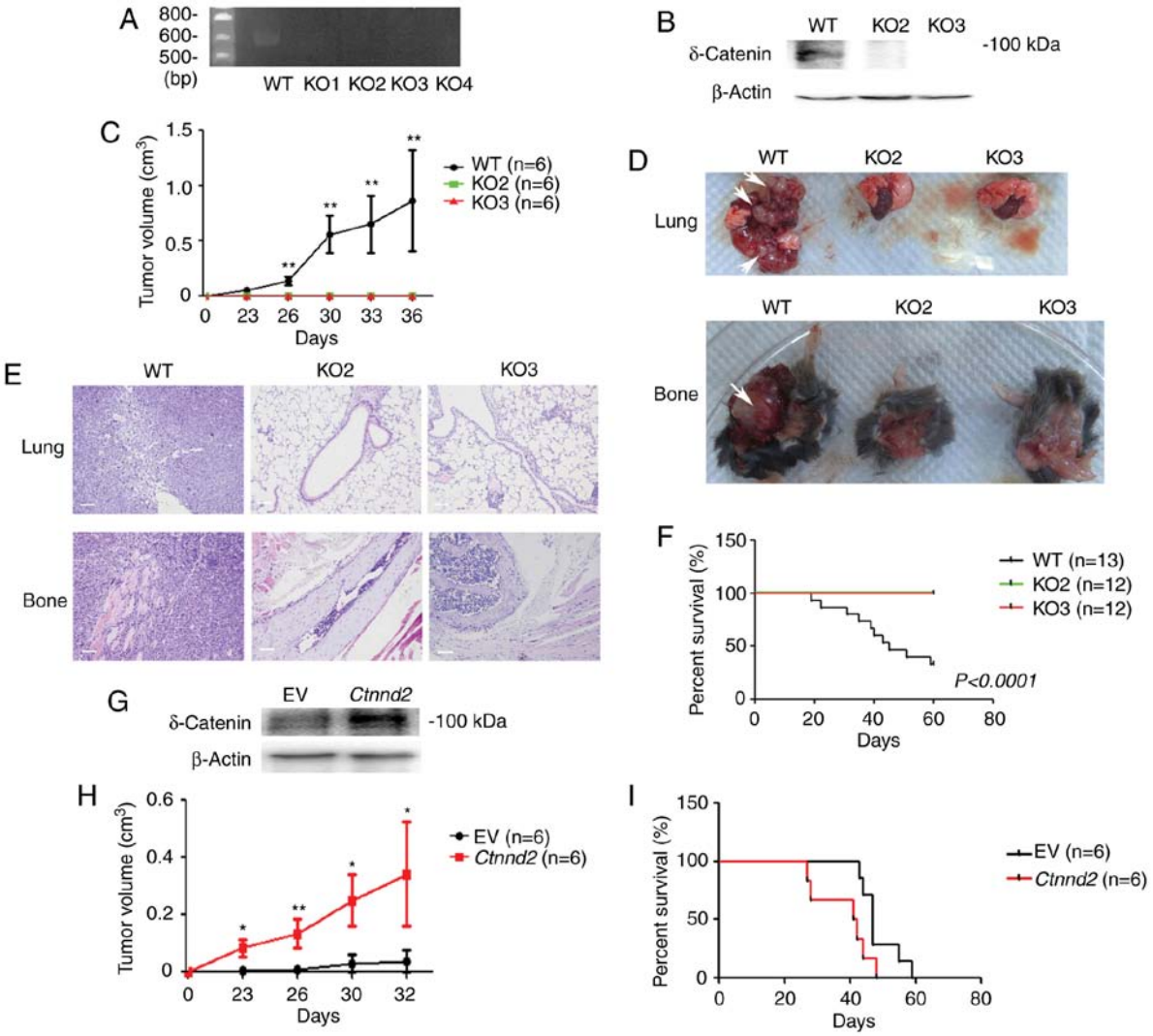

Figure 1. δ-Catenin enhances Lewis lung cell tumorigenesis and metastasis *in vivo*. (A) Genomes of WT and four *Ctnnd2* knockout monoclones of LLC cells were extracted by PCR. The primers used are in Table I. (B) Cell lysates of LLC cells of different genome types by immunoblotting. (C) LLC cells ($5x10^5$) of different genome types were subcutaneously injected into B6/C57 mice. Tumor sizes were measured and tumor volumes were calculated at the indicated time to draw the curves ($^*P<0.05$, $^{**}P<0.01$). (D) LLC cells ($1x10^6$) of different genome types were injected into the tail veins of B6/C57 mice. Tissue samples of lungs and bones from B6/C57 mice were collected. Images of representative lung colonization of LLC cells *in vivo* were captured. White arrows indicated metastatic nodules (upper). Images of representative bone metastasis of LLC cells *in vivo* were captured. White arrows indicate metastatic lesions (lower). (E) Representative histology analyses of lungs and bones taken from WT or *Ctnnd2* knockout LLC cells injected mice. Scale bars, 10 μM. (F) LLC cells ($1x10^6$) of different genome types were injected into the tail veins of B6/C57 mice. Death of mice were recorded and survival curves were drawn ($^*P<0.05$, $^{**}P<0.01$). (G) Cell lysates of empty vector (EV) or *Ctnnd2* overexpressed LLC cells were subjected to immunoblotting. (H) Empty vector (EV) or *Ctnnd2* overexpressed LLC cells ($5x10^5$) were injected subcutaneously into B6/C57 mice, tumor sizes were measured and tumor volumes were calculated at the indicated time to draw the curves ($^*P<0.05$, $^{**}P<0.01$). (I) Empty vector (EV) or *Ctnnd2* overexpressed LLC cells ($1x10^6$) were injected into the tail veins of B6/C57 mice. Death of mice were recorded and survival curves were drawn ($^*P<0.05$, $^{**}P<0.01$).

Table I. Sequences of primers used in genome identification and qRT-PCR.

Name (mouse)	Forward 5'-3'	Reverse 5'-3'
Ctnnd2	CCTCCGAATAGACAATGACC	GAGAAGCAGCCTTGACCAC
c-myc	ATGCCCCTCAACGTGAACTTC	GTCGCAGATGAAATAGGGCTG
p21	GTGATTGCGATGCGCTCATG	TCTCTTGCAGAAGACCAATC
Gapdh	CATGGCCTTCCGTGTTCCTA	CCTGCTTCACCACCTTCTTGAT
Ctnnd2-genome	CGGGAGGAGCCTCGCTCT	CGGCGGTGAGCAAGAGGA

target genes (25). GSK3β is known to be a classical Wnt signaling inhibitor by phosphorylating β-catenin to degradation (26). GSK3β inhibitor, lithium chloride (LiCl), induces the accumulation of β-catenin (27). Our data demonstrated that

