Achyranthes bidentata polysaccharides induce chondrocyte proliferation via the promotion of the G_1/S cell cycle transition

FANGRONG YU*, XIHAI LI*, LIANGLIANG CAI, HUITING LI, JIASHOU CHEN, XIAPING WONG, HUIFENG XU, CHUNSONG ZHENG, XIANXIANG LIU and HONGZHI YE

Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China

Received September 19, 2012; Accepted December 24, 2012

DOI: 10.3892/mmr.2013.1286

Abstract. *Achyranthes bidentata* polysaccharides (ABPS) are the major bioactive constituents of *Radix Achyranthes bidentata* (AB), which has been widely used in traditional Chinese medicine for the treatment of osteoarthritis. However, the molecular mechanisms behind the therapeutic effect of ABPS remain unclear. In the present study, chondrocytes were isolated from Sprague-Dawley rats. The effects of ABPS on the G_1/S cell cycle transition in primary chondrocytes were investigated. The chondrocytes treated with and without ABPS were analyzed and it was observed that ABPS treatment was able to enhance chondrocyte proliferation in a dose- and time-dependent manner and promote the progression of chondrocyte cell cycle proliferation via the promotion of the G_1 to S phase transition. Furthermore, using RT-PCR and western blot analysis, ABPS were observed as significantly upregulating the expression of cyclin D1 and the cyclin-dependent kinases (CDKs) CDK4 and CDK6. These results suggest that ABPS are able to promote chondrocyte proliferation via the promotion of the G_1/S cell cycle transition.

Introduction

Osteoarthritis (OA), a public health concern that causes the most chronic disability in middle-aged and older individuals in modern society, is a degenerative joint disease that is characterized by a progressive loss of articular cartilage (1,2). Chondrocytes, the only types of cell present in cartilage, play a central role in the equilibrium between the anabolism and catabolism of the fundamental component of cartilage, the extracellular matrix (ECM). The stability of chondrocyte function is evidently extremely significant for maintaining the normal activity of cartilage (3,4). As cartilage has a limited capacity to respond to injury and a low potential for self-repair, strengthening the functions of the chondrocytes by promotion of their proliferation may be an effective treatment for OA.

The eukaryotic cell cycle is divided into 4 phases: G_1, S, G_2 and M. Of these stages, the G_1 phase, in which DNA synthesis is prepared, is the director that determines whether the cell is able to continue through the cycle or withdraw. Only once past the G_1/S transition may the cell continue to proliferate (5). The progression through each phase of the cell cycle is delicately controlled by the activity of various cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins (6). During the G_1 phase, cyclin D1 is a key cell cycle regulatory protein that is associated with CDK4 or CDK6 in the control of cell cycle progression (7,8). Therefore, cyclin D1/CDK4 or D1/CDK6 complexes may promote cell proliferation via the promotion of the cell cycle from the G_1 to the S phase.

Radix Achyranthes bidentata (AB), a traditional Chinese medicinal herb, has been extensively used in Chinese medicinal formulations for the clinical treatment of OA (9). AB polysaccharides (ABPS) have a unit composition molecular weight of ~1,400 Da. The ABPS are purified polysaccharides isolated from AB and composed of fructose and glucose residues in the molar ratio of 8:1. In addition, ABPS contain 2,1-linked fructose, 1,2,6-linked fructose, terminal fructose and terminal glucose residues (10,11). Previous studies reported that the medical effects of ABPS were anti-inflammatory, antiviral, immunomodulatory and antitumoral(12-15). In order to explore the activity of ABPS in OA treatment, the present study observed their effects on cultured chondrocytes and attempted to identify the underlying mechanisms. ABPS were subsequently identified as promoting chondrocyte proliferation via the upregulation of cyclin D1, CDK4 and CDK6 expression.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). The type II collagenase was purchased from Sigma (St. Louis, MO, USA) and the cell cycle detection kit was from Nanjing Key Gen Biotech (Nanjing, Jiangsu, China).

