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Original Article

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β-Catenin is important for cancer stem cell generation and tumorigenic activity in nasopharyngeal carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors with poor prognosis and recurrence in South China. The hard eradication of NPC in clinic is predominantly due to cancer stem cells (CSCs). Increasing evidence revealed that the aberrant activation of Wnt/β-catenin was positively correlated with the produce of CSCs. To further investigate the effect of β-catenin on CSCs and tumorigenesis in NPC, a CNE2 cell line (pLKO.1-sh-β-catenin-CNE2) with stably suppressed expression of β-catenin was used in this study. The expressions of biomarkers in CSCs including c-myc, Nanog, Oct3/4, Sox2, EpCAM as well as adhesion-related proteins like E-cadherin and vimentin were analyzed by western blot analysis and immunofluorescent staining. The proliferation and migration abilities were investigated by MTT assay and Transwell assay, respectively. Cell cycle was analyzed by flow cytometry. Finally, xenograft was performed to determine the effect of β-catenin on oncogenesis *in vivo*. Results showed that the expressions of c-myc, Nanog, Oct3/4, Sox2, and EpCAM were all decreased in pLKO.1-sh-β-catenin-CNE2 cells. It was also found that vimentin was downregulated, while E-cadherin was upregulated. Results of MTT and Transwell assays suggested that the proliferation and migration abilities were impaired by silencing of β-catenin, and more cells were arrested in G1 phase when compared with the control. *In vivo* study indicated that the tumor growth was markedly suppressed in experimental group. Based on current findings, β-catenin may function as an essential protein for the maintenance of migration and proliferation abilities of NPC cells, and a complicated network consisting of c-myc, Nanog, Oct3/4, Sox2, EpCAM, E-cadherin, vimentin, and β-catenin may be involved in the inherent regulation mechanisms.

Key words: CNE2 cell line, cancer stem cell, β-catenin, tumorigenic capability

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors and reported as an endemic cancer with high prevalence in Southeast Asia, particularly in South China [1,2]. Thus far, the etiology and pathogenesis mechanism of NPC have not yet been elucidated thoroughly. Recent studies suggested that environmental factors, genetic susceptibility, and Epstein–Barr virus may play critical roles in its progression. Although the 5-year survival rate of NPC has been greatly improved through comprehensive treatment such as radiotherapy and chemotherapy [3], long-term prognosis remains unsatisfactory. About 55% of the NPC metastasis and recurrence occurred in 5 years after conventional treatment. Many patients developed chemotherapy resistance.

Cancer stem cell (CSC) theory suggests that CSC is related to tumor recurrence and metastasis as well as chemotherapy-related tolerance [4,5]. Since the existence of CSCs, the solid tumors cannot be eradicated. Although the size of metastatic tumor can be reduced after regular treatment, it can relapse quickly. Research showed that CSCs are a small population of cell subsets that have high proliferative capacity, self-renewal, and multi-directional differentiation potential,

with the characteristics of resistance to radiotherapy and chemotherapy. These features are similar to ordinary stem cells and embryonic stem cells. Normally, the differentiation and self-renewal of stem cells are tightly regulated by signal transduction pathways. Once the related signal components become aberrant or are destroyed, dysregulated differentiation will occur and cells will grow indefinitely. Thus, CSCs occur, subsequently leading to tumor formation. Recent research suggested that the tumor cells, stem cells, and CSCs have some common signaling pathways, such as Wnt [6,7], Notch, SHH, BMP, PI3K/Akt [8,9], and Bmi1 signal pathway [10]. Ma *et al*. [11] reported that the effects of epidermal growth factor receptor (EGFR) on CSCs were mediated by β-catenin, demonstrating that β-catenin was a crucial downstream factor in signaling pathway concerning CSC generation. However, further and detailed studies about β-catenin were absent in their research. The essential effects of β-catenin on CSCs deserve more attention.

Nasopharyngeal CSCs have been evaluated by analyzing the number of side population (SP) cells, which appear as a dimly stained subgroup by extruding the Hoechst 33342 dye [12–14], as well as the expressions of octamer-binding transcription factor 3/4 (Oct3/4), sex-determining region Y-box 2 (Sox2), c-myc, and Nanog [14]. Sufficient evidence has demonstrated that Oct3/4, Sox2, c-myc, and Nanog function as reprograming factors which can efficiently transform terminally differentiated cells into induced pluripotent stem cells [15]. Wnt/β-catenin signaling pathway exists not only in normal stem cells, playing an important role in maintaining cell self-renewal and inhibiting cell differentiation [6,16,17], proliferation, migration, and apoptosis, but also in immature CSCs, playing important roles in controlling drug resistance and tumor recurrence. Recent studies revealed that β-catenin signaling was important in the maintenance of CSC phenotype [17–19]. Knockdown of *β-catenin* led to the loss of CSC phenotype and tumor degeneration [20]. However, the Wnt/β-catenin signaling pathway is quiescent in normal epithelial cells. This difference may be used to kill CSCs and ultimately cure the cancer. NPC is a kind of malignant tumor occurred in nasopharyngeal epithelium. In our previous work [21], several siRNAs targeting β-catenin were designed and transfected into CNE1, CNE2, and 6–10B cell lines. A CNE2 cell line (pLKO.1-sh-β-catenin-CNE2) with stable downregulation of β-catenin was successfully established. In the present study, this cell line was used to investigate the role of Wnt/β-catenin signaling pathway in CSC of NPC. The results indicated that silencing the expression of *β-catenin* resulted in the elimination of CSC properties and inhibition of NPC *in vitro* and *in vivo*. Our studies may provide a novel approach for molecular targeted therapy for NPC patients.

