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Effects of short-hairpin RNA-inhibited β-catenin expression on the growth of human multiple myeloma cells *in vitro* and *in vivo*

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ABSTRACT

Multiple myeloma (MM) is thrombogenic as a consequence of multiple hemostatic effects. Overexpression of β-catenin has been observed in several types of malignant tumors, including MM. However, the relationship between β-catenin expression and MM remains unclear. In the present study, RNA interference was used to inhibit β-catenin expression in RPMI8226 cells. RT-PCR and Western blotting analyses showed that β-catenin mRNA and protein expression were markedly down-regulated by CTNNB1 shRNA. Western blotting showed that the protein levels of cyclin D1 and glutamine synthetase were downregulated and supported the transcriptional regulatory function of β-catenin. The MTT assay showed that CTNNB1 shRNA could have significant inhibitory effects on the proliferation of RPMI8226 cells. The TOPflash reporter assay demonstrated significant downregulation after CTNNB1 shRNA transfection in RPMI8226 cells. Flow cytometric analyses also showed significantly profound apoptosis in CTNNB1 shRNA cells. We found CTNNB1 silence led to growth inhibition of MM growth *in vivo*. Immunohistochemical analyses showed that c-myc and β-catenin were reduced in CTNNB1 shRNA tumor tissues, but that expression of cleaved caspase-3 was increased. These results show that β-catenin could be a new therapeutic agent that targets the biology of MM cells.

1. Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by the accumulation of clonal malignant plasma cells in the bone marrow. MM is a fetal disease in which malignant plasma cells can migrate and localize in the bone marrow. Cells then disseminate and facilitate the formation of bone lesions. MM is characterized by the production of monoclonal immunoglobulin molecules and painful destruction of bone [1]. MM is incurable, so developing new therapeutic agents that focus on the biology of MM cells is important.

New molecular targeting agents can be developed as better understanding of the pathogenesis underlying MM is achieved. β-Catenin is the central effector molecule of the canonical Wnt signaling pathway, which is important for normal growth and development [2]. The Wnt/β-catenin pathway governs the fate and differentiation of cells during embryogenesis. However, anomalous activation of Wnt signaling in adults is often associated with oncogenesis [3,4]. Aberrant signaling involving the stabilization and nuclear translocation of β-catenin has been observed in MM [5,6] and overexpression of β-catenin has been noted in several types of malignant tumors [7–9].

RNA interference (RNAi) is a gene-silencing mechanism triggered by various double-stranded RNAs [10,11]. RNAi-mediated gene silencing can be achieved by delivering viral vectors that transcribe short hairpin RNA (shRNA), which stably express small interfering RNA (siRNA) in target cells, thereby knocking down the gene of interest [12].

We initiated the present study based on the role of β-catenin in cellular events common to development and oncogenesis, such as proliferation and survival. We used CTNNB1 shRNA directed against β-catenin to examine the impact of successful β-catenin knockdown on the MM cell line RPMI8226, and to demonstrate the indispensable role of β-catenin in the survival and proliferation of tumor cells. We also established a xenograft mouse model *in vivo* to show the inhibitory effect of β-catenin shRNA on the growth of MM cells.

2. Materials and methods

2.1. Acute myeloid leukemia (AML) cell culture

The human cell line RPMI8226 was purchased from the Shanghai Cancer Institute (Shanghai, China). It was maintained in RPMI 1640 medium with 2 mM L-glutamine adjusted to 1.5 g/L of sodium

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bicarbonate, 4.5 g/L of glucose, 10 mM of 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and 1.0 mM sodium pyruvate supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin (all from Invitrogen, Carlsbad, CA, USA) by incubating at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Lentivirus production and transient transfection of cells

