β-Catenin Small Interfering RNA Successfully Suppressed Progression of Multiple Myeloma in a Mouse Model

Eishi Ashihara,[1] Eri Kawata,[1] Yoko Nakagawa,[1] Chihiro Shimazaski,[2] Junya Kuroda,[2] Kyoko Taniguchi,[2] Hitoji Uchiyama,[2] Ruriko Tanaka,[1] Asumi Yokota,[1] Miki Takeuchi,[1] Yuri Kamitsuji,[1] Tohru Inaba,[3] Masafumi Taniwaki,[2] Shinya Kimura,[1] and Taira Maekawa[1]

Abstract

Purpose: β-catenin is the downstream effector of the Wnt signaling pathway, and it regulates cell proliferation. β-catenin overexpression correlates positively with prognosis in several types of malignancies. We herein assessed its effects on growth of multiple myeloma cells using a xenograft model.

Experimental Design: We first investigated the expression of β-catenin in multiple myeloma cell lines and multiple myeloma cells obtained from patients. Next, we investigated the growth inhibitory effects of β-catenin small interfering RNA on the growth of multiple myeloma cells *in vivo*. Six-week-old male BALB/c *nu/nu* mice were inoculated s.c. in the right flank with 5×10^6 RPMI8226 cells, followed by s.c. injections of β-catenin small interfering RNA, scramble small interfering RNA, or PBS/atelocollagen complex twice a week for a total of eight injections.

Results: Significantly higher levels of β-catenin expression were observed in multiple myeloma cell lines and in samples from patients with multiple myeloma than those found in mononuclear cells obtained from healthy volunteers. In *in vivo* experiments, no inhibitory effects were observed following treatment with scramble small interfering RNA or PBS/atelocollagen complexes, whereas treatment with β-catenin small interfering RNA/atelocollagen complex significantly inhibited growth of multiple myeloma tumors ($P < 0.05$).

Conclusions: β-catenin small interfering RNA treatment inhibited the growth of multiple myeloma tumors in a xenograft model. To our knowledge, this is the first report showing that the treatment with β-catenin small interfering RNA produces an inhibitory effects on growth of hematologic malignancies *in vivo*. Because treatment with β-catenin small interfering RNA inhibited growth of multiple myeloma cells, β-catenin is the attractive novel target for treating multiple myeloma.

As we gain a better understanding of the pathogenesis underlying multiple myeloma, new molecular targeting agents can be developed. At present, multiple myeloma remains incurable, so it is important to continue to investigate new therapeutic agents that focus on the biology of multiple myeloma cells. β-catenin is the downstream effector of the Wnt signaling pathway, and it regulates the genes encoding cyclin D1 and c-myc (1–3). Activation of Wnt signaling is closely involved in the process of carcinogenesis (4), and β-catenin overexpression has been observed in several types of malignant tumors, including hematologic malignancies (5–9).

RNA interference is a powerful tool in postgenomic research, and recently, experimentally introduced small interfering RNAs have been used in cancer therapy. The success of small interfering RNA therapy depends upon the development of suitable delivery systems, and several useful drug delivery systems have been developed (10–13). Among the drug delivery systems, atelocollagen represents one of the most attractive nonviral carriers for gene delivery. It is obtained from calf dermis, following the removal of immunogenic telopeptides located at the N- and C-termini of collagen molecules. Because atelocollagen has a positively charged surface, it easily binds negatively charged molecules such as nucleic acids. The small interfering RNA/atelocollagen complex is also resistant to nucleases and is transduced efficiently, resulting in long-term gene silencing (14). Here,

Authors' Affiliations: [1]Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital; [2]Division of Hematology and Oncology, Department of Medicine; and [3]Department of Molecular Genetics and Laboratory Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

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E. Ashihara and E. Kawata contributed equally to this study.

Requests for reprints: Eishi Ashihara, Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-3630; Fax: 81-75-751-4283; E-mail: ash0325@kuhp.kyoto-u.ac.jp.

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Translational Relevance

In this study, we showed that β-catenin small interfering RNA treatment successfully inhibited the growth of myeloma cells *in vivo* and we revealed that β-catenin is an attractive target for multiple myeloma. These findings not only show evidences for efficacy of targeting therapies for Wnt/β-catenin pathway but also encourage the possibilities of small interfering RNA therapies against multiple myeloma.

we use a xenograft model to show the inhibitory effect of the β-catenin small interfering RNA/atelocollagen complex on growth of multiple myeloma cells.

Materials and Methods

Cell lines and human samples. The human AMO-1, RPMI8226, NCI-H929, U226, OPM-2, KMS-12-BM, EJM, LP-1 myeloma cell lines, and IM-9 Epstein-Barr virus–transformed cell line derived from multiple myeloma patient were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The IM-9, OPM-2, RPMI8226, NCI-H929, and U266 were cultured in RPMI1640 (Gibco) containing 10% heat-inactivated FCS (Invitrogen), 2 mmol/L L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). The AMO-1 and KMS-12-BM cell lines were cultured in RPMI1640 containing 20% FCS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin. The EJM and LP-1 cell lines were cultured in Iscove's modified Dulbecco's medium (Gibco) containing 10% FCS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin. All cell lines were maintained at 37°C in a fully humidified atmosphere of 5% CO_2 in air.