Materials and Methods

Cell culture

A CNE2 cell line (pLKO.1-sh-β-catenin-CNE2) with stably suppressed β-catenin expression was established by RNA interference, and verified by western blot analysis [21]. A control cell line transfected with nonsense siRNA (pLKO.1-sh-ctr-CNE2) was simultaneously established. The untreated CNE2 cells were used as the parental cell. All the cell lines were cultured in RPMI-1640 medium (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St Louis, USA) in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

Western blot analysis

Cells were harvested for western blot analysis after 3 days of incubation. Cell lysate was prepared in RIPA buffer containing 1% (v/v) phenylmethane sulfonyl fluoride (PMSF; Sigma). Total protein was quantified using a BCA Protein Assay kit (Pierce, Rockford, USA) according to the manufacturer's instructions. All the protein samples were boiled together with 6× loading buffer, and then an equal amount of each protein sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the separated protein was transferred to a polyvinylidene fluoride membrane and probed with appropriate primary antibodies. The antibodies against β-catenin, c-myc, E-cadherin, vimentin, Oct3/4, and GAPDH as well as the corresponding FITC- and HRP-conjugated secondary antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Protein bands were detected using an Enhanced Chemiluminescence (ECL) Detection kit (Pierce).

Immunofluorescent staining

The pLKO.1-sh-β-catenin-CNE2, pLKO.1-sh-ctr-CNE2, and untreated cells were separately seeded on sterilized cover slides which were placed in six-well culture dishes, and maintained in 2 ml of RPMI-1640 medium supplemented with 10% new born calf serum (NBS; Sigma) in a humidified incubator at 37°C in an atmosphere of 5% CO_2. After incubation for 6–12 h, medium were discarded and cells were washed twice with phosphate-buffered saline (PBS). Cells were then fixed with 3.5% paraformaldehyde for 7 min at room temperature. After rehydration, fixed cells were incubated with monoclonal antibodies against β-catenin, c-myc, Oct3/4, Sox2, Nanog, vimentin, and EpCAM at 4°C overnight, followed by incubation with fluorescein isothiocyanate-conjugated secondary antibodies for 30 min at room temperature. The nuclei were stained with 2,6-diaminopimelic acid (DIPA). Sections were examined with a Nikon Eclipse 80i fluorescent microscope (Nikon Instruments, Inc., Melville, USA).

Cell viability assay

The effects of suppressing β-catenin expression on cell proliferation were evaluated by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma). The pLKO.1-sh-β-catenin-CNE2, pLKO.1-sh-ctr-CNE2, and untreated cells were separately seeded in 96-well culture plates at the density of 1000 cells per well. After 24, 48, and 72 h of incubation, 5 mg/ml of MTT solution was added to each well and the absorbance was determined at 490 nm.

Transwell migration assay

The migration ability of pLKO.1-sh-β-catenin-CNE2, pLKO.1-sh-ctr-CNE2, and untreated cells were measured by Transwell migration assay. A total of 5×10^4 cells were pelleted and resuspended in 500 μl serum-free RPMI-1640 medium, then seeded into the inserts of the Transwell chamber (Corning Costar, Corning, USA). The inserts were then put into the bottom chambers loaded with 500 μl RPMI-1640 medium supplemented with 10% NBS, and the cells were incubated in a humidified incubator at 37°C in an atmosphere of 5% CO_2 for 24 h. The inserts were then taken out, and the cells remaining on the upper surface (non-migrant) were gently removed with a cotton swab. Cells on the lower surface of the inserts (migrant cells) were fixed with 500 μl methanol for 10 min, and stained with hematoxylin for 10 min at room temperature. Images of the migrant cells were captured using a photomicroscope (Axiovert 200 M; Carl Zeiss, Oberkochen, Germany). Three images per well were randomly selected for cell counting, and an average was finally calculated. Each assay was performed in triplicate.

Cell cycle and SP analysis by flow cytometry

The pLKO.1-sh-β-catenin-CNE2, pLKO.1-sh-ctr-CNE2, and untreated cells in exponential growth stage were collected and suspended in RPMI-1640 medium containing 2% FBS to 1×10^6 cells/ml, and subsequently stained with Hoechst33342 dye (1 mg/ml) for 90 min in a light-resistant container at 37°C with continuous mixing. After incubation, the cells were immediately washed with D-Hanks solution, stained with propidium iodide (PI, 1 mg/ml), and maintained on ice for flow cytometry analyses (FACS). Cells for cell cycle analysis were washed twice with D-Hanks solution and resuspended in 300 μl PBS. Then the cells were fixed with pre-cooled alcohol for 12 h at 4°C and washed again with D-Hanks solution, resuspended in D-Hanks solution containing 0.1 mg/ml RNase A, and then stained with PI for 30 min at room temperature for FACS analysis.