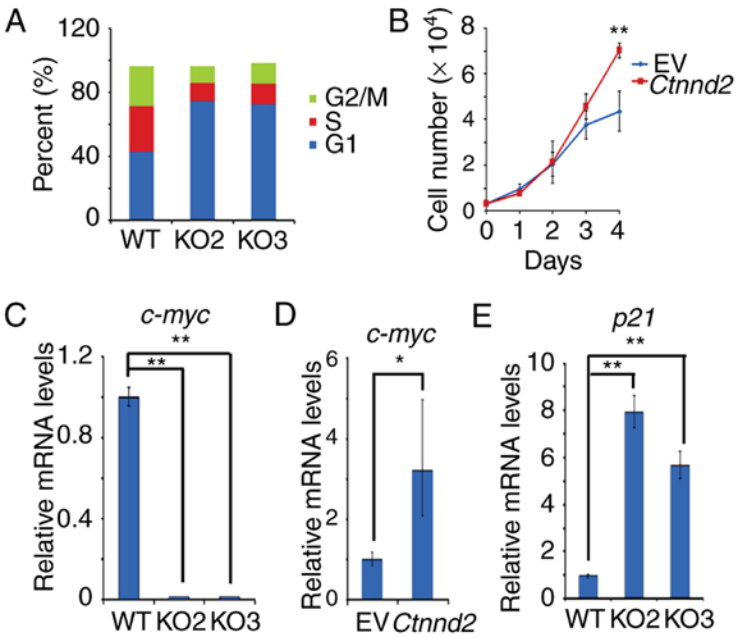

Figure 2. *Ctnnd2* promotes the LLC cell cycle progression and proliferation. (A) The cell cycles of WT and *Ctnnd2* knockout LLC cells were analyzed by flow cytometry. (B) Cells (3,000) of empty vector and *Ctnnd2* overexpressed LLC cells were plated at day 0 and cell numbers were counted every day to draw the proliferation curve ($^{*}P<0.05$, $^{**}P<0.01$). (C) The mRNA levels of c-myc in WT and *Ctnnd2* knockout LLC cells were examined by qRT-PCR. (D) The mRNA levels of c-myc in empty vector (EV) and *Ctnnd2* overexpressed LLC cells were examined by qRT-PCR. (E) The mRNA levels of p21 in WT and *Ctnnd2* knockout LLC cells were examined by qRT-PCR.