Six bone marrow samples, one ascites sample, and two pleural effusion samples were obtained from five multiple myeloma patients. Three bone marrow samples were obtained from healthy volunteers. In accordance with the Declaration of Helsinki recommendations, all procedures were approved by the institutional review board at Kyoto Prefectural University of Medicine, and written informed consent was obtained from every participant.

Expression of β-catenin in multiple myeloma cells. We used Western blotting analysis to investigate the expression of β-catenin in nine human multiple myeloma cell lines, as well as primary multiple myeloma cells. Ficoll-Hypaque density centrifugation was used to separate mononuclear cells from each participant's samples. A magnetic cell sorting separation system (Miltenyi) and anti-CD138 antibody (Miltenyi) were used to enrich multiple myeloma cells and normal plasma cells from bone marrow samples. Cells were analyzed by FACS Calibur using the Cell Quest software (BD Bioscience). The purity of enriched CD138+ cell populations was ≥90%. Multiple myeloma cells from ascites and pleural effusion were shown to express CD138. Cells were lysed with radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 7.4; 0.25 mol/L NaCl; 5 mmol/L EDTA; 20 mmol/L NaF; 1% NP-40) containing freshly prepared phenylmethylsulfonylfluoride (1 mmol/L) and protease inhibitor (10 μg/mL). Cell suspensions were cleared by centrifugation at 14,000 × *g* for 30 mins at 4°C. Nuclear and cytoplasmic protein fractions were obtained using by NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology) according to the manufacturer's instruction. The supernatant (total cell lysate, nuclear, and cytoplasmic protein fractions) was either used immediately or stored at -80°C. Protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Immunoblotting was done as described previously (15). Samples of cell extracts containing 20 μg of protein were analyzed. As the primary antibodies, we used a mouse monoclonal anti–β-catenin antibody (BD Pharmingen), a mouse anti–dephosphorylated β-catenin monoclonal antibody (Alexis Biochemicals), a mouse anti–phosphorylated β-catenin monoclonal antibody (Sigma-Aldrich), a rabbit polyclonal anti–cleaved caspase-3 antibody (Cell Signaling Technology), a rabbit polyclonal anti–caspase-3 antibody (Cell Signaling Technology), a rabbit polyclonal anti–Oct-1 (Santa Cruz Biotechnology), and a rabbit polyclonal anti-actin antibody (Sigma-Aldrich). Horseradish peroxidase–coupled anti–mouse and anti–rabbit immunoglobulin G (Amersham Biosciences) were used as

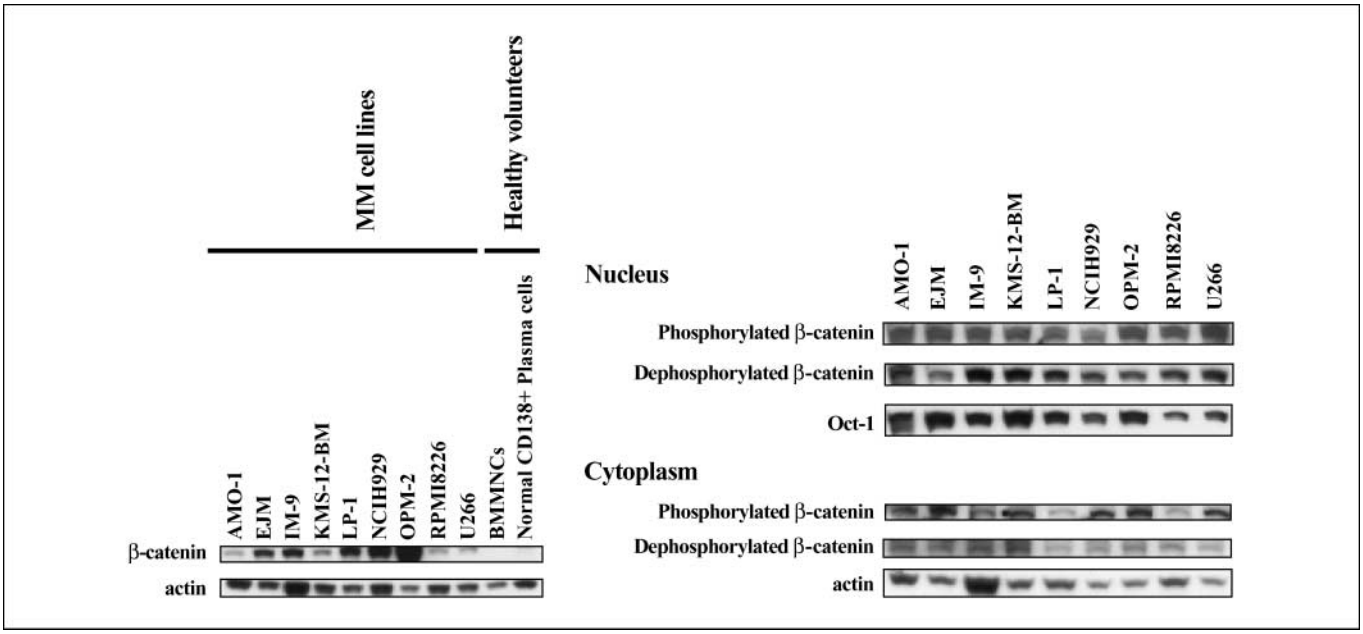

Fig. 1. β-catenin expression in human myeloma cell lines. β-catenin expression was examined by Western blotting. Left, β-catenin was overexpressed in myeloma cell lines and in multiple myeloma patient samples compared with bone marrow mononuclear cells and normal CD138+ plasma cells obtained from healthy volunteers. Actin expression was used as a loading control. Right, phosphorylated and dephosphorylated forms of β-catenin were expressed in multiple myeloma cell lines.