In vivo tumorigenicity assay

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Fudan University Shanghai Cancer Center. For xenograft study, the pLKO.1-sh-β-catenin-CNE2, pLKO.1-sh-ctr-CNE2, and untreated cells were washed with D-Hanks solution and resuspended in serum-free medium. A volume of 100 μl cell solution of the 3 cell lines containing 1×10^6 cells were separately transplanted into the backs of 5 BALB/c-nu mice (4–6 weeks of age) by subcutaneous injection. Mice transplanted with pLKO.1-sh-β-catenin-CNE2 cells were used as experimental group and pLKO.1-sh-ctr-CNE2 cells were used as control. After tumor formation, tumor volumes were measured every 5 days. Tumor volume was calculated as follows: $V = 1/2(\text{width}^2 \times \text{length})$. Four weeks later, all mice were sacrificed for tumor tissue collection. Tumor tissues were fixed in 4% formaldehyde and embedded in paraffin for immunohistochemistry and hematoxylin-eosin (HE) staining.

Immunohistochemistry and HE staining

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded alcohol series, then boiled in 10 mM sodium citrate buffer for antigen retrieval. Subsequently, sections were stained using a VECTASTAIN® ABC kit (Vector Laboratories, Burlingame, USA) according to the manufacturer's protocol. Diaminobenzidine (DBA) was chosen as the substrate for peroxidase. HE staining was performed after the sections were deparaffinized and rehydrated.

Statistical analysis

All data were expressed as the mean ± SD. Statistical evaluation of the data was conducted using SPSS version 19.0. Student's *t*-test was used to determine the significant differences. Graphs summarizing immunohistochemical staining results were analyzed using Fisher's exact test. *P*-values <0.05 were considered to be statistically significant.

Results

Low expression of β-catenin inhibits NPC cell proliferation

As is well known, β-catenin acts as a critical factor in Wnt/β-catenin transduction pathway, which is activated when the abnormal accumulation of β-catenin occurs in the cytoplasm. Previous researches have revealed that tumorigenesis of most organs and tissues is closely related to the aberrant activation of Wnt/β-catenin. In this study, the effect of β-catenin on NPC cell proliferation was investigated. Results of MTT assay indicated that proliferation of pLKO.1-sh-β-catenin-CNE2 cells was significantly suppressed after 2 days of incubation when compared with the parental cells (**Fig. 1A**; $P = 0.001$), while no significant difference was observed between the parental cells and

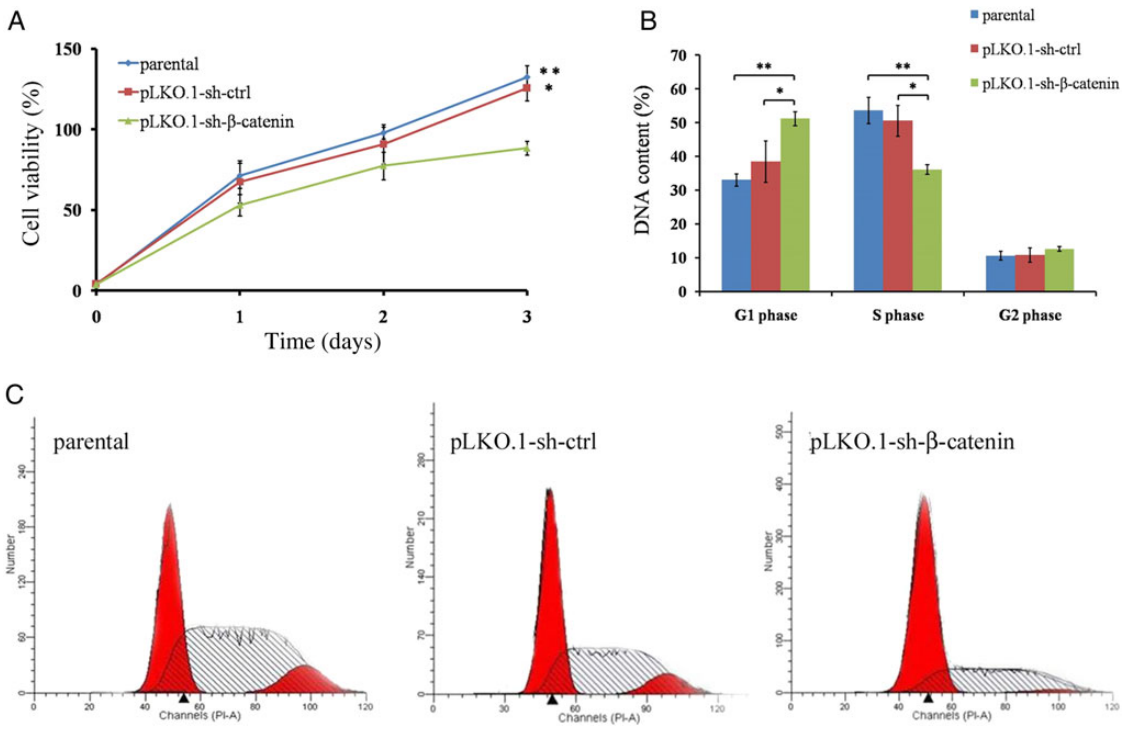

Figure 1. The inhibitory effect of silencing β-catenin on cell proliferation of CNE2 cells (A) Cell viability analysis by MTT. (B,C) Distribution of DNA content among G1, S and G2 phases in three cell lines. Data are presented as the mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$.

control cells ($P = 0.098$). Cell cycle analysis demonstrated that the proportion of pLKO.1-sh-β-catenin-CNE2 cells in G1 phase was significantly higher than that of the control cells ($P = 0.007$) and parental CNE2 cells ($P = 0.001$) (**Fig. 1B,C**), meanwhile lower proportion of cells in S phase was observed in pLKO.1-sh-β-catenin-CNE2 cells (**Fig. 1B,C**; $P < 0.05$). These findings implied that most cells were arrested in G1 phase by knockdown of β-catenin gene expression. And, the results were further confirmed by evidence at the molecular level. C-myc plays a critical role in regulation of cell proliferation. Western blot analysis showed that expression of c-myc was significantly downregulated in pLKO.1-sh-β-catenin-CNE2, in which β-catenin was also downregulated (**Fig. 2A,B**).

