

# PRP-1 significantly decreases the ALDH<sup>high</sup> cancer stem cell population and regulates the aberrant Wnt/ $\beta$ -catenin pathway in human chondrosarcoma JJ012 cells

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**Abstract.** Chondrosarcomas are malignant bone tumors refractory to chemotherapy and radiation treatment; thus, novel therapeutic strategies are required. Proline-rich polypeptide 1 (PRP-1) has previously demonstrated antitumor properties in chondrosarcoma. To further investigate the role of PRP-1 in chondrosarcoma cells, its effects on cancer stem cell (CSC) populations were determined by analyzing aldehyde dehydrogenase (ALDH) activity, an established marker of CSCs, in association with regulation of the Wnt/ $\beta$ -catenin signaling. A significant decrease in ALDH<sup>high</sup> CSCs was observed following treatment of chondrosarcoma JJ012 cells with PRP-1. For RT<sup>2</sup> profiler PCR array analysis of Wnt/ $\beta$ -catenin signaling genes, cells were sorted into: i) Bulk JJ012 cells; ii) ALDH<sup>high</sup> cells sorted from untreated JJ012 cells (ALDH<sup>high-untreated</sup>); and iii) ALDH<sup>low</sup> cells sorted from PRP-1-treated JJ012 cells (ALDH<sup>low-PRP-1</sup>). The expression levels of Wnt/ $\beta$ -catenin signaling genes were determined to be downregulated in the ALDH<sup>high-untreated</sup> cells and upregulated in ALDH<sup>low-PRP-1</sup> cells when compared to the bulk JJ012 cells. Additionally, two important oncogenes involved in this pathway, MMP7 and CCND2, were found to be downregulated in the ALDH<sup>low-PRP-1</sup> cells. Immunocytochemistry demonstrated the localization of  $\beta$ -catenin in the nuclei of the PRP-1-treated cells. Western blotting indicated increased  $\beta$ -catenin expression in the ALDH<sup>low-PRP-1</sup> cells compared with the bulk JJ012 cells. Analysis of the cytoplasmic and nuclear fractions of cells treated with increasing concentrations of PRP-1 and  $\beta$ -catenin nuclear translocation inhibitor CGP57380, suggested the nuclear translocation of  $\beta$ -catenin following PRP-1 treatment. In addition, treatment of JJ012 cells

with a specific ALDH inhibitor, diethylaminobenzaldehyde, and PRP-1 resulted in a significant decrease in cytoplasmic  $\beta$ -catenin protein expression. This indicated that ALDH inactivation may be associated with the nuclear translocation of  $\beta$ -catenin. Derivation of sarcomas from mesenchymal stem cells via inactivation of the Wnt pathway has been previously documented. The findings of the present study support the notion that Wnt/ $\beta$ -catenin activation may serve a differential role in sarcomas, limiting tumor progression in association with decreased CSC activity.

## Introduction

Chondrosarcomas are malignant bone tumors formed from cartilage cells and are the second most common primary bone malignancy, accounting for 25.8% of primary bone cancers (1). Chondrosarcomas are a heterogeneous group of neoplasms; however, all grades and variants are relatively refractory to chemotherapy and radiation therapy (2). Low-to-intermediate grade chondrosarcomas have a good prognosis following surgical management, whereas high-grade tumors have poor outcomes; thus, novel approaches for the management of this disease are required (3). Chondrosarcoma is a cancer of mesenchymal origin, of which the mechanism underlying the mesenchymal transformation of cartilage cells remains unclear.

Proline-rich polypeptide 1 (PRP-1), also known as galarmin, is produced by the neurosecretory cells of the brain (4). The cytostatic, antiproliferative, immunomodulatory, and tumor suppressor properties of PRP-1 suggest its potential as a therapeutic agent in human chondrosarcoma cells resistant to radiation and chemotherapy (4-9).

Aldehyde dehydrogenase (ALDH) is an established marker of cancer stem cells (CSCs) in a variety of neoplasms. Cells exhibiting upregulated expression of ALDH have been isolated from human sarcoma cell lines, including the human chondrosarcoma SW-1353 cell line (10). ALDH1 activity can be used to identify a subpopulation of cells characterized by significant increases in the proliferation rate, colony formation ability and the expression of ABC transporter genes and stemness markers when compared with control cells (10). ALDH1 activity has been reported to be characteristic of cells

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with increased colony formation abilities, invasiveness and the expression of ABC transporters genes (10).

CSCs provide tumors with the capacity for self-renewal. ALDH expression in primary bone sarcomas was found to be associated with metastatic potential; upon culturing with disulfiram, an ALDH inhibitor, sarcoma cells exhibited decreased proliferation (11). Regarding the role of ALDH in self-renewal, proliferation and metastasis, ALDH may be considered as a potential target in the treatment of human chondrosarcoma.

The canonical Wnt/ $\beta$ -catenin signaling pathway plays a critical role in embryonic development and homeostatic stem cell self-renewal in a variety of adult tissues (12). Aberrant activation of the Wnt/ $\beta$ -catenin signaling pathway has been associated with numerous types of cancer, including colorectal cancer and leukemias (12), while loss of  $\beta$ -catenin expression has been linked to disease progression in malignant melanoma (13,14). The role of Wnt/ $\beta$ -catenin signaling has been well reported in the process of epithelial-mesenchymal transition in carcinomas; however, its involvement in the proliferation of bone sarcomas, including osteosarcoma and chondrosarcoma requires further investigation. Bone sarcomas have been associated with the aberrant activation of Wnt/ $\beta$ -catenin signaling (15), which has been demonstrated in a stem-like population of osteosarcoma cells with high tumorigenicity (16). On the contrary, Wnt/ $\beta$ -catenin signaling was proposed as an anti-tumorigenic pathway (17). In addition, the formation of sarcomas from mesenchymal stem cells via inactivation of the Wnt pathway has been reported (18). This suggests that inactivation of Wnt/ $\beta$ -catenin signaling in mesenchymal tumors may initiate sarcomagenesis.

In the present study, an Aldefluor<sup>®</sup> assay was performed to analyze the expression of ALDH in human chondrosarcoma JJ012 cells in the presence or absence of PRP-1. Subsequently, PRP-1-treated and untreated cells were sorted into ALDH<sup>low</sup> and ALDH<sup>high</sup> populations to determine the expression of genes related to the Wnt signaling pathway using a WNT Signaling Pathway RT<sup>2</sup> Profiler PCR Array. Additionally, western blot analysis and immunocytochemistry were performed to determine the differences in the cytoplasmic and nuclear expression of  $\beta$ -catenin in association with the regulation of Wnt/ $\beta$ -catenin signaling.

## Materials and methods

**Tissue culture.** The human chondrosarcoma JJ012 cells were obtained from the laboratory of Dr Joel Block, Rush University, Chicago, IL, USA. The cells were maintained in complete growth medium containing the following: Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX<sup>™</sup>, MEM (minimum essential medium) supplemented with F-12 + GlutaMAX<sup>™</sup> Nutrient mixture (Ham), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA); 25  $\mu$ g/ml ascorbic acid, 100 ng/ml insulin and 100 nM hydrocortisone (Sigma-Aldrich; Merck KGaA). The cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and periodically checked for mycoplasma.

**Mycoplasma detection assay to detect possible contamination of the cells.** With the MycoAlert<sup>®</sup> Mycoplasma Detection

kit (Lonza, Inc., Rockland, ME, USA; cat. no. LT07-118; lot. no. 0000678423) 2 ml of cell culture or culture supernatant was transferred into a centrifuge tube and centrifuged at 1,500 rpm (200 x g) for 5 min. Cleared supernatant (100  $\mu$ l) was transferred into a luminometer well. MycoAlert<sup>™</sup> reagent (100  $\mu$ l) was added to each sample and incubated at room temperature (20°C) for 5 min. The plate was placed in the luminometer and the program was initiated for Reading A. MycoAlert<sup>™</sup> substrate (100  $\mu$ l) was added to each sample and incubated at room temperature for 10 min. The plate was placed in the luminometer for Reading B. The ratio was calculated as Reading B/Reading A. If contamination was detected, cells were seeded in full growth media and treated with Plasmocin<sup>™</sup> (InvivoGen, Inc., San Diego, CA, USA; cat. no. ant-mpt; lot. no. MPT-38-03A) with a concentration of 25  $\mu$ g/ml for 14 days, removing and replacing with fresh Plasmocin<sup>™</sup> treatment containing medium every 3-4 days.