Correspondence to: Professor Xianxiang Liu or Professor Hongzhi Ye, Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, 1 Huatuo, University Town, Minhou Shangjie, Fuzhou, Fujian 350122, P.R. China
E-mail: liuxianxiang@163.com
E-mail: yelin0930@163.com

*Contributed equally

Key words: Achyranthes bidentata polysaccharides, chondrocyte, cell proliferation, osteoarthritis

The reverse transcription system was purchased from the Promega Corporation (Madison, WI, USA). The DNA primers were synthesized by Shengong Biotech (Shanghai, China), while the rabbit anti-rat cyclin D1, CDK4 and CDK6 antibodies and the HRP secondary goat anti-rabbit antibodies were provided by Bioworld Technology Co., Ltd. (Nanjing, China). The study was approved by the ethics committee of Fujian University of Traditional Chinese Medicine.

Preparation of ABPS from AB. The dried and sliced AB was refluxed twice with 80% alcohol for 1 h/time. Subsequent to evaporation to dry the solvent, the residue was refluxed 3 times with distilled water (100 g/l) for 2 h/time and extracted. All extractions were concentrated to 100 ml under hypopiesia conditions. The condensed solution was then purified by being precipitated with anhydrous alcohol whose final content was 80% in the decoction and being stewed overnight. The precipitates isolated by centrifugation were then lyophilized. The crude polysaccharide was dissolved into distilled water (200 g/l), then the protein was removed by Sevag's method. The polysaccharides formed a white power; the ABPS were dissolved in DMEM containing 10% FBS at a density of 10 mg/ml, then the mother liquor was filtered through a 0.22-μm filter and stored at 4˚C.

Isolation and culture of the chondrocytes. Male, 4-week-old, Sprague-Dawley (SD) specific pathogen-free (SPF) rats were purchased from the Super-BK Laboratory Animal, Inc. (Shanghai, China). The rats were sacrificed using cervical dislocation and their knees were stripped and soaked with 75% ethanol for 15 min. The articular cartilages were cut down subsequent to the opening of the joint spaces. then they were transferred to PBS containing penicillin and streptomycin and washed 3 times. The cartilages were cut into 1-mm^3 sections and subsequently digested with 0.2% type II collagenase. The isolated cells were collected every 2 h and cultured in 50-ml culture flasks in 4 ml DMEM containing 10% FBS at 37˚C and 5% CO_2. The culture media were changed every 2 days and the cells were subcultured at 90% confluency.

Evaluation of cell viability by MTT assay. The passage 2 chondrocytes were seeded into 96-well plates at a density of $1.0x10^4$/ml and cultured for 24 h. The cells were treated with varying concentrations of ABPS for 24, 48 and 72 h. At the end of the treatment, 100 μl MTT (1 mg/ml in PBS) was added into each well and subsequent to a 4-h incubation at 37˚C, the supernatant was removed and 150 μl DMSO was added to dissolve the formazane. The solution was agitated for 10 min and the OD_{490} was analyzed using an ELISA reader.

Observation of morphological changes. The passage 2 chondrocytes were seeded into 50-ml culture flasks at a density of $1x10^4$/ml in 4 ml medium and cultured for 24 h. The cells were treated with the varying final concentrations of ABPS (0, 50, 100 and 150 μg/ml) for 48 h. The cell morphology was observed and images were captured by phase-contrast microscopy (x100 magnification).

Detection of cell cycle by flow cytometry. The passage 2 chondrocytes were seeded into 6-well plates at a density of $1x10^4$/ml and cultured to a logarithmic growth phase. The cells were treated with varying concentrations of ABPS for 48 h, collected and the cell density adjusted to $1x10^5$/ml. The suspension was incubated with A, B, C solutions from the cell cycle detection kit (Nanjing Key Gen Biotech) according to the manufacturer's instructions. The percentage of cells in each phase was calculated by ModFit software and the cell numbers from the G_0/G_1, S and G_2/M transition phases were obtained.