Migration of NPC cells is compromised when *β-catenin* gene is silenced

It was reported that most tumor recurrences and poor prognosis were associated with the migration of tumor cells. In this study, the ability of cell migration was diminished by silencing the *β-catenin* gene. Notably, cells that penetrated the Transwell membrane were observed with comparatively lower counts in pLKO.1-sh-β-catenin-CNE2 cells (**Fig. 2C**; $P < 0.001$), while no significant difference was observed between the control cells and parental cells ($P = 0.096$). The increased expression of E-cadherin was found to be negatively correlated with cell migration (**Fig. 2A**). EpCAM, which contributes to cell adhesion, was decreased in pLKO.1-sh-β-catenin-CNE2 cells (**Fig. 3A**).

β-Catenin is necessary for maintaining characteristics of CSCs in NPC

CSCs are reportedly responsible for the recurrence, drug resistance, and metastasis in most tumors. The impacts of β-catenin on the characteristics of CSCs in NPC were explored. C-myc, Nanog, Oct3/4, Sox2, and EpCAM were selected as biomarkers. The decreased expressions of c-myc, Sox2, and Oct3/4 in pLKO.1-sh-β-catenin-CNE2 cells were observed by western blot analysis (**Fig. 2A**). Furthermore, expressions of β-catenin, c-myc, Nanog, Oct3/4, Sox2, EpCAM, and vimentin were analyzed by immunofluorescence stain. The low expressions of c-myc and Oct3/4 were further verified in **Figs. 2B** and **3B**, respectively. Additionally, expressions of Nanog, Sox2, EpCAM, and vimentin were also suppressed in pLKO.1-sh-β-catenin-CNE2 (**Figs. 3** and **4**). The low expression levels of these biomarkers indirectly demonstrated that the CSCs in NPC were reduced by downregulating β-catenin. In order to directly verify that CSCs were decreased, SP cells, a subpopulation of CSCs, were analyzed by FACS. Results showed that the percentage of SP cells were lower than that of the parental and control cells (**Fig. 5**; $P < 0.05$), and the difference between parental and control cells was not statistically significant ($P = 0.255$). The above findings indicated that decreased expression of β-catenin exerted an inhibitory effect on the generation of CSCs. Neoplasm recurrence was reported to be linked to the existence of CSCs in tumor cells. To evaluate the influence of β-catenin on tumor formation ability of CSCs, pLKO.1-sh-β-catenin-CNE2, pLKO.1-sh-ctr-CNE2, and parental cells were