**PRP-1 treatment.** A subset of cultured human JJ012 chondrosarcoma cells were treated with 10  $\mu$ g/ml of PRP-1 and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for a period of 24 h prior to the Aldefluor<sup>®</sup> assay. A subset of cultured human JJ012 chondrosarcoma cells were treated with incrementally increasing doses of 1, 5, 10 and 20  $\mu$ g/ml PRP-1 and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for a period of 24 h prior to cytoplasmic and nuclear fractionation. PRP-1 was initially isolated from the brain hypothalamus (4).

**Aldefluor<sup>®</sup> assay and fluorescence-activated cell sorting (FACS).** To measure cells with ALDH activity, the Aldefluor<sup>®</sup> assay was carried out as described according to manufacturer's protocol (Aldefluor<sup>™</sup> kit, cat. no. 01700; StemCell Technologies, Vancouver, BC, Canada). Briefly, cells were harvested and resuspended in Aldefluor<sup>™</sup> assay buffer at a concentration of 1x10<sup>6</sup>/ml. To activate the Aldefluor<sup>™</sup> reagent, first 25  $\mu$ l of DMSO was added and incubated for 15 min with 25  $\mu$ l of 2N HCl, then 360  $\mu$ l of assay buffer was added to the vial. The cells were then incubated with the activated Aldefluor<sup>™</sup> reagent for 45 min at 37°C. Diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, was added as a negative control. Following incubation, all tubes were centrifuged for 5 min at 250 x g, and the supernatant was removed, and resuspended in Aldefluor<sup>™</sup> assay buffer. The cells were then transferred and strained onto Falcon 5 ml polystyrene round bottom tube with a cell strainer cap (cat. no. 352235). After labeling, the samples were sorted on a BD Biosciences (San Jose, CA, USA) Special Order Research Product (SORP) FACSAria II, using BD FACSDiva software (version 6.1.3) into ALDH<sup>low</sup> and ALDH<sup>high</sup> cells with and without PRP-1 treatment. Data analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR, USA) (version 10).

**RT<sup>2</sup> profiler PCR array.** The human chondrosarcoma JJ012 cells were sorted into cryovials and flash frozen in liquid nitrogen for shipment. Each group was repeated in triplicate. The RT<sup>2</sup> profiler PCR array was carried out by Qiagen, where RNA isolation and quality control were completed (WNT Signaling Pathway RT<sup>2</sup> Profiler PCR Array, cat. no. PAHS-043Z; Qiagen, Inc., Valencia, CA, USA).

*Statistical analysis for RT<sup>2</sup> Profiler PCR array fold-changes.* Fold-change was calculated using the  $\Delta\Delta C_t$  method. Fold-change ( $2^{-\Delta\Delta C_t}$ ) is the normalized gene expression in the test sample divided by the normalized gene expression in the control sample. Fold-regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicated a positive or an upregulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicated a negative or downregulation, and the fold-regulation was the negative inverse of the fold-change. The P-values were calculated based on a Student's t-test of the replicate  $2^{-\Delta\Delta C_t}$  values for each gene in the control group and treatment groups.

*Brief immunocytochemistry protocol.* JJ012 chondrosarcoma cells were cultured and incubated to confluency. A subset of cells was treated with 10  $\mu\text{g/ml}$  of PRP-1 for 24 h. Cells were collected using trypsin and then seeded directly onto coverslips ( $5 \times 10^5$  cells/coverslip) placed into 6-well clusters. Cells were cultured overnight at 37°C in a 5% CO<sub>2</sub> incubator. After 24 h, the medium was removed, and samples were fixed using 1 ml of 4% formaldehyde solution (F8775; Sigma-Aldrich; Merck KGaA) in phosphate-buffered saline (PBS), pH 7.4 1X (Gibco; Thermo Fisher Scientific) (10010-023) for 15 min in the incubator at 37°C. Samples were then washed with PBS twice. Permeabilization of samples was completed with PBS/Triton X-100 1% (T9284; Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. The detergent was removed, and non-specific sites were blocked using PBS containing 2% bovine serum albumin (BSA, A2153; Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. Further incubation of samples was completed adding primary antibodies: Alexa Fluor Conjugate 594 WGA (Thermo Fisher Scientific, Inc.; cat. no. W11262) at a dilution of 1:200 incubated for 10 min at room temperature in the dark; E-cadherin (Abcam; AB1416) at a dilution of 1:100 incubated for 60 min at room temperature in the dark; and  $\beta$ -catenin (Abcam; cat. no. AB223075) at a dilution of 1:100 incubated for 24 h in a cold room (4°C) on a rocker. The following morning, two consecutive washes and incubation were completed with PBS solution. Secondary antibodies anti-rabbit DyLight 488 (Abcam; cat. no. AB96899) at a dilution of 1:500 and anti-mouse DyLight 550 (Abcam; cat. no. AB96872) at a dilution of 1:500 were added and samples were incubated for 60 min at room temperature in the dark. The second fixation step using formaldehyde for 15 min at room temperature was performed followed by two washing steps. 4',6-Diamino-2-phenylindole dihydrochloride (3  $\mu\text{M}$ ) (DAPI; D1306; Thermo Fisher Scientific, Inc.) was used for nuclear staining for 10 min at room temperature. This was followed by two PBS washes. Antifade mounting medium was used to mount the samples on coverslips. ProLong Gold Antifade reagent (p10144; Life Technologies) was applied directly to fluorescently labeled cells on microscope slides to be used as a liquid mount and to protect fading of fluorescent dyes during microscopy.

*Imaging.* Image acquisition was performed by the Analytical Imaging Core Facility at DRI/SCCC, University of Miami (FL, USA). Zeiss 200M, ApoTome fluorescent microscope, DAPI 49, GFP 38HE, Cy3 43, Cy5 50 filter cubes (Carl Zeiss

Microscopy), heated stage, Orca II ERG Hamamatsu b/w 14-bit camera and AxioVision acquisition software were used. The coverslips were placed in regular 35-mm Petri dishes and the cells were grown on them, covered with medium. Once the cells were grown, the coverslips were taken out, and the cells were fixed, stained and mounted on glass slides. For imaging controls secondary antibodies were used without the primaries.

*Gel electrophoresis and western blotting.* JJ012 chondrosarcoma cells were cultured and incubated to confluency. Cells were collected using trypsin and then seeded into Petri dishes at a concentration of  $1 \times 10^6$  cells/ml. The cells were incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. The next day, an ice-cold phosphate-buffered saline wash was performed, and protease inhibitor was added to the cell lysis buffer (C2978; Sigma-Aldrich; Merck KGaA) in a 1:100 ratio. After the collection of cells with a rubber scraper and lysis of cell membranes with an 18-gauge needle, the cells were centrifuged at 15,000  $\times g$  at 4°C. The supernatant was then collected, and protein content was measured using NanoDrop<sup>®</sup> spectrophotometer (Thermo Fisher Scientific, Inc.). The supernatant was frozen at -80°C until loading onto the gels (20  $\mu\text{g/lane}$ ). Polyacrylamide gel electrophoresis and western blotting reagents were supplied by Lonza, Inc. (Allendale, NJ, USA) and related procedures were followed in accordance with the company's protocol. The catalog numbers for the reagents and suppliers are listed below. Pager Gold Precast Gels (59502; 10% Tris-glycine; Lonza, Inc.); ECL reagent (RPN2109; GE Healthcare, Little Chalfont, UK); Western Blocker solution (W0138; Sigma-Aldrich; Merck KGaA); ProSieve Quad Color Protein marker (4.6-300 kDa, 00193837; Lonza, Inc.); 20X reducing agent for ProSieve ProTrack Dual Color Loading buffer (00193861; Lonza, Inc.); ProTrack loading buffer (00193861; Lonza, Inc.); ProSieve ProTrack Dual Color Loading buffer EX running buffer (00200307; Lonza, Inc.); ProSieve EX Western Blot Transfer buffer (00200309; Lonza, Inc.); Immobilon<sup>®</sup>-P polyvinylidene difluoride membranes (P4188; Sigma-Aldrich; Merck KGaA).