The CTNNB1 (β-catenin) shRNA was a double-stranded oligodeoxyribonucleotide (5′-CCG GAA CAG TCT TAC CTG GAC TCT GCT CGA GGA CTT CAT AAG GCG CAT GCT TTT T-3′ and 5′-AAA AAG CAT GCG CCT TAT GAA GTC CTC GAG CAG AGT CCA GGT AAG ACT GTT CCG G-3′). A non-specific sequence was used as a negative control (NS shRNA). Transient or long-term silencing of BCR-ABK alone has been shown to induce the cell-cycle arrest, proliferation, apoptosis and differentiation of leukemic cells (20, 68–76). These were synthesized as a 21-nt inverse repeat separated by a 9-nt loop for each sequence. They were then inserted downstream of the U6 promoter in the lentiviral vector pGCLGFP (GeneChem, Shanghai, China). Lentiviruses were generated by triple transfection of 80%-confluent HEK293T cells with modified pGCL-GFP plasmid and pHelper 1.0 and pHelper 2.0 helper plasmids (GeneChem) using Lipofectamine 2000 (Invitrogen). Lentiviruses were harvested in serum-free medium after 3 d, filtered, and concentrated in primed Centricon Plus-20 filter devices (Millipore, Billerica, MA, USA). RPMI8226 cells were plated in 12-well plates followed by serum starvation for 16 h. When the cells had grown to 70–80% confluence, they were transfected with the lentiviruses of CTNNB1 or NS shRNA at a final concentration of 100 mM in the presence of Lipofectamine 2000 according to manufacturer instructions. Cells were collected at different time points according to the purpose of the experiments.

2.3. Protein extraction and Western blot analyses

Cells treated with CTNNB1 or NS shRNA were used for the preparation of total cell lysates. Homogenization was undertaken in 200 μL of RIPA buffer containing fresh protease and phosphatase inhibitors (Sigma–Aldrich, St. Louis, MO, USA). Protein concentration in lysates was determined by the bicinchoninic acid protein assay, with bovine serum albumin as standard. Twenty micrograms of proteins were loaded onto gels (8–15%) used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose polyvinylidene fluoride (PVDF) membranes (Amersham Bioscience, Piscataway, NJ, USA). The primary antibodies used were against β-catenin, cyclin D1, glutamine synthetase (GS) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Specific protein bands were then developed with ECL Western blotting detection reagents (Amersham Biosciences) and imaged by autoradiography. Differences in protein loading were normalized to the β-actin control.

2.4. Effects of knockdown with β-catenin shRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol™ reagent (Invitrogen) and then subjected to reverse transcription. Levels of human β-catenin mRNA were analyzed using the LightCycler System (Roche Diagnostics, Indianapolis, IN, USA) and FastStart DNA Master SYBER Green I (Roche). Amplicons were validated by evaluating the melting curve and gel electrophoresis. Expression levels of the target mRNAs were normalized to those of the housekeeping gene β-actin. The specific primers used for amplification were: β-catenin, 5′-GCT TGG TTC ACC AGT GGA TT-3′ (forward) and 5′-CCT TCC AGA GGA ACC CTG AG-3′ (reverse); and β-actin, 5′-GGA CTT CGA GCA AGA GAT GG-3′ (forward) and 5′-GAC ATG CGG TTG TGT CAC GA-3′ (reverse).

2.5. β-Catenin/Tcf transcription reporter assay

After CTNNB1 shRNA and NS shRNA stable clones were obtained, cells were transiently transfected with the reporter construct TOPflash or FOPflash (Upstate, Lake Placid, NY, USA). TOPflash has three copies of the Tcf/Lef sites, and is used as a control for measuring non-specific activation of the reporter. All transfections were done using 1.8 μg of TOPflash or FOPflash plasmids and FuGene HD reagent (Roche). To normalize the transfection efficiency in reporter assays, cells were co-transfected with 0.2 μg of the internal control reporter *Renilla reniformis* luciferase driven under the TK promoter (pRL-TK; Promega, Madison, WI, USA). Twenty hours after transfection by TOPflash or FOPflash, the luciferase assay was carried out using a Dual Luciferase Assay System kit in accordance with manufacturer instructions (Promega). Relative luciferase activity (in arbitrary units) was reported as fold induction after normalization for transfection efficiency.

2.6. Cell viability assay

At 24, 48, 72, and 96 h after CTNNB1 shRNA transfection, 10% (*v*/*v*) of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyltetrazolium bromide (MTT; Sigma–Aldrich) diluted in phosphate-buffered saline (PBS) was added to cell cultures. After 30 min of incubation, the medium was aspirated and washed with PBS. Isopropanol (600 mL) was added and shaken gently for 5 min. Two-hundred microliters of this solution were transferred to a 96-well plate, and absorbance measured at 570 nM. Data were normalized to their respective controls and presented as a bar graph.