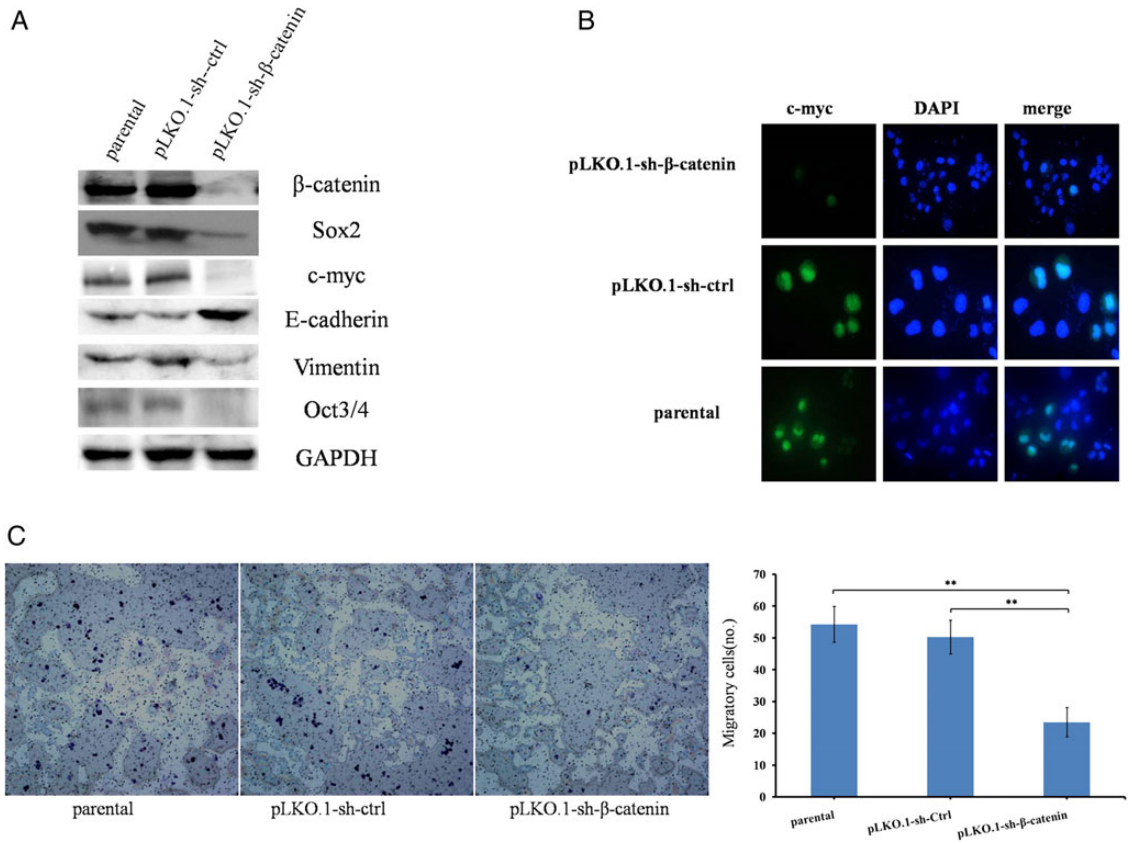

Figure 2. The inhibitory effect of silencing β-catenin on cell migration of CNE2 cells (A) Expression levels of β-catenin, Sox2, c-myc, E-cadherin, vimentin, and Oct3/4 determined by western blot analysis. GAPDH was used as reference. (B) Cellular level of c-myc detected by immunofluorescence staining (original magnification, ×200). (C) Silencing β-catenin reduced migration of CNE2 cells. Data are presented as the mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$.

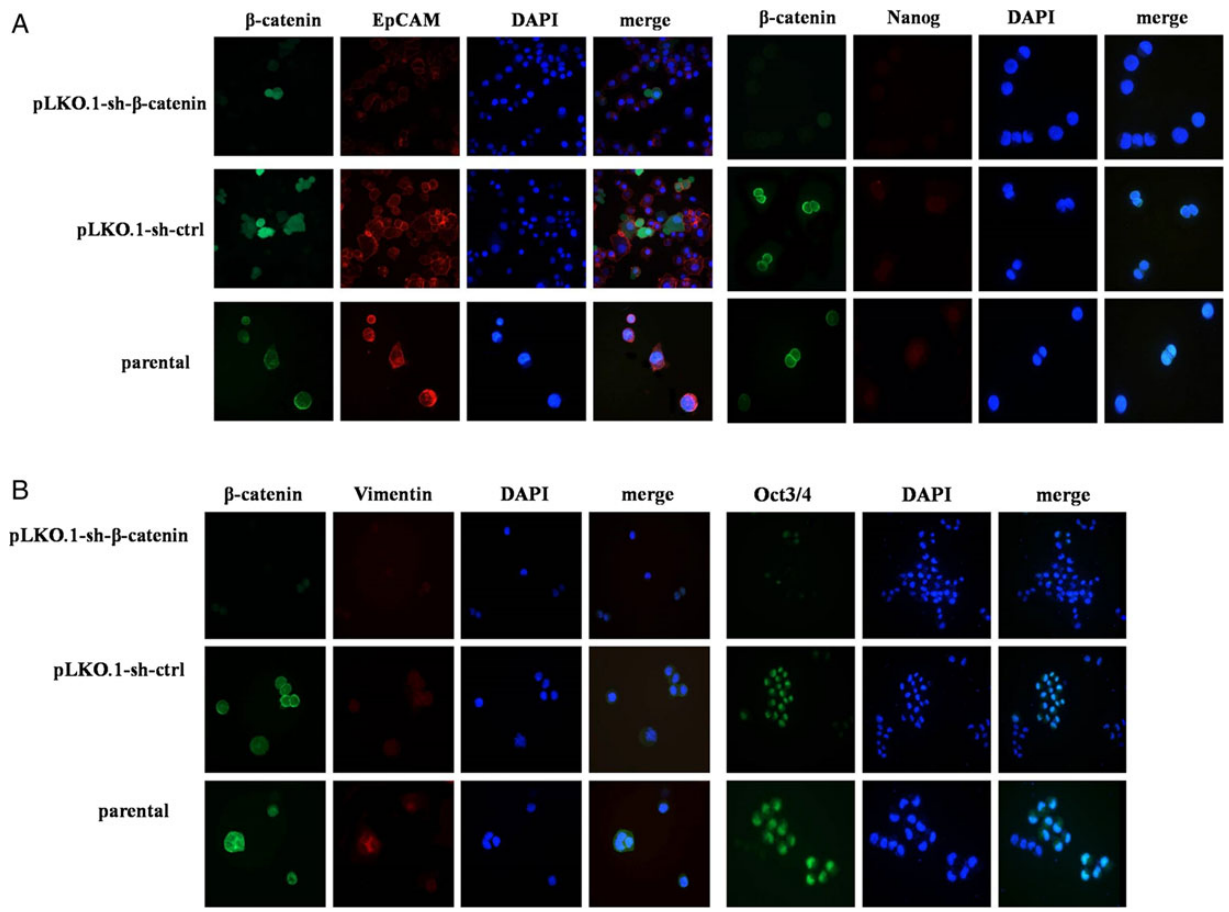

Figure 3. Expression levels of EpCAM and Nanog (A), vimentin and Oct3/4 (B) in three cell lines determined by immunofluorescence EpCAM, Nanog, vimentin, Oct3/4 were all decreased in pLKO.1-sh-β-catenin-CNE2 cell line (original magnification ×200).