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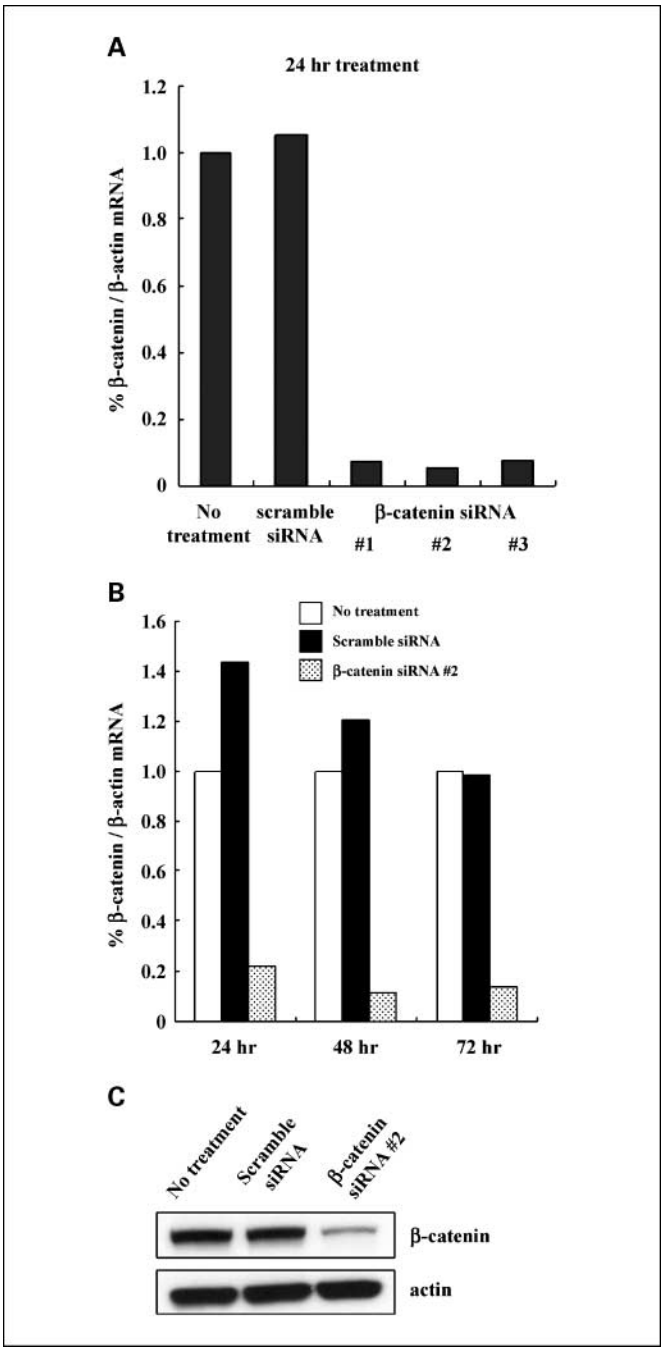

Fig. 2. Effects of β-catenin small interfering RNA *in vitro. A,* RT-PCR analysis. All three β-catenin small interfering RNAs decreased *β-catenin* mRNA levels in SW480 cells after 24-h treatment. No reduction in expression was observed for cells with no treatment or treatment with scramble small interfering RNA. *B,* real-time PCR analysis. Treatment of A549 cells with β-catenin small interfering RNA 2 leads to a decrease in *β-catenin* mRNA levels over a period of 72 h. No reduction in expression was observed for cells with no treatment or treatment with scramble small interfering RNA. *C,* Western blotting analysis of A549 cell lysates. There was a decreased expression of β-catenin small interfering RNA after 72-h treatment with β-catenin 2. No reduction in expression was observed for cells with no treatment or treatment with scramble small interfering RNA. Actin expression was used as a loading control.

secondary antibodies, and signal detection was done with an enhanced chemiluminescence kit (Amersham Biosciences).