*Preparation of the subcellular fraction lysates.* Cytoplasmic and nuclear fractions of cells were isolated following the manufacturer's instructions (cat. no. 40010, lot. no. 22118072; Active Motif) using phosphatase inhibitors, protease cocktail inhibitor and sonicator.

*CGP57380 treatment.* CGP57380 is a cell-permeable selective inhibitor of  $\beta$ -catenin nuclear translocation which was added in incrementally increasing doses of 1, 5, 10 and 20  $\mu\text{M}$  for a period of 24 h prior to the assay (C0993, Sigma-Aldrich; Merck KGaA).

*DEAB treatment.* DEAB, a specific ALDH inhibitor, from the Aldefluor<sup>™</sup> kit was added as a negative control (5  $\mu\text{l/ml}$ ) for a period of 24 h prior to lysate generation.

*Antibodies for western blotting.* Rabbit polyclonal antibody to  $\beta$ -catenin was applied as a primary antibody (ab2365, Abcam) at a dilution of 1:1,000 and goat anti-rabbit IgG peroxidase conjugate as a secondary antibody (A0545;

Sigma-Aldrich; Merck KGaA) at a dilution of 1:5,000. As housekeeping proteins, mouse anti-tubulin antibody was applied for cytoplasmic fractions (T5168; Sigma-Aldrich; Merck KGaA) at a dilution of 1:2,000 and anti-mouse IgG (A4416; Sigma-Aldrich; Merck KGaA) at a dilution of 1:5,000 was applied as a secondary antibody. Mouse anti-TBP was used for nuclear fractions (T1827, Sigma-Aldrich; Merck KGaA) at a dilution of 1:1,000 and anti-mouse IgG (A4416; Sigma-Aldrich; Merck KGaA) at a dilution of 1:5,000 was applied as a secondary antibody. Incubations for all primary antibodies were carried out in a cold room while rocking for a period of 24 h, while secondary antibodies were incubated for 2 h under the same conditions.

*Densitometric analysis for western blot analysis.* Quantitative analysis and densitometry were obtained using integrated density analysis on ImageJ 1.52e (NIH; National Institutes of Health, Bethesda, MD, USA) to calculate relative optical density (OD) of  $\beta$ -catenin to the housekeeping protein. Bulk JJ012 was used as the control.

*Statistical analysis.* Statistical analyses were performed using individual unpaired t-tests for flow cytometry experiments, which were repeated 10 times. Statistical analyses of relative ODs were completed using one-way analysis of variance (ANOVA) with a post hoc Dunnett's multiple comparisons test of all samples to control bulk JJ012 cells expressed as 95% confidence intervals of mean difference. All western blot experiments were repeated 2 times. All statistical analyses were completed using GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). A P-value <0.05 was considered significant. Error bars represent standard error of the mean (SEM) in all graphs with \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 (as indicated in the figures and figure legends).

## Results

*PRP-1 significantly decreases ALDH<sup>high</sup> cells from the bulk JJ012 cell population.* Considering the demonstrated cytostatic, antiproliferative and tumor-suppressor properties of PRP-1 and the established ALDH<sup>high</sup> CSC populations in chondrosarcoma cell lines, we sought to determine whether PRP-1 plays a role in the expression of stem cell characteristics in bulk JJ012 human chondrosarcoma cells. To test this, we cultured a group of bulk JJ012 with PRP-1 and without PRP-1. These cells underwent Aldefluor<sup>®</sup> assay and ALDH expression was detected using FACS. ALDH inhibitor, DEAB, served as the negative control for both treated and untreated cells to ensure the accuracy of the analysis. Unstained untreated and PRP-1-treated cells showed no background fluorescence. A set of untreated cells stained with Aldefluor<sup>®</sup> in the presence of DEAB showed 0.5% ALDH<sup>high</sup> cells (Fig. 1A) and PRP-1-treated cells stained with Aldefluor<sup>®</sup> in the presence of DEAB showed 0.1% ALDH<sup>high</sup> cells (Fig. 1C).

A set of PRP-1-treated cells in the absence of DEAB showed 3.1% ALDH<sup>high</sup> cells (Fig. 1D) compared to a set of untreated cells in the absence of DEAB which showed 75.3% ALDH<sup>high</sup> cells (Fig. 1B). A statistically significant difference was found between the ALDH<sup>low</sup> populations in the untreated

[mean (M)=51.10%, SEM=12.00%, n=10] and PRP-1-treated JJ012 cells (M=88.39%, SEM=2.14%, n=10) stained with Aldefluor<sup>®</sup>; t(18)=3.059, P=0.0068 (Fig. 1E). There was also a statistically significant difference between the ALDH<sup>high</sup> populations in the untreated (M=42.49%, SEM=11.82%, n=10) and PRP-1-treated JJ012 cells (M=6.93%, SEM=1.35%, n=10) stained with Aldefluor<sup>®</sup>; t(18)=2.990, P=0.0079 (Fig. 1E). These results demonstrated that PRP-1 treatment significantly decreased the ALDH<sup>high</sup> CSC population from a bulk JJ012 cell population. Subpopulations were collected using flow cytometry and consisted of: ALDH<sup>high</sup> cells sorted from untreated JJ012 cells (ALDH<sup>high-untreated</sup>), ALDH<sup>low</sup> cells sorted from untreated JJ012 cells (ALDH<sup>low-untreated</sup>) and ALDH<sup>low</sup> cells sorted from PRP-1-treated JJ012 cells (ALDH<sup>low-PRP-1</sup>). ALDH<sup>high</sup> cells were not able to be collected from the PRP-1-treated JJ012 cells due to the exceedingly low levels of these CSC populations after PRP-1 treatment.

*RT<sup>2</sup> profiler PCR array demonstrates differential expression of Wnt signaling genes in ALDH<sup>high-untreated</sup> cells compared to bulk JJ012 cells.* After demonstrating the ability of PRP-1 to eliminate CSCs in JJ012 cells, we sought to understand the pathways involved in this population shift. Studies connecting ALDH expression to Wnt/ $\beta$ -catenin signaling pathway activation in many tumor types, including prostate cancer, liver cancer and breast cancer, drove us to explore this pathway in chondrosarcoma cells (19-21). We used the RT<sup>2</sup> profiler PCR arrays performed by Qiagen to explore the Wnt/ $\beta$ -catenin signaling pathway in PRP-1-treated and untreated cells.

Our first comparison was between ALDH<sup>high-untreated</sup> cells and bulk JJ012 cells. Wnt signaling genes were profiled on three samples with technical triplicates for each group. Notably, ALDH<sup>high-untreated</sup> cells demonstrated significant downregulation of 6 Wnt signaling genes, including *TCF7L1*, *WNT3*, *FZD7*, *FOSL1*, *WNT7B* and *FZD6* and upregulation of only one Wnt signaling gene, *PORCN*, with putative transcription factors involved listed (Table I). *TCF7L1* had the highest fold downregulation (6.33-fold) (P=0.000543). *WNT3*, *FZD7*, *FOSL1*, *WNT7B* and *FZD6* were downregulated in a range from 2.48- to 2.76-fold. *PORCN* showed 2.43-fold upregulation (P=0.006511). miRNAs that regulate these downregulated Wnt signaling genes (*FZD7*, *FZD6*, *FOSL1*, *TCF7L1* and *WNT7B*) in the ALDH<sup>high-untreated</sup> cells were identified (Table II).