RNA extraction and RT-PCR analysis. The total RNA of the chondrocytes treated with the varying concentrations of ABPS for 48 h was extracted with TRIzol reagent and the RNA (2 μg) was reverse transcribed into cDNA. The obtained cDNA was used in PCR to determine the amount of cyclin D1, CDK4 and CDK6 mRNA. β-actin was used as an internal control. The primers used for the amplification of the cyclin D1, CDK4, CDK6 and β-actin transcripts were as follows: cyclin D1 forward, 5'-AAT GCC AGA GGC GGA TGA GA-3' and reverse, 5'-GCT TGT GCG GTA GCA GGA GA-3'; CDK4 forward, 5'-GAA GAC GAC TGG CCT CGA GA-3' and reverse, 5'-ACT GCG CTC CAG ATT CCT CC-3'; CDK6 forward, 5'-TTG TGA CAG ACA TCG ACG AG-3' and reverse, 5'-GAC AGG TGA GAA TGC AGG TT-3'; β-actin forward, 5'-CGT TGA CAT CCG TAA AGA CC-3' and reverse, 5'-GGA GCC AGG GCA GTA ATC T-3'. The DNA bands were examined using a Gel Documentation system.

Western blot analysis. The passage 2 chondrocytes were seeded into 50-ml culture flasks at a density of $1x10^4$/ml in 4 ml medium and cultured for 24 h. Subsequent to treatment with the varying concentrations of ABPS for 48 h, the cells were lysed and the protein concentrations were determined by the BCA assay. The assay proteins were separated by electrophoresis on 12% SDS-polyacrylamide gels and then transferred onto PVDF membranes. The membranes were blocked for 2 h with agitation at room temperature in 5% skimmed milk powder dissolved in TBST. The membranes were washed in TBST and then incubated with the primary antibody solution (1:1,000) at 4˚C overnight. Once the membranes had been washed in TBST, the secondary antibody solution (1:1,500) was added for 1 h at room temperature and then the membranes were washed again in TBST. Finally, the antibody-bound protein bands were detected with ECL and images were captured using a Kodak image station 400R (Kodak, Rochester, NY, USA).

Statistical analysis. The data were analyzed with SPSS 16.0 and expressed as the mean ± standard deviation (SD). A statistical analysis of the data was conducted using a Student's t-test and ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Morphology and characteristics of the chondrocytes. The newly isolated chondrocytes were small, round cells initially grown as a suspension culture. Subsequent to 24 h of proliferation, the cells had gradually attached themselves to the culture flask and formed into halo-like shapes (Fig. 1A). Subsequent to 3 days of proliferation, a number of cells showed an irregular

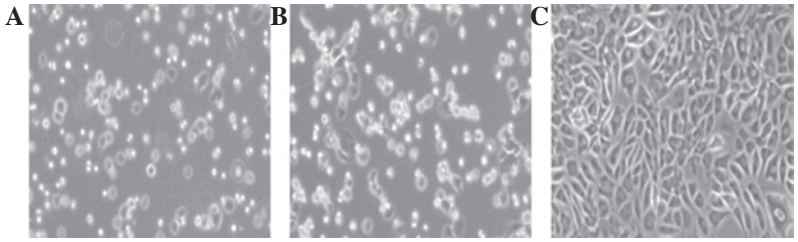

Figure 1. Morphological observation and identification of the chondrocytes. Images of primary cells cultured for (A) 24 h, (B) 3 days and (C) 7 days.

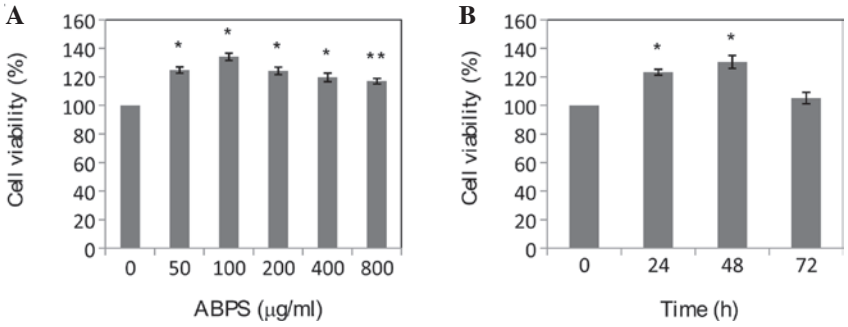

Figure 2. Effect of *Achyranthes bidentata* polysaccharides (ABPS) on the proliferation of the chondrocytes. Cell viability was determined by MTT assay subsequent to the chondrocytes being treated with (A) the indicated concentrations of ABPS for 48 h or (B) with 100 μg/ml ABPS for the indicated time periods. *P<0.01,**P<0.05, compared with the control cells.