Effects of knockdown with β-catenin small interfering RNA. Three types of β-catenin (Gene Bank accession number NM_001904) small interfering RNA and one scramble small interfering RNA with the following sense and antisense sequences were used: β-catenin small interfering RNA 1, 5′-CCAGGAUGAUCCUAGCUAUTT-3′ (sense), 5′-AUAGCUAGGAUCAUCCUGGTT-3′ (antisense); β-catenin small interfering RNA 2, 5′- GUAUUUGAAGUAUACCAUATT-3′ (sense), 5′-UAUGGUAUACUUCAAAUACTT-3′ (antisense); β-catenin small interfering RNA 3, 5′- CCAUUACAACUCUCCACAATT-3′ (sense), 5′-UUGUGGAGAGUUGUAAUGGTT-3′ (antisense); and scramble small interfering RNA 2, 5′- GGAAGAUAAUCUUUUCUAATT-3′ (sense), 5′-UUAGAAAAGAUUAUCUUCCTT-3′ (antisense). All small interfering RNAs were synthesized by Takara Bio, Inc. We first examined the effects of small interfering RNA-mediated knockdown using real-time reverse transcription-PCR (RT-PCR) and Western blotting analysis. Following transfection of β-catenin small interfering RNA into SW480 colon cancer cells and A549 lung cancer cells with Lipofectamine 2000 (Invitrogen), total RNA was extracted using the Micro-to-Midi Total RNA Extraction Kit (Invitrogen) and then subjected to reverse transcription (16). The levels of human *β-catenin* mRNA were analyzed using the LightCycler System (Roche Diagnostics) and FastStart DNA Master SYBER Green I (Roche). Amplicons were validated by melting curve and gel electrophoresis. The expression levels of the target mRNAs were normalized to those of the housekeeping gene *β-actin*. The specific primers used for amplification were as follows: *β-catenin*, 5′-GCTTGGTTCACCAGTGGATT (forward) and 3′- CCTTCCAGAGGAACCCTGAG (reverse); and *β-actin*, 5′-GGACTTCGAGCAAGAGATGG (forward) and 3′-GACATGCGGTTGTGTCACGA (reverse). Transfected cells were also examined using Western blotting analysis as described above.

In vivo *effects of β-catenin small interfering RNA on myeloma tumors.* After 3 Gy irradiation, specific pathogen-free 6- to 7-wk old male BALB/c *nu/nu* mice (SLC) were inoculated s.c. in the right flank with 5×10^6 RPMI8226 myeloma cells in 100 μL PBS. Palpable tumors (100 mm^3 in volumes) developed within 3 or 4 wks. Mice were then treated with s.c. (around tumors) injections of β-catenin small interfering RNA (2.5 μmol/L)/1% atelocollagen complex (final atelocollagen concentration, 0.5%), scramble small interfering RNA (2.5 μmol/L)/1% atelocollagen complex, β-catenin small interfering RNA (2.5 μmol/L)/PBS, or PBS/1% atelocollagen, twice a week for a total of eight injections. Tumor size was measured in two dimensions using a caliper, and tumor volume (mm^3) was calculated as $a^2 \times b/2$ mm^3 (a, minor axis; b, major axis).

Real-time RT-PCR and immunohistochemical examinations were used to examine the effects of β-catenin small interfering RNA–mediated knockdown in s.c. multiple myeloma tumors. Real-time RT-PCR was done as described above. For immunohistochemical 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). Primary antibodies were visualized using the conventional avidin-biotin-peroxidase complex method (VECTASTATIN Elite ABC kit, Vector Laboratories, Inc.). Sections were counterstained with hematoxylin and mounted. Detection of apoptosis was done using the terminal uridine deoxynucleotide nick end labeling (TUNEL) method and an ApopTag plus peroxidase *in situ* apoptosis detection kit (Millipore), according to the manufacturer's instructions. Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

Statistical analysis. The *in vivo* effects of small interfering RNA treatment were analyzed using the Student's t test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Expression of β-catenin in myeloma cells. Firstly, we examined human multiple myeloma cell lines, all of which expressed

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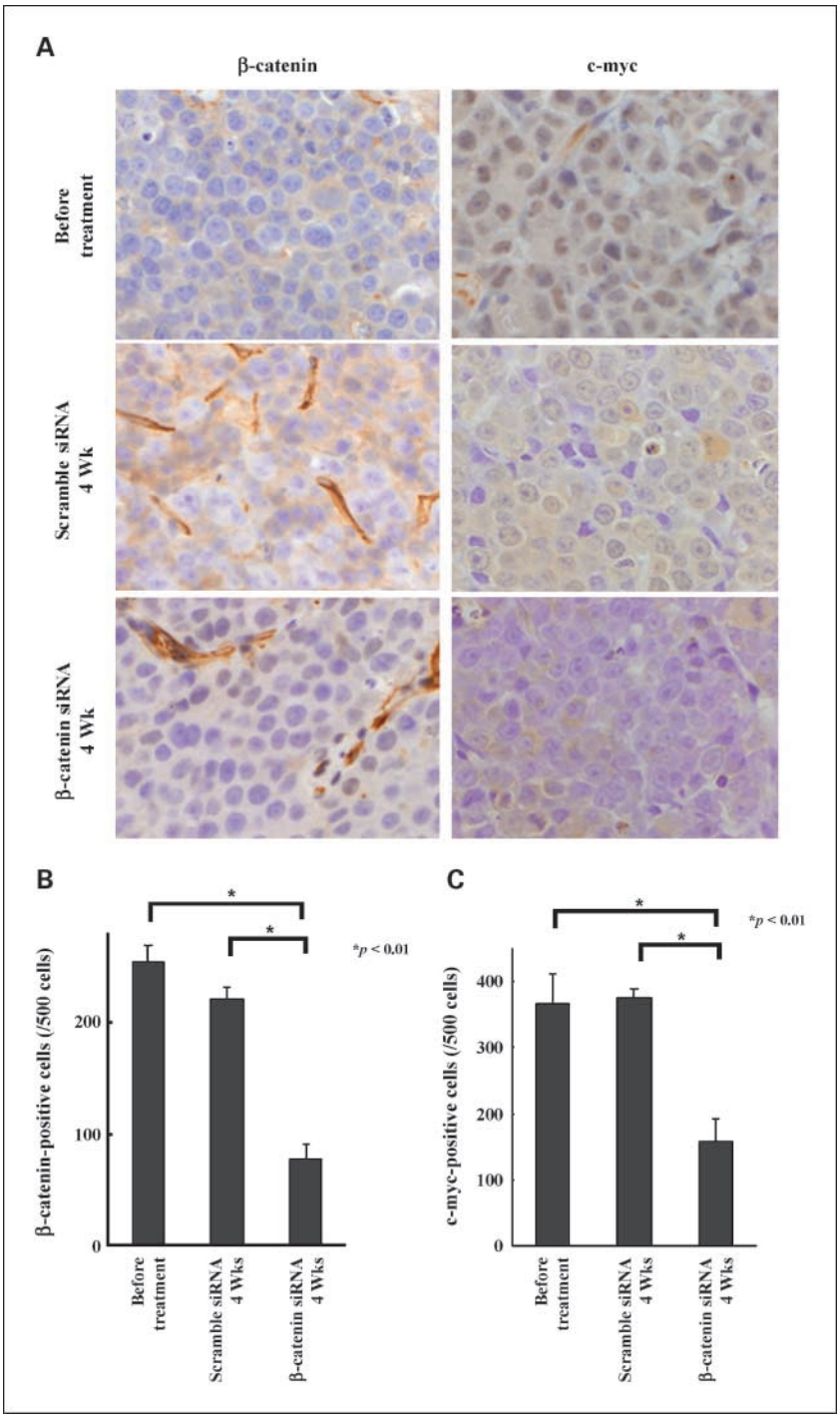