*RT<sup>2</sup> profiler PCR array shows differential expression of Wnt signaling genes in ALDH<sup>low-PRP-1</sup> cells compared to bulk JJ012 cells.* In the present study, we compared ALDH<sup>low-PRP-1</sup> cells to bulk JJ012 cells to determine whether addition of PRP-1 results in a differential expression of Wnt genes compared to an untreated and unsorted JJ012 population using a RT<sup>2</sup> profiler PCR array. We identified significant upregulation of 5 Wnt signaling genes in the PRP-1-treated ALDH<sup>low</sup> cells, including *BCL9*, *PORCN*, *RHOA*, *FZD2* and *RPLP0*, with putative transcription factors involved listed (Table III). *MMP7* was 3.50-fold down-regulated (P=0.018004) in the ALDH<sup>low-PRP-1</sup> cells compared to the bulk JJ012 cells. Notably, *PORCN* was 2.43-fold upregulated (P=0.006511) in the ALDH<sup>high-untreated</sup> cells (Table I) while *PORCN* was 3.54-fold upregulated (P=0.030693) in the ALDH<sup>low-PRP-1</sup> cells (Table III) compared to the bulk JJ012 cells. miRNAs that regulate the upregulated

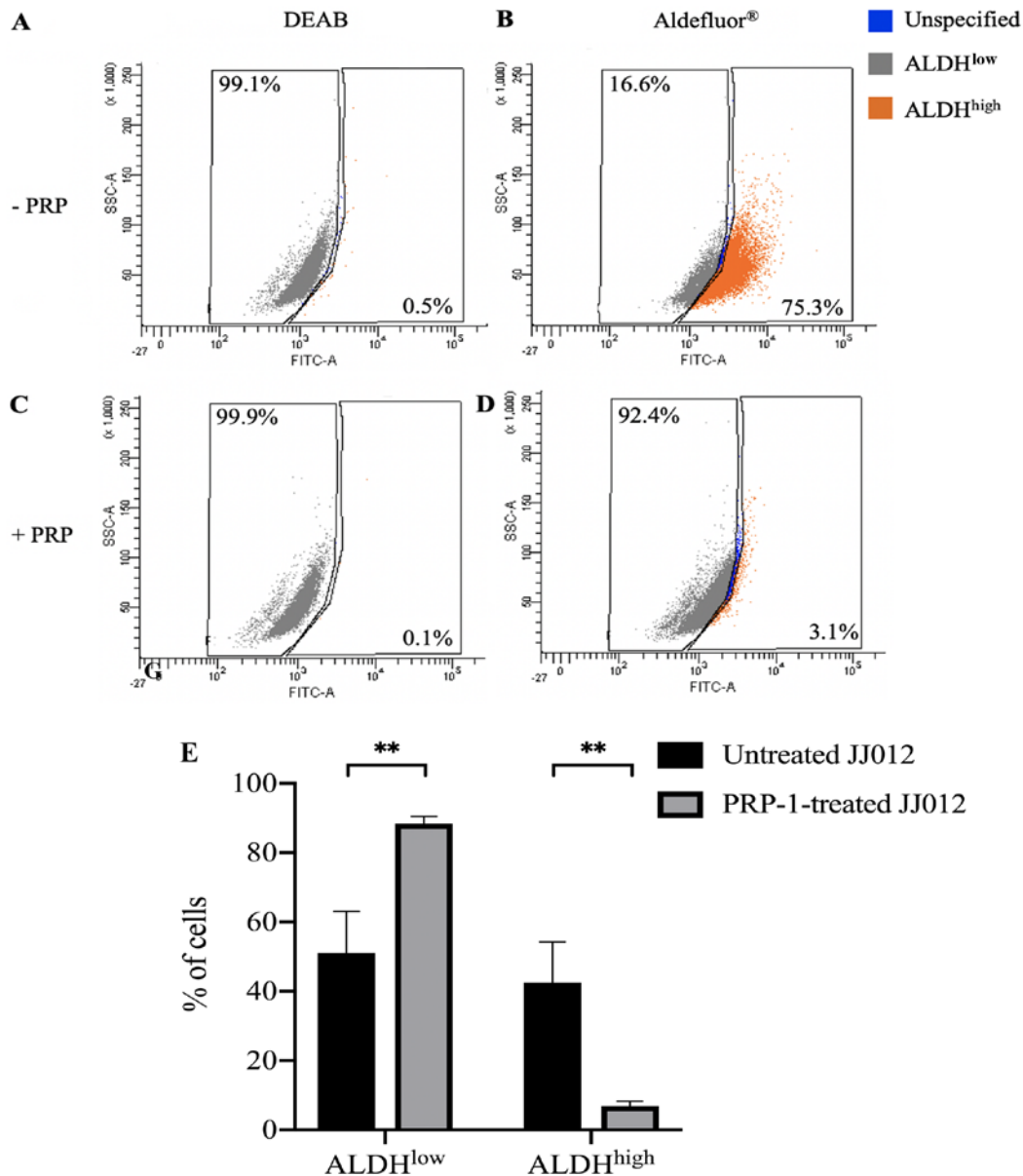


Figure 1. Flow cytometry results representing ALDH activity in untreated and PRP-1-treated JJ012 cells. Flow cytometry gating results for ALDH activity in (A) PRP-1-untreated bulk human chondrosarcoma JJ012 cells treated with DEAB, a specific ALDH inhibitor, and (B) the analysis of ALDH activity in PRP-1-untreated bulk JJ012 cells stained with Aldefluor®. Established gating for the analysis of ALDH activity in (C) PRP-1-treated cells also treated with DEAB, a specific ALDH inhibitor, and (D) analysis of ALDH activity in PRP-1-treated JJ012 cells stained with Aldefluor®. (E) Distribution of ALDH<sup>low</sup> and ALDH<sup>high</sup> activity in untreated bulk JJ012 cells compared to PRP-1-treated JJ012 cells across 5 separate experiments. \*\*P<0.01; n=10; error bars represent SEM. ALDH, aldehyde dehydrogenase; PRP-1, proline-rich polypeptide 1.

Wnt signaling genes *RHOA* and *BCL9* in ALDH<sup>low-PRP-1</sup> cells were identified (Table IV).

*RT<sup>2</sup> profiler PCR array shows differential expression of Wnt signaling genes in the ALDH<sup>low-PRP-1</sup> cells compared to the ALDH<sup>high-untreated</sup> cells.* After showing PRP-1 treatment eliminates CSCs in JJ012 cells, we aimed to determine the differences in Wnt signaling genes between ALDH<sup>high-untreated</sup> cells and ALDH<sup>low-PRP-1</sup> cells using a RT<sup>2</sup> profiler PCR array.

The arrays identified two significantly downregulated cancer genes from the Wnt pathway in the ALDH<sup>low-PRP-1</sup> cells, including the *CCND2* gene, encoding G1/S specific cyclin D2 and the *MMP7* gene, encoding a matrix metalloproteinase, with putative transcription factors involved listed (Table V).

*CCND2* was downregulated 4.51-fold (P=0.004252) and *MMP7* was downregulated 3.25-fold (P=0.000044). miRNAs that regulate the downregulated Wnt signaling gene *CCND2* in the ALDH<sup>low-PRP-1</sup> cells were identified (Table VI). Numerous studies have demonstrated the importance of miRNA regulators, their downregulation, and their role in overexpression of *CCND2* in association with high-grade osteosarcomas and resistance to chemotherapy (22-25). In fact, *CCND2* was found to be upregulated in metastatic osteosarcoma compared to the primary tumor (26). Together, these experimental results demonstrate the important role that *CCND2* plays in the development of the progression of cancer, chemoresistance and metastasis that may also be present in chondrosarcoma.

Table I. Genes differentially expressed in ALDH<sup>high-untreated</sup> vs. bulk JJ012 human chondrosarcoma cells.

Gene symbol	Fold regulation	P-value	Transcription factors
<i>TCF7L1</i>	-6.33	0.000543	Pax-5, ER- $\alpha$ , E47, MAZR, MZF-1, Olf-1, AP-2 $\alpha$ , AP-2 $\alpha$ A, AP-2 $\beta$ , AP-2 $\gamma$
<i>WNT3</i>	-2.76	0.032755	HNF-4 $\alpha$ 1, HNF-4 $\alpha$ 2, p53, Pax-4a, AP-2 $\alpha$ , AP-2 $\alpha$ A, AP- $\beta$ , AP-2 $\gamma$ , c-Myb, Pax-2, Pax-2a, Pax-2b, Gfi-1, CBF(2), CBF-A, CBF-B, CBF-C, CP1A, CP1C, NF-Y, NF-YA, NF-YB, NF-YC, AP-2 $\gamma$ , LCR-F1, Gfi-1, Arnt, MAZR, STAT5A, LUN-1, Pax-5, ARP-1, E47, POU2F1, POU2F1a, STAT1, STAT1 $\alpha$ , STAT1 $\beta$ , STAT3, STAT5B, STAT5A, c-Myb
<i>FZD7</i>	-2.66	0.011918	HNF-4 $\alpha$ 1, MAZR, Sp1, Olf-1, GATA-1, SRY, HSF2, Brachyury, CREB, delta-CREB, C/EBP $\alpha$ , ATF-2, CRE-BP1, E4BP4, CUTL1, Egr-1, HEN1
<i>FOSL1</i>	-2.64	0.025993	ATF-2, Ik-3, ATF, CRE-BP1, CREB, delta-CREB, SRF, SRF (504 AA), Hlf
<i>WNT7B</i>	-2.64	0.019711	p53, Nkx5-1, Olf-1, c-Myc, Max, Bach2, CREB, delta-CREB, AhR, Arnt, Pax-5, CP2, RREB-1, MAZR, MZF-1, CP2, E47, AREB6, MyoD, Zic1, ZIC2/Zic2, Zic3
<i>FZD6</i>	-2.48	0.044088	Pax-4a, Pax-2, Pax-2a, E2F, E2F-1
<i>PORCN</i>	2.43	0.006511	CREB, kx2-5, Ik-3, RFX1, LCR-F1, c-Jun, AP-1, c-Fos, GATA-1, GATA-3, XBP-1

*TCF7L1*, transcription factor 7 like 1; *WNT3*, Wnt family member 3; *FZD7*, frizzled class receptor 7; *FOSL1*, FOS like 1, AP-1 transcription factor subunit; *WNT7B*, Wnt family member 7B; *FZD6*, frizzled class receptor 6; *PORCN*, porcupine O-acyltransferase.