2.7. Detection of apoptosis by flow cytometry

Parental cells and stable subline cells were trypsinized, collected, washed and stained with annexin V-fluorescein isothiocyanate (FITC; BD Pharmingen, Franklin Lakes, NJ, USA) and propidium iodide (PI; Sigma–Aldrich) for 10 min at 4 °C according to the standard protocol from BD Pharmingen. Apoptotic cells were identified using a FACScan Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.8. A mouse xenograft model of human RPMI8226 tumors

Nude mice (4–6 weeks; 18–22 g) were fed on a super-clean biological laminar flow shelf for 1 week. They were inoculated subcutaneously with 100 μL of a mixture containing 3×10^6 parental cells, NS shRNA cells, and CTNNB1 shRNA cells. Tumors were allowed to grow until they reached a diameter of 5–7 mm. Tumor growth was monitored by measuring the largest (a) and smallest (b) two perpendicular diameters with a caliper. Tumor volume (V) was calculated using the formula $V = a \times b^2 \times 0.5$.

2.9. Immunohistochemical (IHC) analyses

For IHC examinations, paraffin-embedded tumor sections were immunolabeled with primary antibodies. That is, mouse β-catenin or anti-c-myc monoclonal antibodies (Santa Cruz Biotechnology) or rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA). Primary antibodies were visualized using the conventional avidin–biotin–peroxidase complex method (Vectastatin Elite ABC kit, Vector Laboratories, Burlingame, CA, USA).

2.10. Statistical analyses

The *in vivo* effects of shRNA treatment were analyzed using the Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. CTNNB1 inhibits β-catenin expression and downregulates the known target genes in the RPMI8226 cell line

The human MM cell line RPMI8226 was grown to ≈75% confluence, followed by transfection with CTNNB1 or NS shRNA lentiviruses. After CTNNB1 shRNA transfection, stable clones were obtained. CTNNB1 shRNA dramatically reduced β-catenin expression at mRNA and protein levels. The inhibition of shRNA was 82.5% and 79.7%, respectively, in RPMI8226 cells compared with the mock and NS shRNA groups at the mRNA level ($P < 0.01$, Fig. 1A). The inhibition of shRNA was 78.3% and 77.2%, respectively, in RPMI8226 cells compared with the mock and NS shRNA groups at the protein level ($P < 0.01$, Fig. 1B). However, no difference was found between the two control groups ($P > 0.05$). To ascertain whether the decrease in total β-catenin protein in RPMI8226 cells after shRNA treatment also affected the transcriptional regulatory function of β-catenin, protein lysates were examined for the protein expression of two known targets: cyclin D1 and GS. After CTNNB1 shRNA transfection, a decrease in cyclin D1 and GS was detectable in cells, supporting the notion of a functional decrease in β-catenin protein in cells (Fig. 1C).