Fig. 3. Changes to β-catenin expression in myeloma tumors following treatment with β-catenin small interfering RNA. *A,* mice (n = 3 per group) were treated with s.c. injections of β-catenin small interfering RNA (2.5 μmol/L)/1% atelocollagen complex (final atelocollagen concentration, 0.5%) or scramble small interfering RNA (2.5 μmol/L)/1% atelocollagen complex. Immunohistochemical studies revealed that β-catenin and c-myc expression diminished following 4 wks of treatments with β-catenin small interfering RNA/atelocollagen complex relative to before treatment and with the scramble small interfering RNA/atelocollagen complex. *B,* β-catenin small interfering RNA/atelocollagen complex treatment significantly decreased β-catenin expression in multiple myeloma tumors relative to no treatment and treatment with the scramble small interfering RNA/atelocollagen complex. Results are means ± SEs. *C,* β-catenin small interfering RNA/atelocollagen complex treatment significantly decreased c-myc expression in multiple myeloma tumors relative to no treatment and treatment with the scramble small interfering RNA/telocollagen complex. Results are means ± SEs.

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significantly higher β-catenin levels than normal human mononuclear cells and normal plasma cells (Fig. 1, *left*). We investigated phosphorylated and dephosphorylated forms of β-catenin. Both forms of β-catenin were expressed in multiple myeloma cell lines and primary multiple myeloma cells (Fig. 1, *right*). Total β-catenin levels in the cell lines did not correlate with phosphorylated and dephosphorylated β-catenin levels in the nuclear and cytoplasmic fractions. We speculate that the localizations of both β-catenin forms are different and that the degradation rates caused by proteasome are different in various cell lines. Moreover, we found significantly elevated expression of both forms of β-catenin in myeloma cells obtained from patients, relative to cells obtained from healthy volunteers (Supplementary Fig. S1).

Effects of knockdown with β-catenin small interfering RNA. We then examined knockdown of endogenous level of β-catenin protein levels using the three types of β-catenin small interfering RNA. Following transfection β-catenin or scramble small interfering RNA (100 nmol/L) into SW480 cells and A549 cells, we examined β-catenin expression using real-time RT-PCR and Western blot analysis. SW480 cells were examined 24 hours after treatment with the three β-catenin