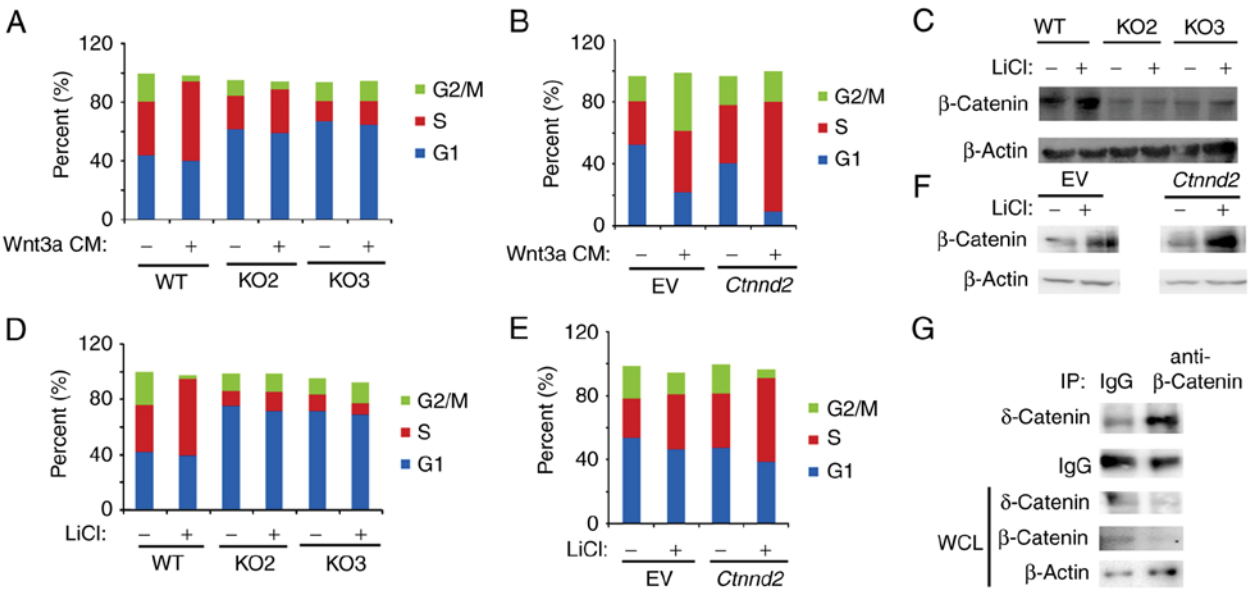

Figure 3. δ-Catenin facilitates G1-S phase transition of Lewis lung cells in cooperation with canonical Wnt signaling. (A) After Wnt3a conditional medium (CM) treatment for 24 h, the cell cycles of WT and *Ctnnd2* knockout LLC cells were analyzed by flow cytometry. (B) After Wnt3a conditional medium (CM) treatment for 24 h, the cell cycles of empty vector (EV) and *Ctnnd2* overexpressed LLC cells were analyzed by flow cytometry. (C) After 1 μM LiCl treatment for 24 h, cell lysates of WT and *Ctnnd2* knockout LLC cells were subjected to immunoblotting. (D) After 1 μM LiCl treatment for 24 h, the cell cycles of WT and *Ctnnd2* knockout LLC cells were analyzed by flow cytometry. (E) After 1 μM LiCl treated for 24 h, the cell cycles of empty vector (EV) and *Ctnnd2* overexpressed LLC cells were analyzed by flow cytometry. (F) After 1 μM LiCl treatment for 24 h, cell lysates of empty vector (EV) and *Ctnnd2* overexpressed LLC cells were subjected to immunoblotting. (G) LLC cell lysates were subjected to IP with control IgG or anti-β-catenin antibody, followed by anti-δ-catenin IB. Protein expression was verified with whole cell lysates (WCL).