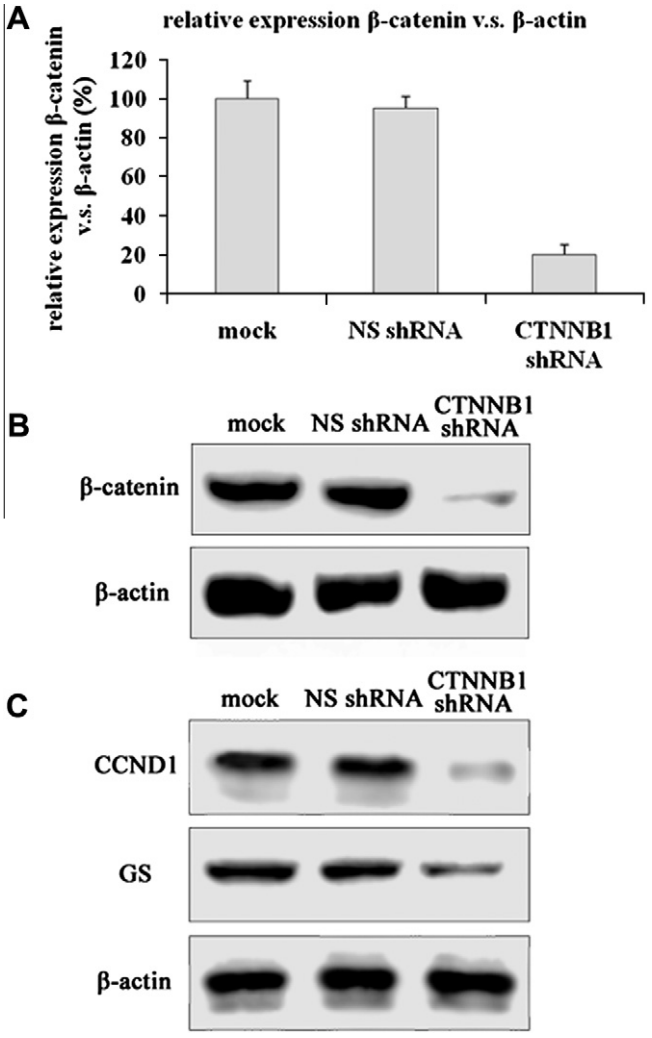

Fig. 1. CTNNB1 inhibits β-catenin expression and downregulates the known target genes in the RPMI8226 cell line. (A) The inhibition of shRNA was 82.5% and 79.7%, respectively, in RPMI8226 cells compared with the mock and NS shRNA groups at the mRNA level ($P < 0.01$); (B) the inhibition of shRNA was 78.3% and 77.2%, respectively, in RPMI8226 cells compared with the mock and NS shRNA groups at the protein level ($P < 0.01$), whereas no difference was found between the two control groups ($P > 0.05$); (C) after transfection with CTNNB1 shRNA, a decrease in cyclin D1 and GS in cells was detectable.

3.2. β-Catenin/Tcf reporter assay confirms CTNNB1 shRNA-mediated loss of β-catenin activity in RPMI8226 cells

We examined the effect of β-catenin knockdown on its activity with the TOPflash reporter assay (which is a direct and reliable measure of β-catenin/Tcf-dependent transcriptional activity). TOPflash and FOPflash activities were measured after CTNNB1 shRNA transfection for 48 h as described in the Material and Methods section. A significant downregulation in TOPflash reporter activity, without any FOPflash activity, was apparent after CTNNB1 shRNA transfection in RPMI8226 cells (Fig. 2). This finding clearly demonstrated a pronounced loss of β-catenin function in tumor cells.

3.3. Effect of CTNNB1 shRNA on the proliferation of RPMI8226 cells

To address the biological relevance of the loss of β-catenin function, we investigated the effect of CTNNB1 shRNA on the survival of RPMI8226 cells. Cell proliferation was monitored and analyzed by the MTT assay for 4 d after RPMI8226 cells were stably transfected with mock, NS shRNA, and CTNNB1 shRNA. The results of the MTT assay suggested that relative cell proliferation in mock and NS shRNA groups was gradually increased from 2 d (0.53 ± 0.07 and 0.52 ± 0.05) to 6 d (1.64 ± 0.11 and 1.65 ± 0.13) in a time-dependent manner, but remained virtually unchanged in cells transfected with CTNNB1 shRNA from 2 d (0.51 ± 0.07) to 6 d (0.71 ± 0.11). Statistical analyses showed that the plasmid CTNNB1 shRNA could have significant inhibitory effects on the proliferation of RPMI8226 cells compared with the mock ($P < 0.001$) and NS shRNA groups ($P < 0.001$). However, the NS shRNA group had no significant inhibitory effects upon cell proliferation compared with those seen in the mock group ($P > 0.05$, Fig. 3A).