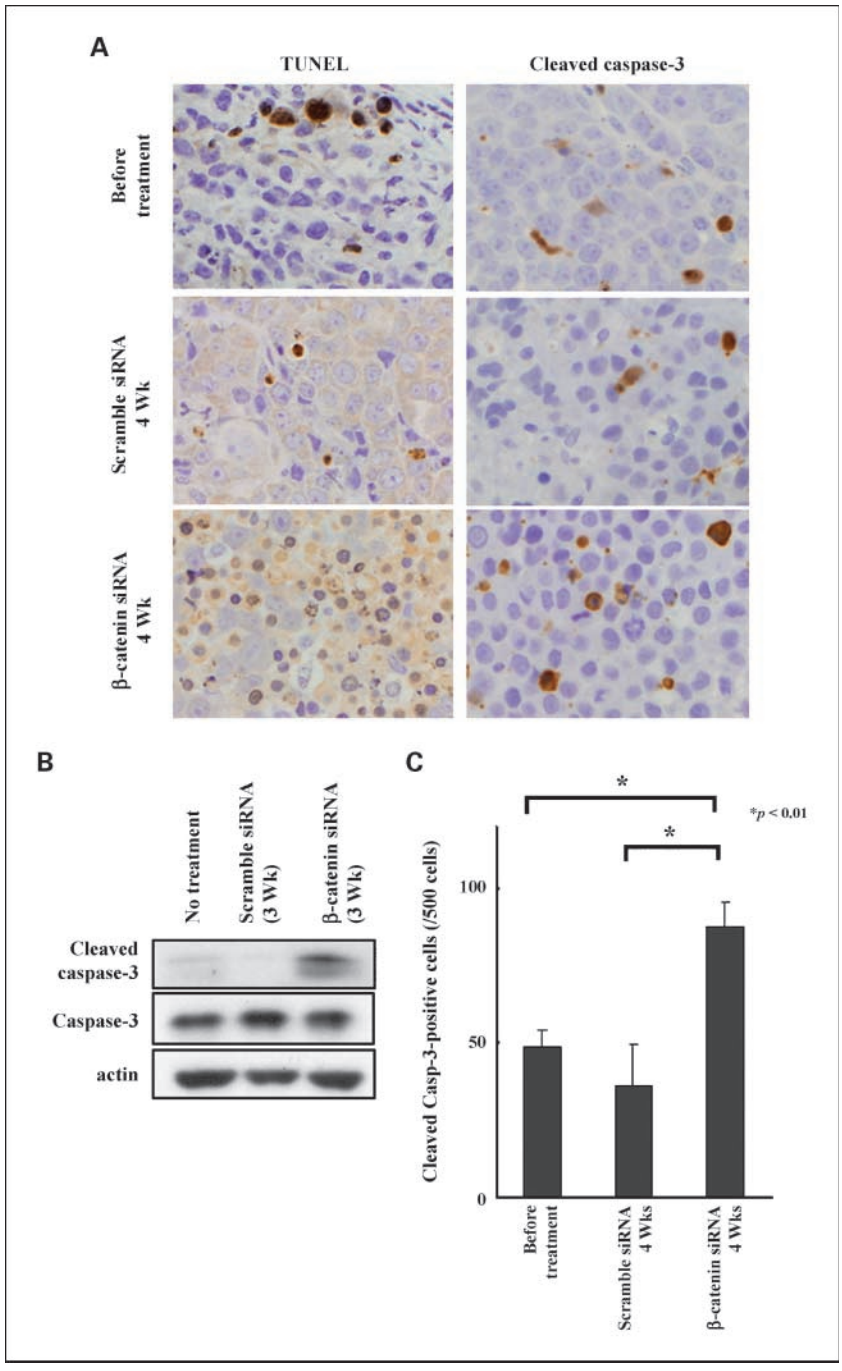

Fig. 4. Apoptotic cells and cleaved caspase-3 expression in myeloma tumors following treatment with β-catenin small interfering RNA. *A,* apoptotic cell (TUNEL-positive cell) numbers and cleaved caspase-3 expression increased following 4 wks of treatment with the β-catenin small interfering RNA/atelocollagen complex relative to before treatment and with the scramble small interfering RNA/telocollagen complex treatment. *B,* the expression of cleaved capsase-3 was increased in tumors after 3-wk treatment with β-catenin small interfering RNA/atelocollagen complex. *C,* cleaved caspase-3 positive cell numbers significantly increased in tumors of mice treated with β-catenin small interfering RNA/atelocollagen complex compared with those before treatment and those treated with scramble small interfering RNA/atelocollagen complex. Results are means ± SEs.

small interfering RNAs. In comparison with no treatment or treatment with scramble small interfering RNA, all three β-catenin small interfering RNAs caused a marked decrease in *β-catenin* mRNA levels (Fig. 2A). There were no significant differences in knockdown effects among three small interfering RNAs; therefore, β-catenin small interfering RNA 2 was used in further experiments. In A549 cells treated with β-catenin small interfering RNA 2, expression of *β-catenin* mRNA was reduced even at 72 hours after treatment, whereas no reduction was observed in non- or scramble small interfering RNA–treated cells (Fig. 2B). Similarly, β-catenin protein levels decreased after 72-hour treatment, whereas no reduction was observed in non- or scramble small interfering RNA–treated cells (Fig. 2C). These data showed that our β-catenin small interfering RNAs can diminish β-catenin expression successfully. Next, we investigated the antimyeloma effects of β-catenin small interfering RNA using a xenograft model.