Ctnnd2 knockout could abolish the accumulation of β-catenin induced by LiCl in Lewis lung cells (Fig. 3C). Such as Wnt3a CM, LiCl enhanced G1-S phase progression in Lewis lung cells, which was diminished when *Ctnnd2* was knocked out (Fig. 3D). However, overexpression of *Ctnnd2* resulted in β-catenin proteins and S phase accumulation of Lewis lung cells induced by LiCl (Fig. 3E and F). δ-Catenin may enhance the Lewis lung cell response to Wnt3a signaling through the interaction between β-catenin and δ-catenin (Fig. 3G), which needs further investigation.

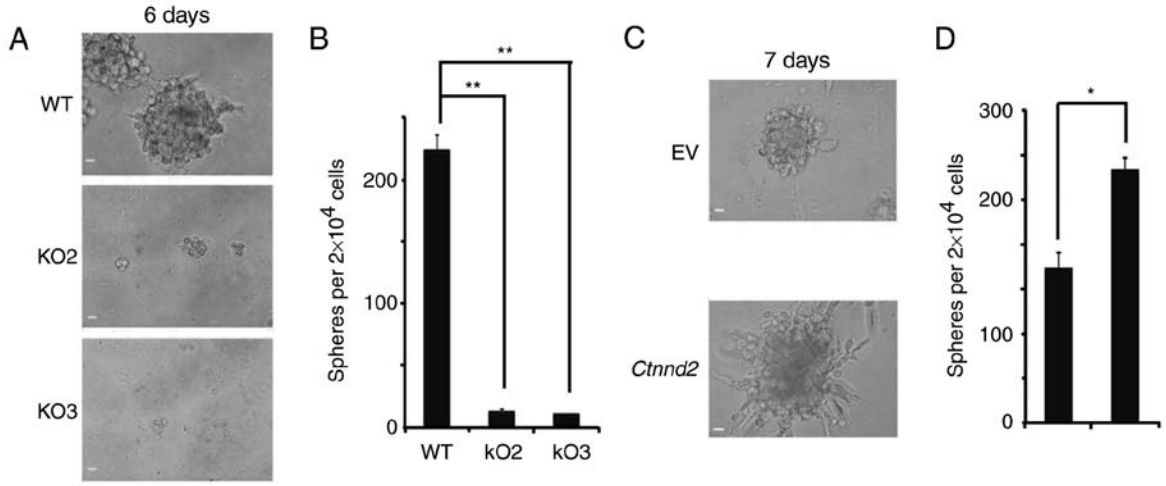

Figure 4. δ-Catenin enhances the oncosphere formation of Lewis lung cells. (A) LLC cells of different genome types were cultured in Matrigel to form spheres. Images were captured after six days. Scale bars, 10 μM. (B) Numbers of spheres in (A) were counted (*P<0.05, **P<0.01). (C) Empty vector (EV) and *Ctnnd2* overexpressed LLC cells were cultured in Matrigels to form spheres. Images were captured after seven days. Scale bars, 10 μM. (D) Number of spheres in (C) was counted (*P<0.05, **P<0.01).