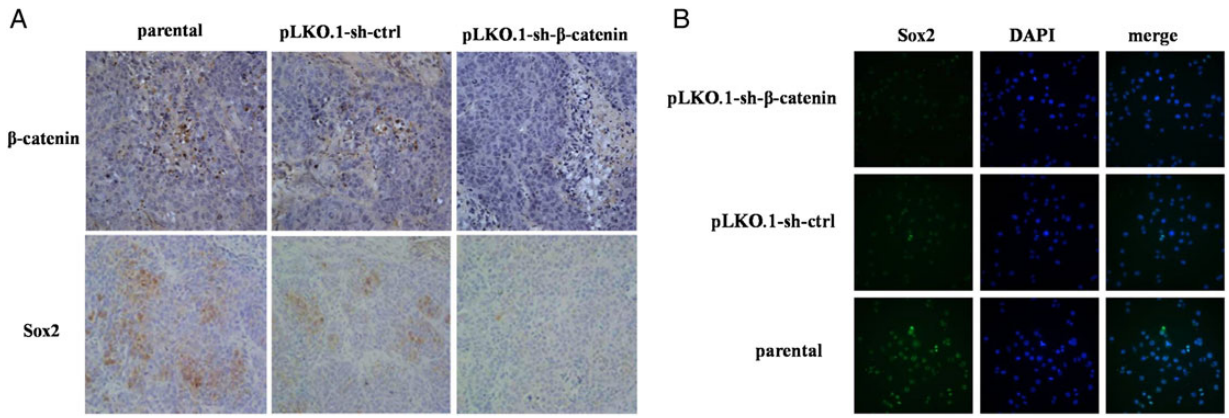

Figure 4. Expression of Sox2 in tumor tissues determined by immunohistochemistry (A) and in cells by immunofluorescence (B) Sox2 was downregulated both *in vivo* and *in vitro* experiments (original magnification ×100).

delivered subcutaneously into nude mice. Tumor growth was monitored by measuring the volume of solid tumors. As shown in **Fig. 6A**, the size of tumors in mice transplanted with pLKO.1-sh-β-catenin-CNE2 was evidently smaller than that in control and parental groups ($P < 0.001$), while the difference between the control group and parental group was not statistically significant ($P = 0.882$). After 21 days of growth, the average tumor volume was 70.342 ± 46.448 mm^3 in the experimental group and 1014.91 ± 430.624 and 1185.77 ± 364.965 mm^3 in the control group and parental group, respectively (**Fig. 6A**). Histological analysis of subcutaneous tumors by HE staining demonstrated more seriously disorganized tumor cells, hyperchromatic nuclei, and higher nuclear–cytoplasmic ratio in control and parental groups than in the experimental group (**Fig. 6B**), indicating a more advanced stage of

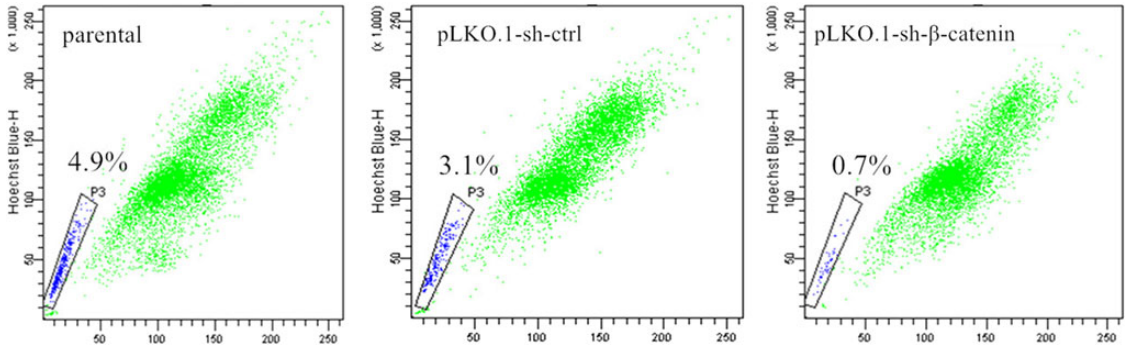

Figure 5. SP cells analysis of three CNE2 cell lines by FACS SP cells were obviously reduced in pLKO.1-sh-β-catenin-CNE2 cells, indicating the decreased production of CSC.