In vivo *effects of β-catenin small interfering RNA on myeloma tumors.* We assessed β-catenin small interfering RNA–mediated growth inhibition *in vivo* using a mouse model (n = 3 per group). We administered β-catenin and scramble small interfering RNA/atelocollagen complexes twice a week for a total of eight injections and then compared expression of β-catenin and c-myc, as well as relative numbers of apoptotic

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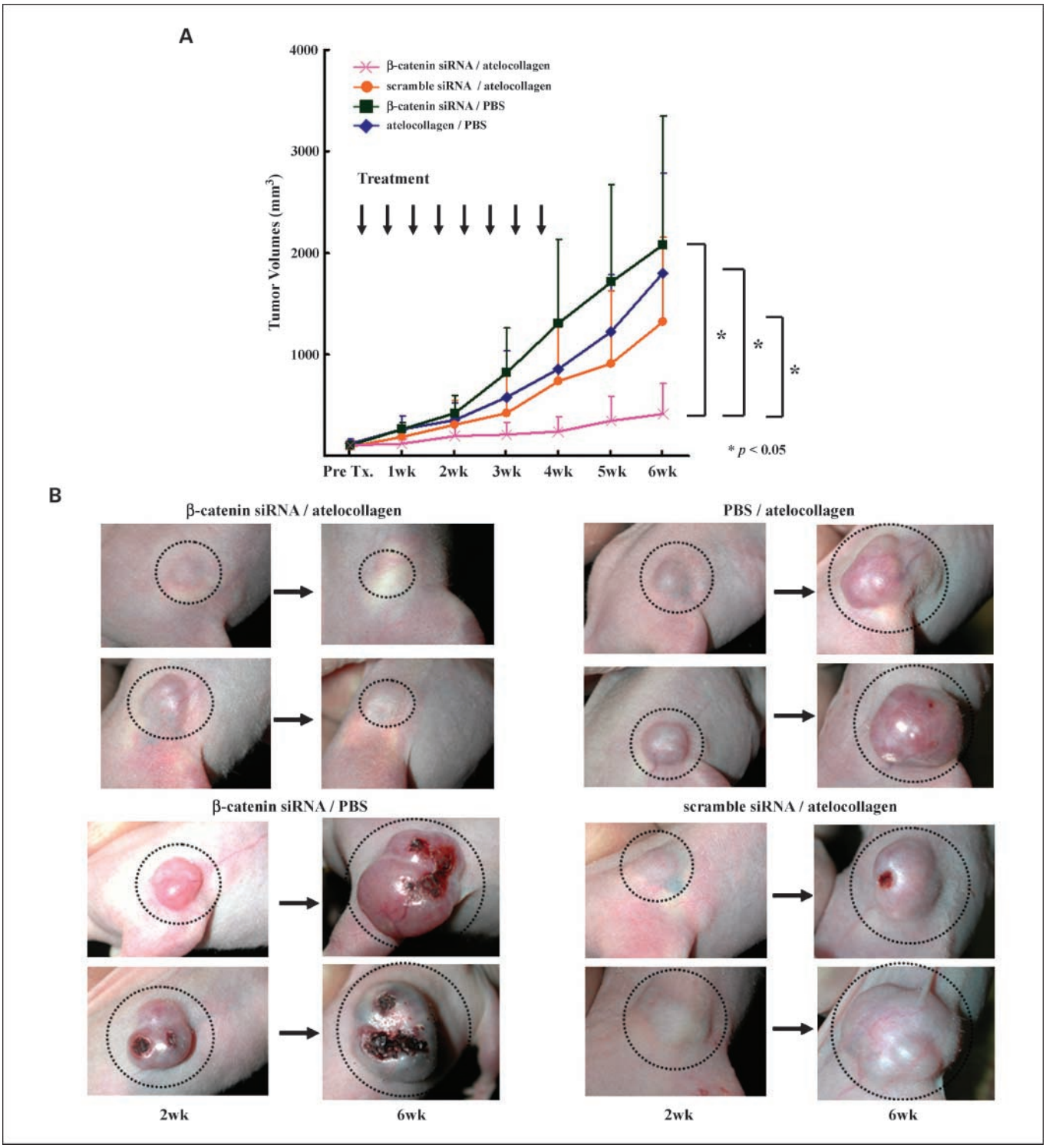

Fig. 5. Antimyeloma effects of β-catenin small interfering RNA in RPMI8226 xenografts. *A,* tumor growth curves. Palpable myeloma tumors (~100 mm^3 in volumes) were treated with s.c. (around the tumors) injections of β-catenin small interfering RNA (2.5 μmol/L)/1% atelocollagen complex (final atelocollagen concentration, 0.5%), scramble small interfering RNA (2.5 μmol/L)/1% atelocollagen complex, β-catenin small interfering RNA (2.5 μmol/L)/PBS, or PBS/1% atelocollagen twice a week for a total of eight injections (n = 5 per group). Treatment with β-catenin small interfering RNA/atelocollagen complex significantly reduced tumor volumes ($P < 0.05$). Results are means ± SEs. *B,* representative photos of tumor grafts after 2 and 6 wks of treatment. Dashed circles, myeloma tumors.

and cleaved caspase-3–positive cells. After RPMI8226 tumors had been treated for 1 or 2 weeks with the β-catenin small interfering RNA/atelocollagen complex, a significant decrease in *β-catenin* mRNA was observed (data not shown). After treatment for 4 weeks, β-catenin expression was decreased immunohistologically whereas expression was observed in tumors treated with the scramble small interfering RNA/atelocollagen complex (Fig. 3A, *left*). Because c-myc is a

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target of β-catenin, we examined its expression in these 4-week-treated tissues (Fig. 3A, *right*). We found that, like β-catenin, c-myc expression was reduced significantly by treatment with the β-catenin small interfering RNA/atelocollagen complex (Fig. 3B and C). At this time point, we observed that the β-catenin small interfering RNA/atelocollagen complex–treated cells showed a significant increase in apoptotic cells using a TUNEL assay (Fig. 4A, *left*). To clarify the whether caspase was activated by the depletion of β-catenin, we investigated the expression of cleaved caspase-3 in multiple myeloma tumors by β-catenin small interfering RNA/atelocollagen complex treatment. Cleaved capase-3–positive cells were significantly increased in myeloma tumors treated with β-catenin small interfering RNA/atelocollagen complex treatment (Fig. 4A, *right*, and C). In Western analysis, cleaved caspase-3 was overexpressed in multiple myeloma tumors (Fig. 4B). Taken together, our results indicate that treatment with the β-catenin small interfering RNA/atelocollagen complex induced apoptosis of multiple myeloma cells by activating caspase-3.