δ-Catenin enhances the oncosphere formation of Lewis lung cells. Tumor cells are highly heterogeneous in both phenotypes and functions. Implanting tumors usually derive from subpopulations in the highly tumorigenic state. These subsets of tumor cells, called cancer stem cells, can proliferate asymmetrically, sustain tumorigenesis and inherent heterogeneity (28,29). As Lewis lung cells totally lost their tumorigenic and metastatic ability after *Ctnnd2* was knocked out, we supposed that δ-catenin protein contributed to the maintenance of cancer stem cells. 3D cultures of tumor cells can mimic the *in vivo* growth environment for tumors. Oncospheres in 3D cultures can show the activity of cancer stem cell subsets (30,31). WT Lewis lung cells could form oncospheres under non-adhesive 3D culture conditions, but when *Ctnnd2* was knocked out, Lewis lung cells formed hardly any oncospheres in Matrigel (Fig. 4A and B). With *Ctnnd2* overexpressed, Lewis lung cells could form more and larger oncospheres (Fig. 4C and D). Moreover, we observed that overexpression of *Ctnnd2* made Lewis lung cells form highly protrusive structures with a compact spherical core, indicating *Ctnnd2* overexpressed cancer cells had higher invasive ability (32) (Fig. 4C). Results above revealed that, δ-catenin enhanced Lewis lung cell colonization and invasion. Overexpression of δ-catenin indicated higher tumorigenic and metastatic ability in Lewis lung cells.

δ-Catenin enhances the expression of cancer stem cell markers in Lewis lung cells. Notably, *Ctnnd2* was upregulated in the oncospheres compared to monolayer cultures of the same samples (Fig. 5A). Oncospheres in 3D cultures can show the activity of cancer stem cell subsets (30,31). We supposed that the expression of *Ctnnd2* may be associated with the expression of cancer stem cell markers. Later, we measured the expression of lung cancer stem cell maker CD133 by flow cytometry when *Ctnnd2* was knocked out or overexpressed (33). CD133 in *Ctnnd2* knockout cells was downregulated (Fig. 5B). *Aldh1*, another cancer stem cell marker, was also downregulated in *Ctnnd2* knockout cells (Fig. 5C). In monolayer cultures, *Ctnnd2* overexpression could not increase the expression of CD133 (Fig. 5D). However, the expression of CD133 in *Ctnnd2* overexpressed cells was increased in 3D cultures (Fig. 5E). Collectively, *Ctnnd2* coded δ-catenin was associated with maintenance of cancer stem cell markers in Lewis lung cells.

δ-catenin promotes the malignancy of human lung adenocarcinoma. To test whether δ-catenin played roles in human lung adenocarcinoma as it did in mice, we overexpressed CTNND2 in human lung adenocarcinoma cell line A549 (34) (Fig. 6A). With CTNND2 overexpressed, A549 cells grew faster, indicating that δ-catenin enhanced the proliferation of A549 cells (Fig. 6B). Furthermore, we measured the migration of A549 cells affected by δ-catenin via scratch wound assay. We found that the wound shrunk rapidly when CTNND2 was overexpressed, suggesting that CTNND2 benefited cell migration (Fig. 6C). Moreover, ectopic expression of CTNND2 caused more and larger oncosphere formation by A549 cells (Fig. 6D and E). Collectively, CTNND2 contributed to the proliferation, migration and oncosphere formation of A549 cells, indicating that CTNND2 played similar roles in human lung adenocarcinoma cells. In general, our results demonstrated that δ-catenin promoted the malignancy of human lung adenocarcinoma.