Table II. miRNAs that regulate the downregulated genes in the ALDH<sup>high-untreated</sup> vs. the bulk JJ012 human chondrosarcoma cells.

miRNA name	Target gene
hsa-miR-338-5p	<i>FZD7</i>
hsa-miR-519b-3p	<i>FZD6</i>
hsa-miR-519c-3p	<i>FZD6</i>
hsa-miR-519a-3p	<i>FZD6</i>
hsa-miR-593-3p	<i>FOSL1</i>
hsa-miR-101-3p	<i>FZD6</i>
hsa-miR-199b-5p	<i>FZD6</i>
hsa-miR-199a-5p	<i>FZD6</i>
hsa-miR-568	<i>FOSL1, FZD7</i>
hsa-miR-1283	<i>TCF7L1</i>
hsa-miR-130b-3p	<i>FOSL1, FZD6</i>
hsa-miR-1294	<i>TCF7L1</i>
hsa-miR-301a-3p	<i>FOSL1, FZD6</i>
hsa-miR-301b	<i>FOSL1, FZD6</i>
hsa-miR-646	<i>FOSL1</i>
hsa-miR-505-3p	<i>WNT7B</i>
hsa-miR-130a-3p	<i>FOSL1, FZD6</i>
hsa-miR-548d-5p	<i>FZD7</i>
hsa-miR-548a-5p	<i>FZD7</i>
hsa-miR-548b-5p	<i>FZD7</i>

*Immunocytochemistry demonstrates increased nuclear  $\beta$ -catenin expression in the PRP-1-treated JJ012.* Immunofluorescent stained cell images demonstrated that  $\beta$ -catenin was present in the nuclei in the PRP-1-treated JJ012 cells vs. in the cytoplasm of untreated JJ012 cells.

Single-cell images are depicted (Fig. 2A-D) and a wider field of multiple cells is shown (Fig. 2E and F).

*Western blot analysis indicates that  $\beta$ -catenin protein expression is increased in the ALDH<sup>low-PRP-1</sup> cells.* To confirm the findings from the RT<sup>2</sup> profiler PCR arrays and immunocytochemistry, western blot analysis was carried out. One-way ANOVA found significant differences in the mean relative optical density (OD) of  $\beta$ -catenin between bulk JJ012 cells, ALDH<sup>low-untreated</sup> and ALDH<sup>low-PRP-1</sup> cells [F(2,3)=70.60, P=0.0030]. Post hoc Dunnett's tests determined no significance difference between ALDH<sup>low-untreated</sup> and bulk JJ012 cells, but a significant increase in relative OD of  $\beta$ -catenin was found between ALDH<sup>low-PRP-1</sup> and bulk JJ012 cells (1.834 $\pm$ 0.611, P=0.0023) (Fig. 2G and H). This supports the finding that PRP-1 regulates  $\beta$ -catenin protein expression.

*Western blot analyses of cytoplasmic and nuclear fractions of PRP-1-treated JJ012 cells demonstrate nuclear translocation of  $\beta$ -catenin.* To determine whether PRP-1 has an effect on subcellular regulation of the Wnt/ $\beta$ -catenin pathway, western blot analysis was performed on cytoplasmic and nuclear fractions using bulk JJ012 cells as a control compared to various combinations of PRP-1, CGP57380 (CGP; a  $\beta$ -catenin nuclear translocation inhibitor) and DEAB (a direct ALDH inhibitor). One-way ANOVA of the western blot experiments with incrementally increasing PRP-1 concentrations found a statistically significant difference in relative OD of  $\beta$ -catenin between the groups [F(4,5)=67.93, P=0.0002]. Post hoc Dunnett's test demonstrated a significant decrease in the mean relative OD in cells treated with 1  $\mu$ g PRP-1 (-0.1867 $\pm$ 0.0762, P=0.0011), 5  $\mu$ g PRP-1 (-0.1923 $\pm$ 0.0763, P=0.0010), 10  $\mu$ g PRP-1 (-0.2555 $\pm$ 0.0762, P=0.0003) and 20  $\mu$ g PRP-1 (-0.3485 $\pm$ 0.0762, P<0.0001) compared to the

Table III. Genes differentially expressed ALDH<sup>low-PRP-1</sup> vs. bulk JJ012 human chondrosarcoma cells.

Gene symbol	Fold regulation	P-value	Transcription factors
<i>BCL9</i>	3.86	0.035605	STAT1, STAT1 $\alpha$ , STAT1 $\beta$ , STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6, MZF-1, RFX1, HNF-1, HNF-1A, Cdc5, Meis-1, Meis-1a, Meis-1b, FOXL1
<i>PORCN</i>	3.54	0.030693	CREB, kx2-5, Ik-3, RFX1, LCR-F1, c-Jun, AP-1, c-Fos, GATA-1, GATA-3, XBP-1
<i>MMP7</i>	-3.50	0.018004	HNF-4 $\alpha$ 1, Bach1, c-Jun, AP-1, c-Fos, FosB, Fra-1, JunB, JunD, Bach2, POU2F1, POU2F1a, Oct-B1, oct-B2, oct-B3, POU2F2, POU2F2 (Oct-2.1), POU2F2B, POU2F2C
<i>RHOU</i>	2.68	0.019367	
<i>FZD2</i>	2.64	0.005308	
<i>RPLP0</i>	2.21	0.008985	

*BCL9*, BCL9 transcription coactivator; *PORCN*, porcupine O-acyltransferase; *MMP7*, matrix metalloproteinase 7; *RHOU*, Ras homolog family member U; *FZD2*, frizzled class receptor 2; *RPLP0*, ribosomal protein lateral stalk subunit P0.

Table IV. miRNAs that regulate overexpressed genes in PRP-1-treated ALDH<sup>low</sup> vs. the bulk JJ012 human chondrosarcoma cells.

miRNA name	Target genes
hsa-miR-525-5p	<i>RHOU</i>
hsa-miR-520a-5p	<i>RHOU</i>
hsa-miR-767-3p	<i>RHOU</i>
hsa-miR-101-3p	<i>BCL9</i>
hsa-miR-218-5p	<i>BCL9</i>
hsa-miR-124-3p	<i>RHOU</i>
hsa-miR-506-3p	<i>RHOU</i>
hsa-miR-562	<i>RHOU</i>
hsa-miR-1284	<i>BCL9</i>
hsa-miR-204-5p	<i>BCL9</i>
hsa-miR-1301-3p	<i>BCL9</i>
hsa-miR-211-5p	<i>BCL9</i>
hsa-miR-559	<i>BCL9</i>
hsa-miR-548a-5p	<i>BCL9</i>
hsa-miR-548i	<i>BCL9</i>
hsa-miR-548d-5p	<i>BCL9</i>
hsa-miR-548h-5p	<i>BCL9</i>
hsa-miR-548c-5p	<i>BCL9</i>
hsa-miR-548j-5p	<i>BCL9</i>
hsa-miR-548b-5p	<i>BCL9</i>