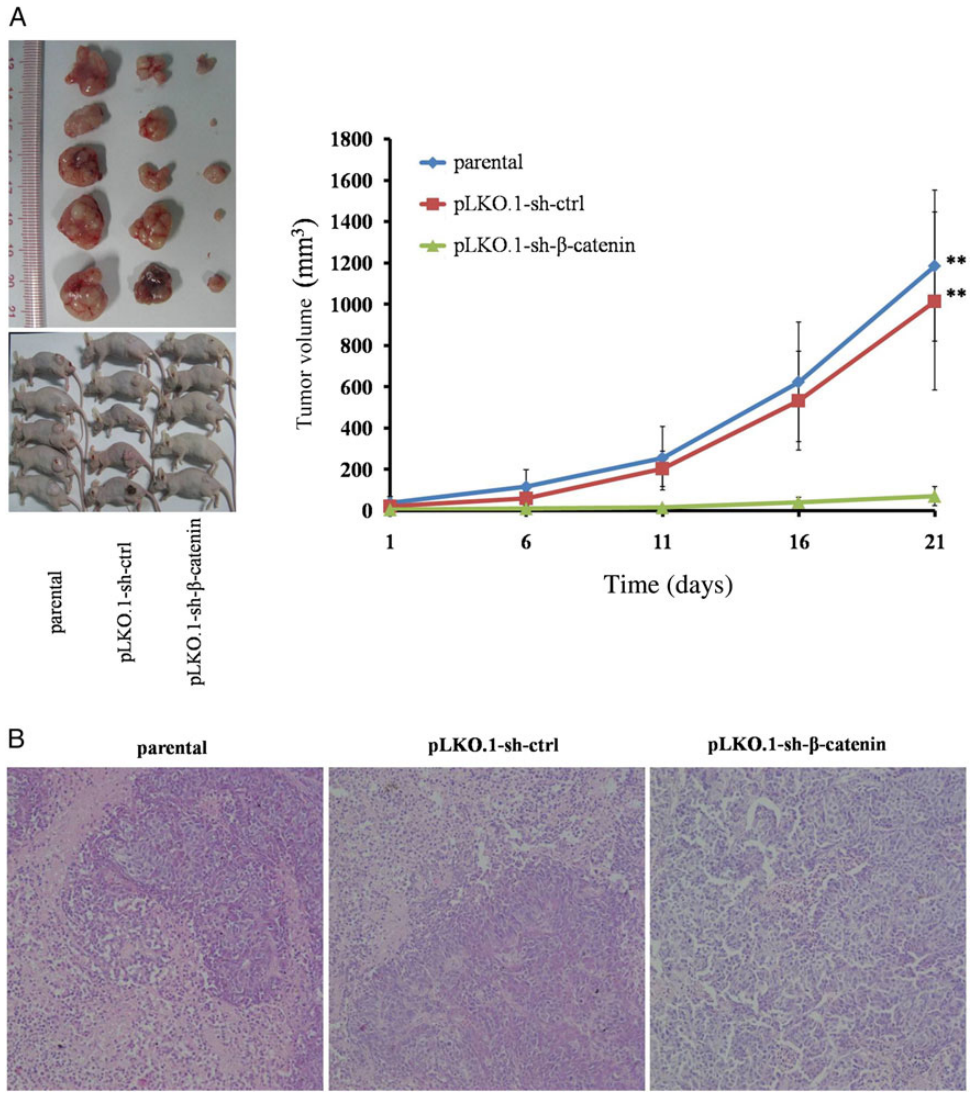

Figure 6. The attenuation effect of downregulated β-catenin on tumor growth in nude mice (A) The tumor growth was significantly suppressed by inoculating pLKO.1-sh-β-catenin-CNE2 cells. (B) HE staining of tumor tissues among three groups (original magnification ×100). Data are presented as the mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{*}P < 0.05$.

NPC in the control and parental groups than in the experimental group. Immunohistochemistry analysis showed a relatively lower expression of Sox2 in pLKO.1-sh-β-catenin-CNE2 group (**Fig. 4A**), indicating a reduced generation of CSCs. Therefore, the limited tumor formation ability of NPC cells might lead to the decreased CSCs due to low endogenous expression of β-catenin.

Discussion

CSC, as a subset cell within the majority tumors including brain tumor, breast cancer, ovarian cancer, and NPC, has long been suggested to be involved in tumor relapse, metastasis, and resistance to chemoradiotherapy [22,23]. β-Catenin, which acts as a pivotal protein in Wnt/β-catenin pathway, was found to be overexpressed in CSCs in many tumors including NPC, and it was determined with lower level in cells from normal tissues. Jang *et al.* [24] demonstrated that blocking Wnt/β-catenin signaling would reverse CSC-like phenotypes in breast cancer. In previous studies, cell lines with overexpression of β-catenin were constructed to assess the effect of β-catenin on the fate of CSCs and tumorigenesis, and the results showed that up-regulated endogenous β-catenin may promote the proliferation, migration, and tumor formation of CSC and also promote tumorigenesis in mice. Increasing evidence demonstrated that β-catenin was of great importance in the maintenance of CSC properties in NPC, as well as in the development of cancers. However, whether the high expression level of β-catenin is necessarily required for stemness maintenance of CSC is still not clear. Some studies have suggested that the CSC characteristics in NPC cells with high level of β-catenin are attenuated when the Wnt/β-catenin signaling pathway is blocked by inhibitors targeting β-catenin [16,25]. In addition, the antineoplastic activities of some microRNAs like miR-200a were reported to be mediated by their inhibitory effect on β-catenin pathway [26–28].