Discussion

δ-Catenin has been reported to be involved in the malignancy of various types of cancers. It may be a promising biomarker for the clinical diagnosis and therapy of cancer (3,5-7). According to our results, δ-catenin contributes to the malignancy of lung adenocarcinoma, owing to its important effects on tumorigenesis and metastasis. Previous research has proved the higher expression of δ-catenin in lung cancer (5). The preliminary functional mechanisms of δ-catenin were also proposed. However, our results throw light upon new working mechanisms of δ-catenin. δ-catenin promotes cell proliferation and cell cycle progression via canonical Wnt signaling pathway in

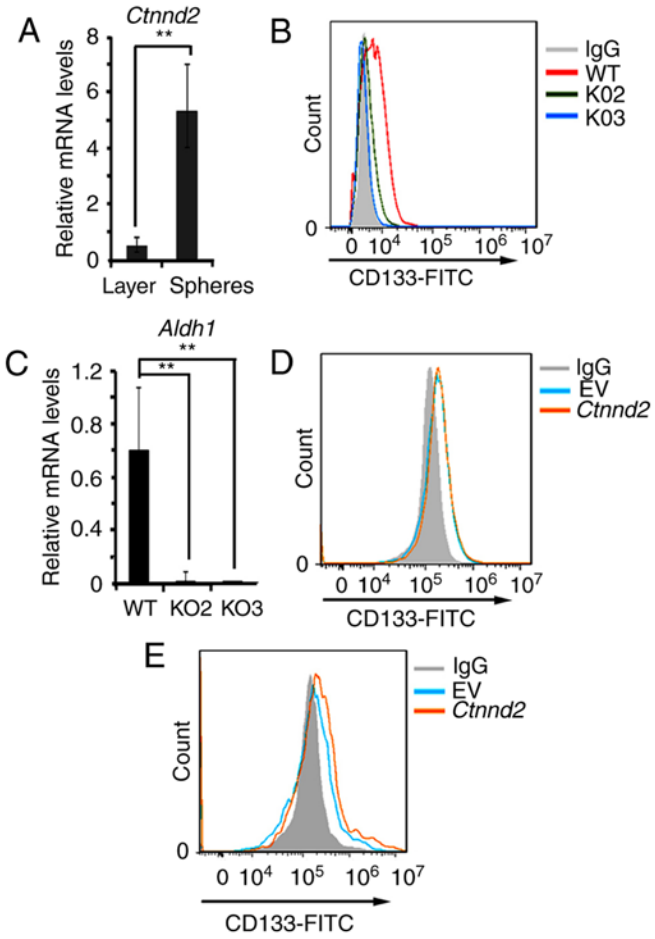

Figure 5. δ-Catenin enhances the expression of cancer stem cell markers in Lewis lung cells. (A) *Ctnnd2* expression levels increased in 3D spheres. The mRNA levels of *Ctnnd2* of LLC cells in 2D cultures and 3D cultures were examined by qRT-PCR. (B) The expression of CD133 on surfaces of LLC cells of different genome types were measured by flow cytometry. (C) The mRNA levels of *Aldh1* in WT and *Ctnnd2* knockout LLC cells were examined by qRT-PCR. (D) The expression of CD133 on surfaces of empty vector (EV) and *Ctnnd2* overexpressed LLC cells in monolayer cultures were measured by flow cytometry. (E) The expression of CD133 on surfaces of empty vector (EV) and *Ctnnd2* overexpressed LLC cells in 3D cultures were measured by flow cytometry.