bulk JJ012 control cells (Fig. 3A and F). One-way ANOVA of experiments with the addition of  $\beta$ -catenin nuclear translocation inhibitor CGP57380 (27,28) in increasing concentrations demonstrated no significant differences in mean relative OD of cytoplasmic  $\beta$ -catenin protein between the groups [F(4,5)=2.649, P=0.1570] (Fig. 3B and G). When adding CGP57380 (CGP) with increasing PRP-1 concentrations, one-way ANOVA demonstrated significant differences in mean relative OD of cytoplasmic  $\beta$ -catenin protein between

the groups [F(4,5)=16.71, P=0.0043]. Post hoc analysis using Dunnett's test demonstrated a significant increase in relative OD of cytoplasmic  $\beta$ -catenin with 5  $\mu$ g PRP-1 + 5  $\mu$ M CGP (2.076 $\pm$ 1.1198, P=0.0040), 10  $\mu$ g PRP-1 + 10  $\mu$ M CGP (1.881 $\pm$ 1.0504, P=0.0062) and 20  $\mu$ g PRP-1 + 20  $\mu$ M CGP (1.754 $\pm$ 1.1199, P=0.0083) compared to the bulk JJ012 control cells (Fig. 3C and H). One-way ANOVA analysis of JJ012 cells treated with DEAB found significant differences in the mean relative OD of cytoplasmic  $\beta$ -catenin between groups [F(3,4)=62.33, P=0.0008]. Post hoc Dunnett's test found a significant decrease in relative OD of cytoplasmic  $\beta$ -catenin following treatment with DEAB alone (-0.2969 $\pm$ 0.2876, P=0.0452), 10  $\mu$ g PRP-1 alone (-0.7082 $\pm$ 0.2876, P=0.0020) and 10  $\mu$ g PRP-1 + DEAB (-1.006 $\pm$ 0.2873, P=0.0005) compared to the bulk JJ012 control cells (Fig. 3D and I). When examining the relative OD of nuclear  $\beta$ -catenin in the JJ012 cells treated with PRP-1 and CGP, one-way ANOVA found significant differences in means between groups [F(3,4)=1182, P<0.0001]. Post hoc analysis using Dunnett's test demonstrated a significantly increased intensity of nuclear  $\beta$ -catenin in the high-dose (20  $\mu$ g) PRP-1 (0.2612 $\pm$ 0.0797, P=0.0007) and significantly decreased intensity of nuclear  $\beta$ -catenin following 20  $\mu$ g PRP-1 + 20  $\mu$ M CGP (-0.9474 $\pm$ 0.0797, P<0.0001) compared to the bulk JJ012 control cells (Fig. 3E and J).

### Discussion

Chondrosarcomas represent a heterogeneous group of cartilaginous malignancies that exhibit both radioresistance and chemoresistance (2). In order to investigate potential novel therapies for the treatment of this disease, improved understanding of the cellular mechanisms driving the proliferation and metastasis of chondrosarcoma is required. PRP-1 has been previously reported as a potential therapeutic agent with cytostatic, antiproliferative and tumor-suppressive properties (5-9). In the present study, PRP-1 was observed to notably decrease the abundance of ALDH<sup>high</sup> CSCs in a population of JJ012 cells. ALDH, a marker of chondrosarcoma stem cells,

Table V. Genes differentially expressed in ALDH<sup>low-PRP-1</sup> vs. ALDH<sup>high-untreated</sup> human JJ012 chondrosarcoma cells.

Gene symbol	Fold regulation	P-value	Transcription factors
<i>CCND2</i>	-4.51	0.004252	GATA-1, STAT1, STAT1 $\alpha$ , STAT1 $\beta$ , STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6, Pax-3, c-Rel, HEN1, PPAR- $\gamma$ 1, PPAR- $\gamma$ 2, HNF-4 $\alpha$ 1, HNF-4 $\alpha$ 2, E47, Lmo2
<i>MMP7</i>	-3.25	0.000044	HNF-4 $\alpha$ 1, Bach1, c-Jun, AP-1, c-Fos, FosB, Fra-1, JunB, JunD, Bach2, POU2F1, POU2F1a, Oct-B1, Oct-B2, Oct-B3, POU2F2, POU2F2 (Oct-2.1), POU2F2B, POU2F2C

*CCND2*, cyclin D2; *MMP7*, matrix metalloproteinase 7.

Table VI. miRNAs that regulate the downregulated gene *CCND2* in the ALDH<sup>low-PRP-1</sup> cells vs. the ALDH<sup>high-untreated</sup> human chondrosarcoma cells.

miRNA name	Target gene
hsa-miR-200a-3p	<i>CCND2</i>
hsa-miR-141-3p	<i>CCND2</i>
hsa-miR-548d-3p	<i>CCND2</i>
hsa-miR-506-3p	<i>CCND2</i>
hsa-miR-124-3p	<i>CCND2</i>
hsa-miR-548p	<i>CCND2</i>
hsa-miR-154-5p	<i>CCND2</i>
hsa-miR-19b-3p	<i>CCND2</i>
hsa-miR-19a-3p	<i>CCND2</i>
hsa-miR-656-3p	<i>CCND2</i>
hsa-miR-1183	<i>CCND2</i>
hsa-miR-18b-5p	<i>CCND2</i>
hsa-miR-18a-5p	<i>CCND2</i>
hsa-miR-29b-3p	<i>CCND2</i>
hsa-miR-520h	<i>CCND2</i>
hsa-miR-520g-3p	<i>CCND2</i>
hsa-miR-29c-3p	<i>CCND2</i>
hsa-miR-29a-3p	<i>CCND2</i>
hsa-miR-1269a	<i>CCND2</i>
hsa-miR-634	<i>CCND2</i>

correlated with increases in their proliferative capacity and colony formation abilities of tumor cells (10). The potential of PRP-1 to almost completely eliminate ALDH<sup>high</sup> CSCs suggests the application of PRP-1 in inhibiting the formation of chondrosarcoma (11,29). The present study proposed the potential of PRP-1 in reducing the population of ALDH<sup>high</sup> CSCs in monolayer culture; however, the viability of ALDH<sup>low</sup> cancer cells was not analyzed. Thus, further investigation is required to establish the effects of PRP-1 on the viability of chondrosarcoma cells and on three dimensional models of chondrosarcoma.

Additionally, the role of the Wnt/ $\beta$ -catenin signaling pathway in various populations of JJ012 chondrosarcoma cells was investigated. ALDH<sup>high</sup> JJ012 cells exhibited significantly downregulated Wnt signaling gene expression compared to bulk JJ012 cells. When comparing ALDH<sup>low-PRP-1</sup> cells with bulk

JJ012 cells, significant upregulation in the expression of Wnt signaling genes was found in the ALDH<sup>low-PRP-1</sup> cells, with certain exceptions. These results suggest that the activation and inactivation of the Wnt/ $\beta$ -catenin signaling pathway serve differing roles in chondrosarcomas, similar to findings in human mesenchymal stem cells and malignant fibrous histiocytoma (18). PRP-1 was reported to be involved in the regulation of Wnt/ $\beta$ -catenin pathway; however, the role of certain dysregulated Wnt/ $\beta$ -catenin signaling genes was not identified, which poses as a limitation to our study. To confirm the findings of the PCR array analysis, immunocytochemistry and western blotting were conducted to determine the cellular expression of  $\beta$ -catenin. Increased nuclear  $\beta$ -catenin expression in the PRP-1-treated JJ012 cells as demonstrated in immunocytochemistry and upregulated  $\beta$ -catenin protein expression in ALDH<sup>low-PRP-1</sup> cells as determined by western blotting suggest that PRP-1 may directly induce the nuclear translocation of  $\beta$ -catenin.

PRP-1 decreased the cytoplasmic expression levels of  $\beta$ -catenin; however, opposing effects were observed following treatment with the nuclear translocation inhibitor CGP57380, indicating the nuclear translocation of  $\beta$ -catenin following PRP-1 treatment. Additionally, a dose-dependent increase in nuclear  $\beta$ -catenin expression was reported in response to PRP-1; however, treatment with CGP57380 significantly decreased the nuclear expression of  $\beta$ -catenin protein. Of note, treatment of JJ012 cells with DEAB, a specific ALDH inhibitor, followed by the administration of PRP-1 revealed reductions in cytoplasmic  $\beta$ -catenin protein expression. This indicated that decreased ALDH expression, and therefore CSC activity, may induce the nuclear translocation of  $\beta$ -catenin in chondrosarcoma cells. Activation of the Wnt/ $\beta$ -catenin signaling pathway has been reported to serve an important role in the normal maintenance of mesenchymal tissue (30). Additionally, inhibition of Wnt signaling by a known Wnt inhibitor, Dickkopf-related protein 1 (Dkk-1), has been determined to prevent osteogenesis under conditions of bone repair (31). In addition, it was demonstrated that upregulated levels of Dkk-1 serve a role in the pathogenesis of osteosarcoma by inhibiting the repair of the surrounding bone (32). These studies suggest that inactivation of the Wnt/ $\beta$ -catenin signaling pathway may be involved in the development and progression of sarcomas, which opposes the effects of activation of this pathway in a variety of carcinomas and other types of tumors (12).