In the present study, whether β-catenin is necessarily involved in the stemness maintenance of CSCs in NPC was studied by silencing β-catenin in CNE2 cells (pLKO.1-sh-β-catenin-CNE2), and we hypothesized that the lower level of β-catenin would reverse the CSC traits of NPC cells. Subsequently, the proliferation and migration of CNE2 cells in which β-catenin had been silenced were reduced as expected. C-myc, one of numerous downstream proliferation signals activated by β-catenin, was also found to be downregulated in pLKO.1-sh-β-catenin-CNE2 cells by western blot analysis and immunofluorescent staining. And, the cell line possessed a relatively higher ratio of cells that were arrested in G1 phase compared with the control cells. These findings may partly explain a relatively weak proliferation of cells with reduced β-catenin expression at molecular level. Simultaneously, E-cadherin was inversely up-regulated in pLKO.1-sh-β-catenin-CNE2 cells. E-cadherin was reported to contribute to the cell adhesion and was negatively correlated with tumor cell migration [29,30]. E-cadherin and the interaction between its intracellular domain and cytoplasmic β-catenin are essential for cell adhesion. However, the expression of E-cadherin was found to be negatively correlated with intracellular β-catenin expression in this study. The inhibitory effect of E-cadherin expression was potentially caused by hypomethylation of *E-cadherin* gene resulted from activation of signaling pathway, such as JNK pathway [10,31]. The translocation of β-catenin to the nucleus had a validated negative relationship with E-cadherin expression, and the intracellular β-catenin localized in the nucleus of pLKO.1-sh-β-catenin-CNE2 cell was markedly reduced. Fang *et al.* [1] found that when E-cadherin and β-catenin were up-regulated by (−)-epigallocatechin-3-gallate, the nuclear translocation of β-catenin was suppressed. More efforts should be made to figure out the detailed underlying mechanisms between E-cadherin expression and nuclear translocation of β-catenin. Reduced ability of cell migration in cells with stably suppressed expression of β-catenin was presumably attributed to the changes of E-cadherin expression. The results implied that the inhibitory effect of β-catenin via indirectly suppressing E-cadherin expression was the main influence of cellular β-catenin on cell migration, and the regulatory mechanisms of cell migration in NPC mediated by β-catenin were more complicated than expected. Vimentin (an intermediate filament protein) and EpCAM (a transmembrane protein) [32], two proteins related to cell adhesion and migration, were both decreased in β-catenin-silenced cells. Vimentin expression was speculatively correlated with the progression of NPC, which was supported by an investigation in 122 NPC patients showing that high nuclear vimentin expression was correlated significantly with advanced clinical stage [33]. Up-regulated vimentin and downregulated E-cadherin are the typical phenotypes of epithelial–mesenchymal transition (EMT) which contributes to tumor invasion and metastasis [34]. Involvement of E-cadherin, vimentin, EpCAM, and β-catenin in cell adhesion and migration implied an intricate mechanism in tumor invasion and metastasis. More investigations are needed to further understand the regulatory mechanisms of cell migration in NPC.

Reduced production of CSC may also contribute to the impaired proliferation and migration. The lower expressions of c-myc, Nanog, Oct3/4, Sox2, and EpCAM in β-catenin-silenced cells suggested that the production of CSC was suppressed. The enrichment of Sox2 in CSC-like population in NPC has already been reported, and Sox2 has also found to be involved in regulating the growth of the CSC-like population in NPC [4,35]. In the present study, we demonstrated that silencing β-catenin would inversely influence the expression of Sox2 both *in vitro* and *in vivo*. EpCAM is a tumor-associated protein involved in regulating the functions of cell adhesion, migration, and proliferation [36]. In our study, low β-catenin level resulted in decreased EpCAM expression, which was in agreement with the fact found in breast cancer cells that high EpCAM expression was closely related to the activation of the Wnt pathway [31]. Nanog, Sox2, and Oct3/4, which function as critical transcriptional factors in cell self-renewal and pluripotency, were downregulated when β-catenin was decreased. According to recent studies, Nanog was positively regulated by Sox2 and Oct3/4 in NPC, and the inhibitory effect of reduced β-catenin on Nanog was potentially mediated by Sox2 and Oct3/4 [17]. Nanog was also found to be associated with chemoresistance in head and neck cancer cells [37], and reduced Nanog expression implied a weakened resistance in β-catenin-silenced cells. Therefore, low levels of c-myc, Nanog, Oct3/4, Sox2, and EpCAM proved that the production of CSCs was indeed reduced, which was also confirmed by SP analysis. Based on the above findings, the complicated regulation network consisting of Nanog, Oct3/4, Sox2, and β-catenin was of great significance for the maintenance of pluripotency in CSCs. Since CSCs were responsible for tumor recurrence and metastasis, and the biomarkers of CSC were found to be highly expressed in most patients with advanced-stage NPC or recurrence, decreased CSCs caused by silencing β-catenin would limit the oncogenesis. Our xenograft experiment results showed that the growth of tumors was indeed limited. In addition, prevention of Wnt/β-catenin transduction pathway can also inhibit the expressions of angiogenesis-related genes, such as MMP9, MMP2, and VEGF [25,38], which were very important in solid tumor growth. Some studies have already proved that suppressing the function or expression of β-catenin by corresponding inhibitors and microRNA ultimately leads to the limited growth of tumors in mice. Therefore, β-catenin may be a potential target for the treatment of NPC and its recurrence. However, our understanding of the pathologies of neoplasm recurrence and metastasis is still limited, and more efforts are needed for the final cure of NPC.

The poor prognosis of NPC found in patients was mostly associated with CSCs. The migration and proliferation of CSC can assist the growth and invasion of tumors. The activation of Wnt/β-catenin

signal pathway by accumulation of endogenous β-catenin was partly responsible for the characteristics of CSC. Our study demonstrated that β-catenin acts as an indispensable factor for the maintenance of migration and proliferation abilities in NPC cells. Reduced CSC capacities in NPC cells were ascribed to the decreased CSC production caused by downregulated β-catenin. Furthermore, the inhibitory effect on CSC was presumably regulated by a comprehensive signal network consisting of c-myc, Nanog, Oct3/4, Sox2, EpCAM, E-cadherin, vimentin, and β-catenin. It has been proposed that β-catenin may be a promising target for tumor therapy, and the anti-tumor effect of reduced expression of β-catenin was verified *in vivo* in this study. The development of specific inhibitors of β-catenin and drugs that can suppress β-catenin expression may potentially improve the therapeutic effects and prognosis of NPC.

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