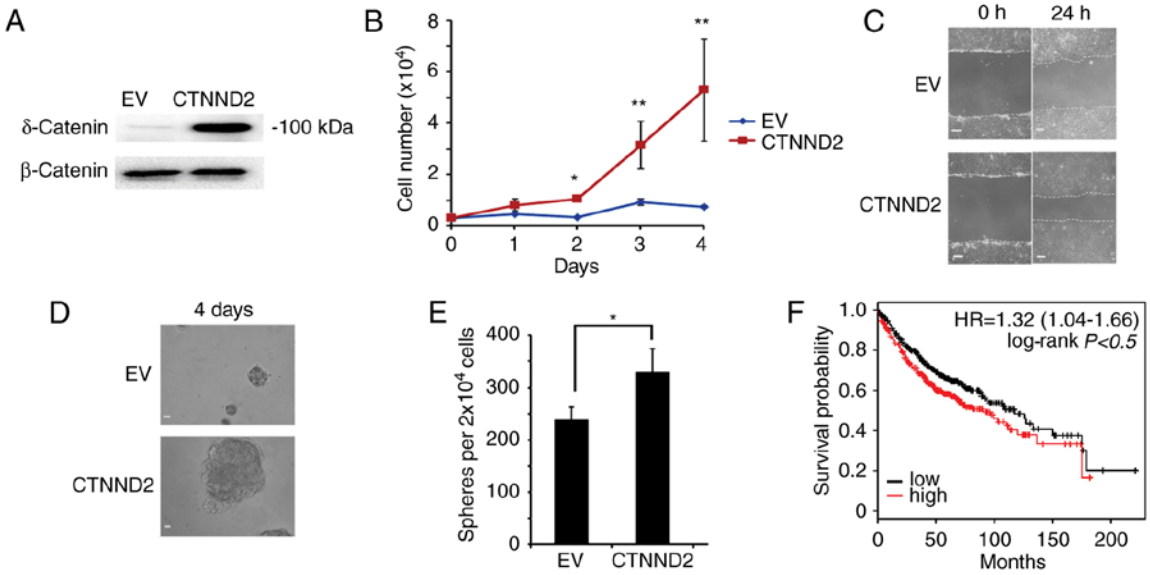

Figure 6. δ-Catenin promotes the malignancy of human lung adenocarcinoma. (A) Cell lysates of empty vector (EV) or CTNND2 overexpressed A549 cells were subjected to immunoblotting. (B) Cells (3,000) of empty vector (EV) and CTNND2 overexpressed A549 cells were plated at 0 day and cell numbers were counted every day to draw the proliferation curve (*P<0.05, **P<0.01). (C) Subconfluent A549 cells were wounded by a 20-μl size pipette tip. Initial boundaries of wound were marked with white lines. Scale bars, 100 μM. (D) Empty vector (EV) and CTNND2 overexpressed A549 cells were cultured in Matrigel to form spheres. Images were captured after four days. Scale bars, 10 μM. (E) Numbers of spheres in (D) were counted (*P<0.05, **P<0.01). (F) Higher CTNND2 expression indicates worse prognosis in human lung adenocarcinoma. Kaplan-Meier plots of lung adenocarcinoma patients, stratified by expression of CTNND2. Data obtained from the Kaplan-Meier plotter database of Gyorffy *et al* (35). The P-value was calculated by a log-rank test.

Lewis lung cells. Moreover, the deletion of δ-catenin results in almost complete loss of cancer stem cells in Lewis lung cells, indicating a significant role of δ-catenin in tumorigenesis and metastasis (28,33). From a public clinical microarray database of lung adenocarcinoma from 1,157 patients (35), we estimated that the prognosis of the patients with higher CTNND2 expression was worse than that of these with lower CTNND2 expression (Fig. 6F). In summary, our current research provides more evidence to prove that δ-catenin is a promising biomarker and therapy target for lung cancer.