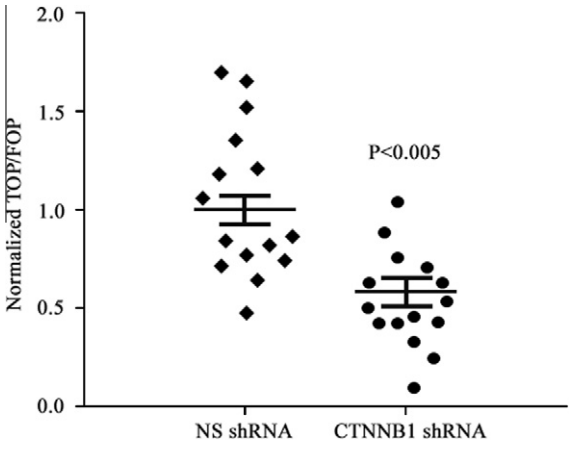

Fig. 2. The β-catenin/Tcf reporter assay confirms CTNNB1 shRNA-mediated loss of β-catenin activity in RPMI8226 cells. TOPflash and FOPflash activities were measured after CTNNB1 shRNA transfection for 48 h. A significant downregulation in TOPflash reporter activity, without any FOPflash activity, was apparent after CTNNB1 shRNA transfection in RPMI8226 cells.

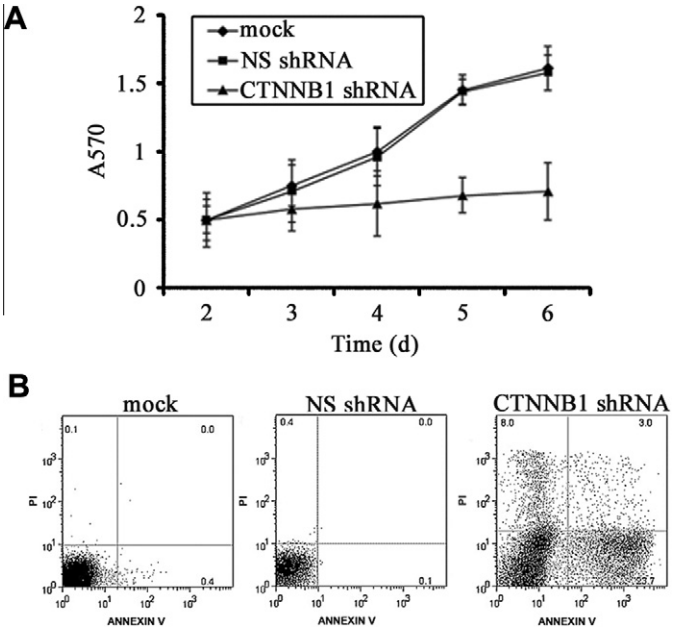

Fig. 3. Effect of CTNNB1 shRNA on the proliferation and apoptosis of RPMI8226 cells. (A) Effect of CTNNB1 shRNA on cell proliferation. The plasmid CTNNB1 shRNA could have significant inhibitory effects on the proliferation of RPMI8226 cells compared with the mock ($P < 0.001$) and NS shRNA groups ($P < 0.001$). The NS shRNA group had no significant inhibitory effects on cell proliferation compared with the mock group ($P < 0.05$); (B) effect of CTNNB1 shRNA on apoptosis. Significantly profound apoptosis was found in CTNNB1 shRNA-expressing cells compared with mock ($P < 0.001$) and NS shRNA control groups ($P < 0.001$). The rate of apoptosis in RPMI8226 cells and by stable transfection of NS shRNA control groups showed no significant difference ($P < 0.05$, in B).

3.4. Effect of CTNNB1 shRNA on cell apoptosis

To investigate if CTNNB1 shRNA stable sublines increased the rate of apoptosis of RPMI8226 cells, apoptosis was assessed using the flow cytometry-based annexin V and PI binding assay. Flow cytometric analyses also showed that the rate of apoptosis of the mock, the NS shRNA control and their stable sublines were 0.96% ± 0.65%, 0.43% ± 0.35%, and 24.57% ± 4.5%, respectively. Statistical analyses showed significantly profound apoptosis in CTNNB1 shRNA-expressing cells compared with mock ($P < 0.001$) and NS shRNA control groups ($P < 0.001$). However, there was no significant difference in the rate of apoptosis in RPMI8226 cells and by stable transfection of NS shRNA control groups ($P > 0.05$, Fig. 3B).