The present study identified several dysregulated genes involved in the Wnt/ $\beta$ -catenin signaling pathway, including

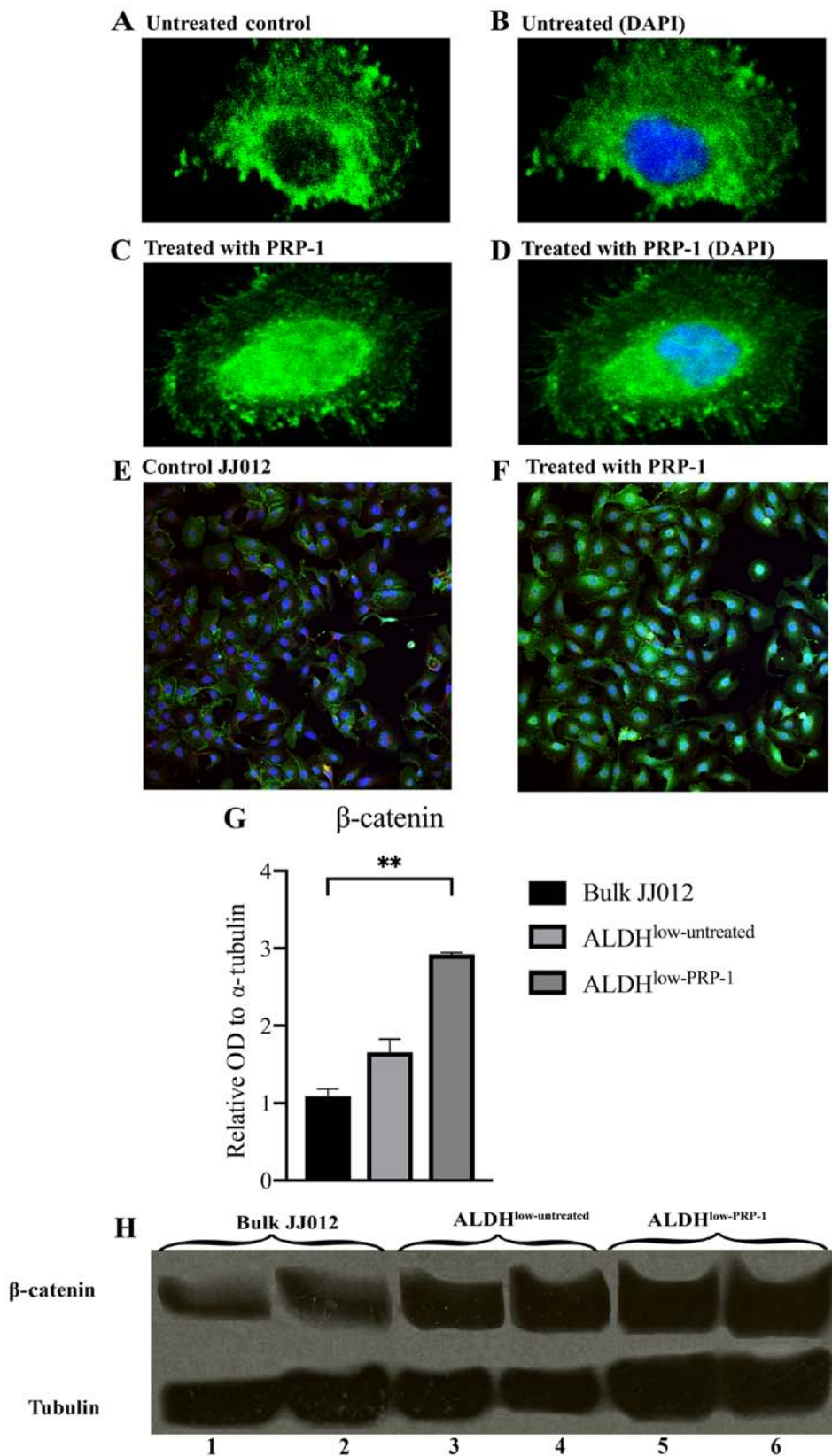


Figure 2. Immunocytochemistry and western blot results indicating localization and expression of  $\beta$ -catenin in untreated and PRP-1-treated human chondrosarcoma JJ012 cells. (A) Untreated JJ012 cells showing  $\beta$ -catenin (green). (B) Untreated JJ012 cells with DAPI to stain the nucleus (blue) show  $\beta$ -catenin (green) outside of the nucleus. (C) PRP-1-treated JJ012 cells showing  $\beta$ -catenin (green). (D) PRP-1-treated cells with DAPI to stain the nucleus (blue) show  $\beta$ -catenin (green) overlapping with the nucleus. (E) Composite image of untreated cells demonstrating  $\beta$ -catenin (green) outside of the nucleus (blue) in the majority of cells. (F) Composite image of PRP-1-treated cells demonstrating  $\beta$ -catenin (green) overlapping with nucleus (blue) in the majority of cells. (G) Densitometry measurements of western blot experiments using bulk JJ012 as a control with a significant difference found between ALDH<sup>low</sup>-PRP-1 cells and bulk JJ012 cells, but no significant difference was detected between ALDH<sup>low</sup>-PRP-1 cells and ALDH<sup>low</sup>-untreated cells or ALDH<sup>low</sup>-untreated cells and bulk JJ012 cells. (H) Western blots demonstrating increasing  $\beta$ -catenin protein expression in whole JJ012 cells in ALDH<sup>low</sup>-PRP-1 cells. Lanes 1 and 2, bulk JJ012 cells; lanes 3 and 4, ALDH<sup>low</sup>-untreated cells; lanes 5 and 6, ALDH<sup>low</sup>-PRP-1 cells. Tubulin was used as a housekeeping protein control. \*\*P<0.01 vs. bulk JJ012 control cells; n=2; error bars represent SEM. ALDH, aldehyde dehydrogenase; PRP-1, proline-rich polypeptide 1.