Knockdown of genes by siRNA or shRNA has been used for years to silence genes. The shortcoming of knockdown is that it can only partly silence the expression of one gene but the residual mRNAs and proteins can still have partial functions (36,37). The CRISP/Cas9 system, developed in recent years, is a new method for the gene knockout in mammalian cells (38,39). Compared with knockdown by siRNA or shRNA, knockout via the CRISP/Cas9 system can absolutely eliminate residual expression of proteins. Therefore, the gene knockout via CRISP/Cas9 system is a more reliable method for research on the loss of the gene function. In our research, the CRISP/Cas9 system was used for *Ctnnd2* knockout. The following experiments were based on the *Ctnnd2* knockout cell lines. Thus, the roles of δ-catenin in lung adenocarcinoma cells can be revealed more directly and reliably. Compared with WT Lewis lung cells, empty vector overexpressed Lewis lung cells in our experiments had slightly weaker tumorigenesis, stemness and proliferation ability (Figs. 1H, 3B and E, and 4C and D), which may be due to the load of vectors or the G418 selection process. As the gene *Ctnnd2* was subcloned to the same vector and *Ctnnd2* overexpressed Lewis lung cells underwent the same selection process, we came to the opinion that results from *Ctnnd2* overexpressed Lewis lung cells should be compared with empty vector overexpressed Lewis lung cells in our experiments.

Dai *et al* reported that the expression of δ-catenin is increased in lung cancer tissues (5), but our research provide direct evidence that δ-catenin contributes to tumorigenesis and metastasis of lung adenocarcinoma in mouse models. Moreover, in their data from 70 patients, higher expression levels of δ-catenin alone cannot indicate worse prognosis (5). However, in our data from 1,157 patients in Kaplan-Meier plotter database, the higher expression levels of δ-catenin indicate worse patient prognosis. The differences may be due to the size of samples. Furthermore, the effects of δ-catenin on cell proliferation and invasion, which can support δ-catenin to promote metastasis at the cellular level, are seriously missing in previous reports. In our research, δ-catenin was proved to have effects on cell proliferation and cancer stem cell maintenance.

Different variants with modifications of δ-catenin are expressed in different cancers (3). It has been reported, in lung cancer cell line, NIC-H1299, that fragments with low molecular weight of δ-catenin are expressed (3). According to our data, δ-catenin expressed in Lewis lung cells and A549 cells migrate faster than the 100 kDa protein marker (Figs. 1B and G, and 6A). Furthermore, the CMV promoter-driven exogenously expressed δ-catenin has the same molecular weight with the endogenous expressed δ-catenin. Collectively, probably post-translational modifications regulate the expression of δ-catenin fragments in lung cancer cells.

The mechanisms how δ-catenin promotes the malignant progression of cancer may be complicated. Mutations of δ-catenin have been proven to contribute to its oncogenic functions. In prostate cancer, -9 G>A mutation in 5'-UTR promotes δ-catenin expression (6). Moreover, truncated mutations of CTNND2 enhance metabolic reprogramming, hypoxia pathways and Wnt signaling response to promote cancer cell survival in prostate cancer (8). In our research, mutations of CTNND2 in lung cancer were not tested. Oncogenic roles of CTNND2 mutations in lung cancer development may be a spot to be focused on in the future. However, based on our research, CTNND2 coded protein, δ-catenin enhanced canonical Wnt3a signaling by reinforcing the accumulation of β-catenin, but the detailed mechanism is not clear. CTNND1 has been reported to facilitate β-catenin to shuttle from the cytoplasm to the nuclear (40). The interaction between β-catenin and δ-catenin is confirmed in our research. Other mechanisms can be further investigated in the future.

δ-Catenin can be a clinical diagnosis marker and therapy target for cancer (3). According to our results and former reports, mutants or high expression of δ-catenin indicate poorer prognosis (5-8). Therefore, examination of CTNND2 mutations or immunohistochemistry of δ-catenin in tumor tissues can be applied to anticipate the patients prognosis or select treatment plans. Deletion of CTNND2 attenuates tumor growth and metastasis. Thus, inhibitors or antibodies of δ-catenin can be developed for cancer therapy. Currently, neither inhibitors nor antibodies have been developed for the abolishment of δ-catenin, which can be a promising future research area.

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