3.5. In vivo effects of CTNNB1 shRNA on myeloma tumors

We evaluated tumor size during 4 weeks ($n = 8$ per group). Tumor sizes were monitored every 2 d with a caliper. Tumor volumes were markedly increased 10 d in mock and NS-shRNA groups. Xenografts with CTNNB1 shRNA showed a very slow growth pattern; this finding suggested that CTNNB1 silence led to growth inhibition of MM *in vivo* (Fig. 4A, $P < 0.01$). To understand the underlying mechanism, tumor sections were subjected to IHC analyses of β-catenin, c-myc and cleaved caspase-3 (Fig. 4B). β-Catenin expressions were dramatically reduced in the CTNNB1 shRNA group compared with those in the NS shRNA and mock groups. c-myc is a target of β-catenin, so we examined its expression in tumor tissues: c-myc expressions were also reduced significantly in the CTNNB1 shRNA group. To clarify if caspase was activated by β-catenin depletion, we investigated the expression of cleaved caspase-3-positive cells: cell number was significantly increased in the CTNNB1 shRNA group compared with that seen in the NS shRNA and mock groups.

4. Discussion

The Wnt signaling pathway plays a major part in the maintenance of the phenotypes of stem cells, and is highly active during organogenesis and body patterning. Aberrant signaling of Wnt is also observed in many types of cancer, including MM. MM is a malignant tumor of bone whose incidence has been increasing due to an increased prevalence of risk factors. β-Catenin is an independent prognostic factor for survival from MM, but its precise role in the physiology of MM cells and disease establishment *in vivo* is undetermined. The present study described the delineation of the role of β-catenin in MM through specific downregulation of a protein with a shRNA expression lentiviral vector.

We examined the effect of the inhibition of β-catenin by CTNNB1 shRNA in human MM cell lines. We used the RPMI8226 cell line, which demonstrates appreciable expression of β-catenin [13]. Western blotting analyses revealed a dramatic decrease in β-catenin expression in RPMI8226 cells compared with the mock group and NS shRNA group. RT-PCR results also showed that β-catenin mRNA levels were inhibited by CTNNB1 shRNA compared with those in the mock group and NS shRNA group. Cyclin D1 and GS are two known targets of β-catenin. After CTNNB1 shRNA transfection, a decrease in expressions of cyclin D1 and GS were detectable in RPMI8226 cells, supporting the notion of a functional decrease of β-catenin. The TOPflash reporter assay demonstrated a significant decrease in β-catenin transcriptional activity in RPMI8226 cells after CTNNB1 shRNA transfection for 48 h. Luciferase activity in FOPflash remained unaffected, confirming a lack of non-specific activity of the reporter system. In addition, there was a significant decrease in the viability and proliferation of tumor cells after CTNNB1 shRNA transfection for 48 h, as shown by the MTT assay. The rate of apoptosis in the CTNNB1 shRNA group showed a substantial increase compared with the mock group and NS shRNA group.

Based on the findings detailed above, β-catenin suppression in RPMI8226 cells appears to have advantages. We wanted to ascertain if β-catenin could influence the viability and proliferation of tumor cells *in vivo*. We established an animal model in which RPMI8226 cells were allowed to proliferate in nude mice. We observed that RPMI8226 cells transfected with CTNNB1 shRNA grew slowly *in vivo* in nude mice compared with the growth seen in mice in mock and NS shRNA groups. β-Catenin is a downstream effector of the Wnt signaling pathway. When the Wnt signaling pathway stabilizes β-catenin, and its non-phosphorylated form accumulates in the cytoplasm, β-catenin translocates to the nucleus, where it interacts with T-cell factors, driving the transcription of target genes encoding c-myc [14–16]. We confirmed that CTNNB1 shRNA could diminish the expression of β-catenin and c-myc in IHC analyses. In addition, expressions of cleaved caspase-3-positive cells were observed in MM tumors. Taken together, these results suggest that depletion of β-catenin induces apoptosis by activating caspase-3 and inhibiting the growth of MM cells.