Next, we evaluated the size of tumors during the 6 weeks following the treatment ($n = 5$ per group). At 6 weeks after treatment, the mean tumor volumes were as follows: β-catenin small interfering RNA/atelocollagen complex, 412.2 mm^3; scramble small interfering RNA/atelocollagen complex, 1,317.9 mm^3; β-catenin small interfering RNA/PBS, 2,075.9 mm^3; and PBS/1% atelocollagen, 1,802.3 mm^3. Treatment with the β-catenin small interfering RNA/atelocollagen complex significantly reduced tumor burdens and retarded tumor growth as measured by tumor volumes ($P < 0.05$; Fig. 5A and B). Therefore, these data show that the treatment with the β-catenin small interfering RNA/atelocollagen complex inhibits the proliferation of multiple myeloma tumors.

Discussion

In the present study, we used a mouse xenograft model to show that β-catenin small interfering RNA inhibits growth of multiple myeloma cells. To our knowledge, this is the first report showing that, for hematologic disorders, β-catenin small interfering RNA has growth inhibitory effects *in vivo*. Multiple myeloma cells are maintained and proliferate in the bone marrow through interactions between the bone marrow microenvironment and several cytokine growth factors for multiple myeloma cells, such as Wnt3a, Wnt5a, and Wnt10b (17). Activation of the canonical Wnt signaling pathway stabilizes β-catenin, and its nonphosphorylated form accumulates in the cytoplasm. β-catenin then translocates to the nucleus, where it interacts with T cell factor, driving transcription of target genes such as *c-myc* and *cyclin D1*. The mechanisms underlying aberrant β-catenin expression in multiple myeloma cells remain unclear. However, we have confirmed previous findings that suggested that multiple myeloma cells exhibit higher levels of β-catenin expression than normal hematopoietic cells (7, 9).

In various cancer therapies, RNA interference has been introduced experimentally and numerous methods for small interfering RNA transfection have been developed. At present, methods using viral vectors are the most efficient (18, 19). However, their utility is limited because of their potential to cause mutagenesis and develop cancers (20, 21). Several nonviral carriers have been developed for gene delivery, and atelocollagen is one of the most attractive of these novel carriers. It provides a clinically safe and readily available biomaterial (22).

Because atelocollagen has been developed as an *in vivo* drug delivery system (12, 14, 23, 24), we firstly confirmed the efficacy of three types of β-catenin small interfering RNAs *in vitro* using lipofection reagents as described previously (12, 24). Although small interfering RNAs could not be effectively transfected into myeloma cells *in vitro* (data not shown), our β-catenin small interfering RNAs decreased mRNA and protein levels of β-catenin in A549 and SW480 cells. We confirmed that our β-catenin small interfering RNAs could effectively induced RNA interference against β-catenin. Next, we evaluated the growth inhibition of myeloma cell tumors *in vivo* by β-catenin small interfering RNA/atelocollagen complex. After being administered around the tumors (enveloping the tumors), the β-catenin small interfering RNA/atelocollagen complex releases β-catenin small interfering RNA slowly, allowing it to diffuse into the tumors, where it silences β-catenin expression. We confirmed that treatment with the β-catenin small interfering RNA/atelocollagen complex diminished β-catenin and c-myc expression in immunohistological examinations. In addition, the expression of cleaved capsase-3 in multiple myeloma tumors was increased by the treatment of β-catenin small interfering RNA/atelocollagen complex, and significant increases in apoptotic and cleaved caspase-3–positive cells were observed in multiple myeloma tumors. Taken together, these results indicate that depletion of β-catenin induces apoptosis by activating capsase-3 and inhibit the growth of multiple myeloma cells.

In this study, we showed that the β-catenin small interfering RNA/atelocollagen complex inhibited proliferation of multiple myeloma tumors and that β-catenin might represent a molecular target for therapy against multiple myeloma. Small β-catenin inhibitor molecules have been developed and investigated, and preliminary findings have implicated β-catenin as a novel target for a cancer therapy (3, 9). Our data support these findings. However, because β-catenin is an important molecule for stem cell systems (25–27), systemic administration of β-catenin inhibitors might induce severe adverse effects. Moreover, Wnt/β-catenin signaling is essential for skeletogenesis, and it promotes osteoblast differentiation (28–30). Inhibition of Wnt/β-catenin signaling has been reported to result in the development of multiple myeloma bone disease(31, 32), whereas activation of the Wnt pathway suppresses the disease development (33). Supported by these results, a specific targeting strategy against these cells, such as antibody-combined small interfering RNA, is under investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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