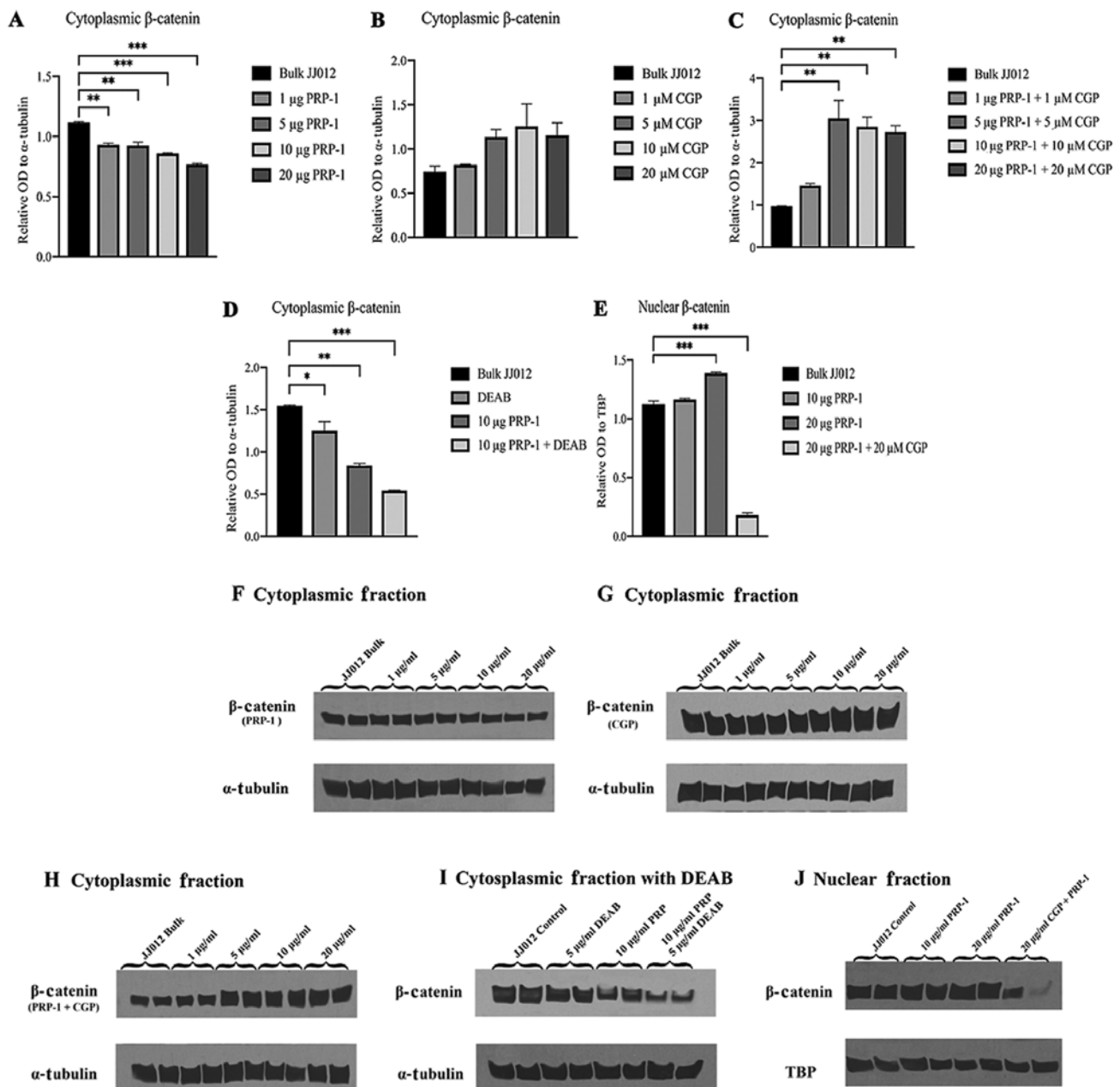


Figure 3. Fractionated cell western blot results indicating  $\beta$ -catenin expression in untreated and PRP-1-treated JJ012 cells. (A) Significant decrease in relative optical density (OD) of cytoplasmic  $\beta$ -catenin to  $\alpha$ -tubulin housekeeping protein was found with incrementally increasing dosages of PRP-1 at all doses compared to the bulk JJ012 control cells; (F) image of the western blot. (B) No significant changes were noted in the relative OD of cytoplasmic  $\beta$ -catenin to  $\alpha$ -tubulin housekeeping protein with incrementally increasing concentrations of CGP57380 (CGP), a direct inhibitor of  $\beta$ -catenin nuclear translocation; (G) image of the western blot. (C) Significant increase in relative OD of cytoplasmic  $\beta$ -catenin to  $\alpha$ -tubulin housekeeping protein was found with incrementally increasing dosages of PRP-1 + CGP at all doses compared to the bulk JJ012 control cells; (H) image of the western blot. (D) Significant increase in the relative OD of cytoplasmic  $\beta$ -catenin to  $\alpha$ -tubulin housekeeping protein with 10  $\mu$ g PRP-1 and combination treatment with DEAB (5  $\mu$ l/ml) compared to the bulk JJ012 control cells, DEAB only and 10  $\mu$ g PRP-1 only cells; (I) image of the western blot. (E) Significant increase in relative OD of nuclear  $\beta$ -catenin to TBP housekeeping protein with 20  $\mu$ g PRP-1 and significant decrease in relative OD of nuclear  $\beta$ -catenin to TBP housekeeping protein with 20  $\mu$ g and CGP combination compared to bulk JJ012 control cells; (J) image of the western blot. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  vs. bulk JJ012 control cells;  $n = 2$ ; error bars represent SEM. ALDH, aldehyde dehydrogenase; PRP-1, proline-rich polypeptide 1.

*FOSL1*, *FZD1*, *RHO* and *BCL9* (33-36). Of note, when comparing ALDH<sup>low-PRP-1</sup> cells with ALDH<sup>high-untreated</sup> cells, two important cancer genes, *MMP7*, a matrix metalloproteinase, and *CCND2*, coding for cyclin D2 involved in G1/S phase progression were reported to be downregulated in ALDH<sup>low-PRP-1</sup> cells. The results of the present study indicated that PRP-1 may downregulate certain Wnt genes and assume the role as a regulator of the Wnt/ $\beta$ -catenin pathway. In addition, microRNAs (miRNAs) that were associated with these

dysregulated genes were identified; however, their role in the activation or suppression of these genes was not determined. Thus, further investigation is required to elucidate the roles of these specific miRNAs in the regulation of the Wnt/ $\beta$ -catenin signaling pathway in human chondrosarcoma cells.

Considering that chondrosarcomas are of mesenchymal origin, previously reported derivation of sarcomas from mesenchymal stem cells as determined by inactivation of the Wnt/ $\beta$ -catenin signaling pathway support the findings of the present study (18).

*CCND2* has been associated with the progression of sarcomas, particularly osteosarcoma (37). Numerous studies have demonstrated the importance of miRNA regulators, their down-regulation and role in overexpression of *CCND2* in high-grade osteosarcomas and resistance to chemotherapy (22-25). Additionally, *CCND2* was reported to be upregulated in metastatic osteosarcoma compared with primary tumor samples (26). Collectively, these results suggest the important role served by *CCND2* in cancer progression, chemoresistance and metastasis that may also occur in chondrosarcoma.

*MMP7* has been associated with an increased level of invasiveness of endothelial cells infected by Kaposi's sarcoma herpesvirus (36). Inactivation of Wnt signaling has been demonstrated to increase the expression of *MMP7* in osteosarcoma, which opposes the aforementioned findings reported in carcinomas (37). Investigation into chondrosarcoma cells revealed that *MMP7* upregulation promoted cell motility and invasion, leading to increased lung metastasis *in vivo* (38). Considering these findings, reductions in the expression of *MMP7* in ALDH<sup>low-PRP-1</sup> cells indicate the potential of PRP-1 to inhibit the progression and metastasis of chondrosarcoma.

This present study reported the role of PRP-1 in activating the Wnt pathway and the translocation of  $\beta$ -catenin to the nucleus, in addition to downregulating the expression of oncogenes *MMP7* and *CCND2*. It is likely that in these cases, PRP-1 also normalized the expression of unexpected targets of the noncanonical pathway. For example, overexpression of *MMP7* was reported to be induced by the noncanonical WNT signaling pathway (39). Wnt/ $\beta$ -catenin signaling cascades often intersect with other signaling pathways, resulting in synergistic or antagonistic effects on stem cell behavior; thus, various  $\beta$ -catenin co-activators may lead to different outcomes. The interaction of  $\beta$ -catenin with different transcription factors and the potential effects of these interactions for the direct crosstalk between the Wnt/ $\beta$ -catenin and non-Wnt signaling pathways require further investigation (40,41). Collectively, our results support that inactivation of the Wnt/ $\beta$ -catenin signaling pathway in mesenchymal tumors may initiate sarcomagenesis in chondrosarcoma (18). Furthermore, Wnt signaling was proposed to promote or inhibit tumor initiation, metastasis and drug resistance in a cancer stage-specific and a cancer type-specific manner (42).

The findings of the present study suggest that activation of the Wnt/ $\beta$ -catenin signaling pathway may serve an antitumor role in sarcomas as demonstrated by the upregulated expression of genes associated with this pathway in ALDH<sup>low-PRP-1</sup> cells. ALDH activity and reductions in the abundance of CSCs were demonstrated to promote  $\beta$ -catenin translocation into the nucleus. PRP-1 was determined to be involved in regulating the Wnt/ $\beta$ -catenin signaling targets; however, whether this regulation alone or in combination with other signaling events underlies the depletion of the CSC population requires further investigation.

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#### Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

#### Authors' contributions

AKH, AM and KAG conceived and designed the study. AKH, AM, CG, AS, SS and JB performed the experiments. AKH, AS and SS performed the statistical analyses and produced the figures. AKH and AM wrote the manuscript. AKH, AM, CG and KAG reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring the accuracy or integrity of any part of the work are appropriately investigated and resolved.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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