We showed that CTNNB1 shRNA inhibited proliferation of MM tumors, and that β-catenin might represent a molecular target for therapy against MM. Small β-catenin inhibitor molecules have been developed and investigated [9,15]. However, systemic administration of β-catenin inhibitors might induce severe adverse effects because β-catenin is an important molecule for stem cells [17–19]. Moreover, inhibition of Wnt/β-catenin signaling has been reported to result in the development of MM-mediated bone disease [20–22]. Hence, Wnt/β-catenin signaling could be essential for skeletogenesis and osteoblast differentiation [23–25]. Therefore,

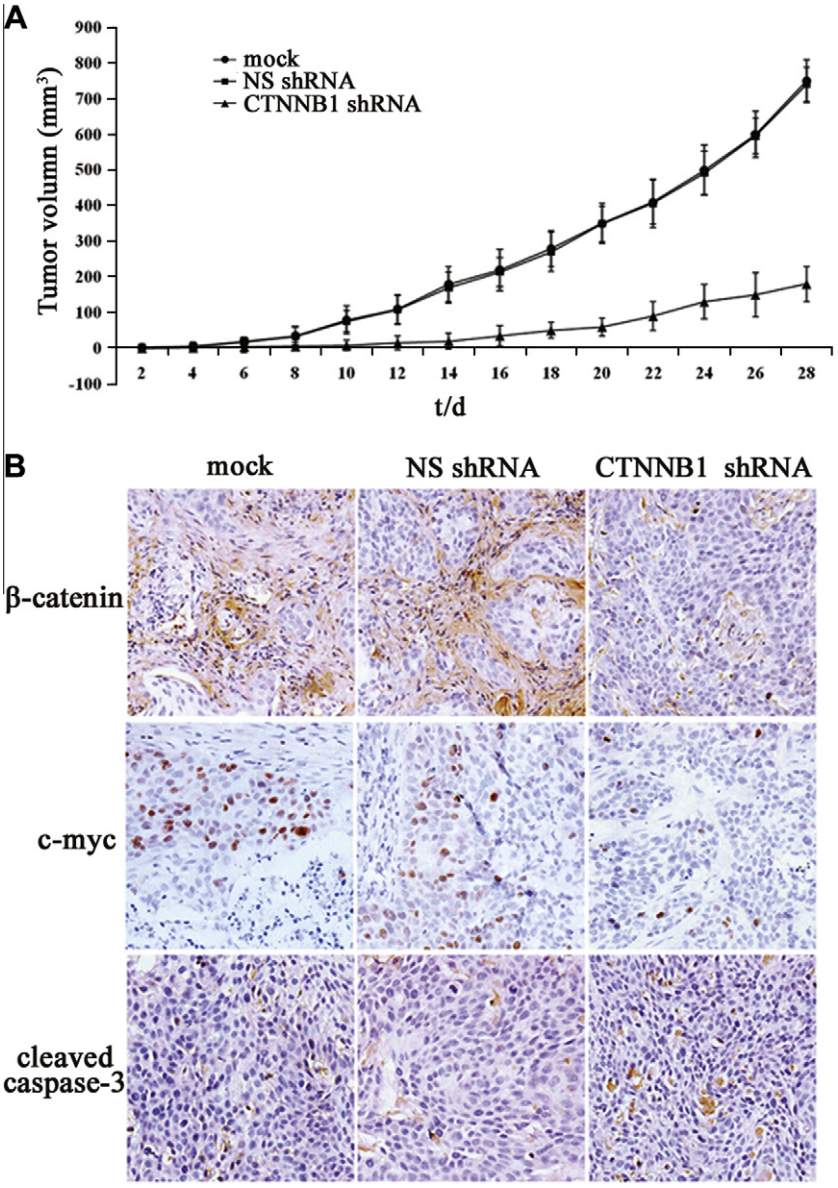

Fig. 4. Effects of CTNNB1 shRNA on MM tumors *in vivo*. (A) Tumor volumes during 4 weeks. Tumor sizes were monitored every 2 d with a caliper. Tumor volumes were markedly increased 10 d in mock and NS-shRNA groups. Xenografts with CTNNB1 shRNA showed a very slow growth pattern; these results suggested that CTNNB1 silence led to growth inhibition of MM *in vivo*; (B) immunohistochemical analyses of β-catenin, c-myc and cleaved caspase-3 of tumor sections. Expressions of β-catenin and c-myc were dramatically reduced in the CTNNB1 shRNA group compared with those in the NS shRNA and mock groups, whereas the expression of cleaved caspase-3-positive cells was significantly increased in the CTNNB1 shRNA group compared with that in the NS shRNA and mock groups.

the CTNNB1 shRNA-targeting β-catenin strategy needs further investigation.

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