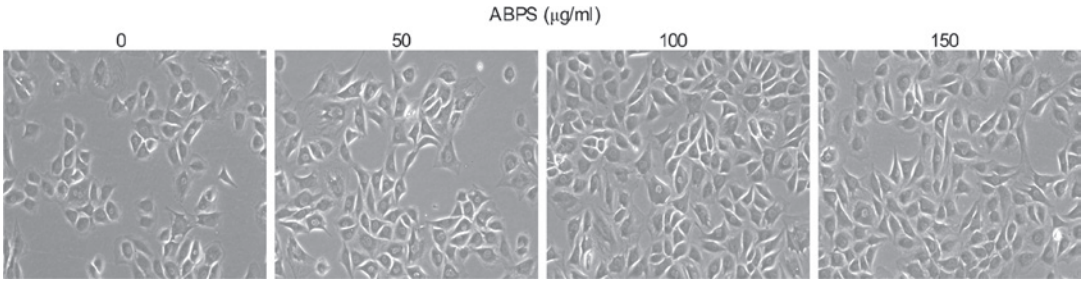

Figure 3. Effect of *Achyranthes bidentata* polysaccharide (ABPS) treatment on the morphological changes of the chondrocytes (x200 magnification). Chondrocytes were treated with or without ABPS for 48 h, the morphological changes of cultured chondrocytes were observed using phase microscopy.

flagstone stereo shape growth and certain cells exhibited a fibroblast-like morphology (Fig. 1B). Subsequent to 8 days of proliferation, the cells spread across the flask in long spindle lines and demonstrated clear boundaries and distinct nuclei (Fig. 1C). In the subculture, chondrocytes proliferated markedly faster than the primary generation and usually reached 80 or 90% density in ~5 days.

ABPS promote the proliferation of the chondrocytes. The effect of ABPS on the viability of the chondrocytes was measured by MTT assay. As shown in Fig. 2A, treatment with 50, 100, 200, 400 and 800 μg/ml ABPS for 48 h increased cell viability by 24.82±2.11, 34.14±2.50, 23.21±2.62, 11.92±3.08 and 11.73±1.92%, respectively, compared with the 0-μg/ml group (P<0.01). The data in Fig. 2B showed that treatment with 100 μg/ml ABPS for 24, 48 and 72 h increased cell viability by 23.44±2.41 (P<0.01), 34.09±4.53 (P<0.01) and 5.23±4.24% (P=0.085), respectively. As shown in Fig. 3, when compared with the 0-μg/ml group, there were no significant differences in the morphological changes of the treated groups, but the number of chondrocytes in the ABPS-treated groups was markedly greater and with an evident time dependence. Taken together, it may be suggested that ABPS treatment promotes the growth of chondrocytes in a dose- and time-dependent manner within appropriate ranges.

Effects of ABPS on the chondrocyte cell cycle. Prior to treatment with ABPS, all cells were cultured in DMEM without FBS for 24 h to synchronize the cell cycle stage. As shown in Fig. 4, the percentage proportion of cells in the G_0/G_1 phase was lower in all the ABPS-treated groups (74.88±6.20, 67.48±2.63 and 74.89±3.48; 50, 100, 150 μg/ml, respectively), with levels in the 100-μg/ml group significantly decreased compared with the 0-μg/ml group (77.78±5.59, P<0.05). The percentages of cells in the S phase from the ABPS-treated groups were 16.28±2.81, 24.72±3.88 and 16.78±3.01% (50, 100, 150 μg/ml, respectively), with levels in the 100-μg/ml group significantly higher than in the 0-μg/ml group (13.90±3.05, P<0.01). This showed an opposite trend to the G_0/G_1 phase. These results suggested that ABPS treatment is able to promote the progression of the cell cycle in the transition from G_1 to S phase.

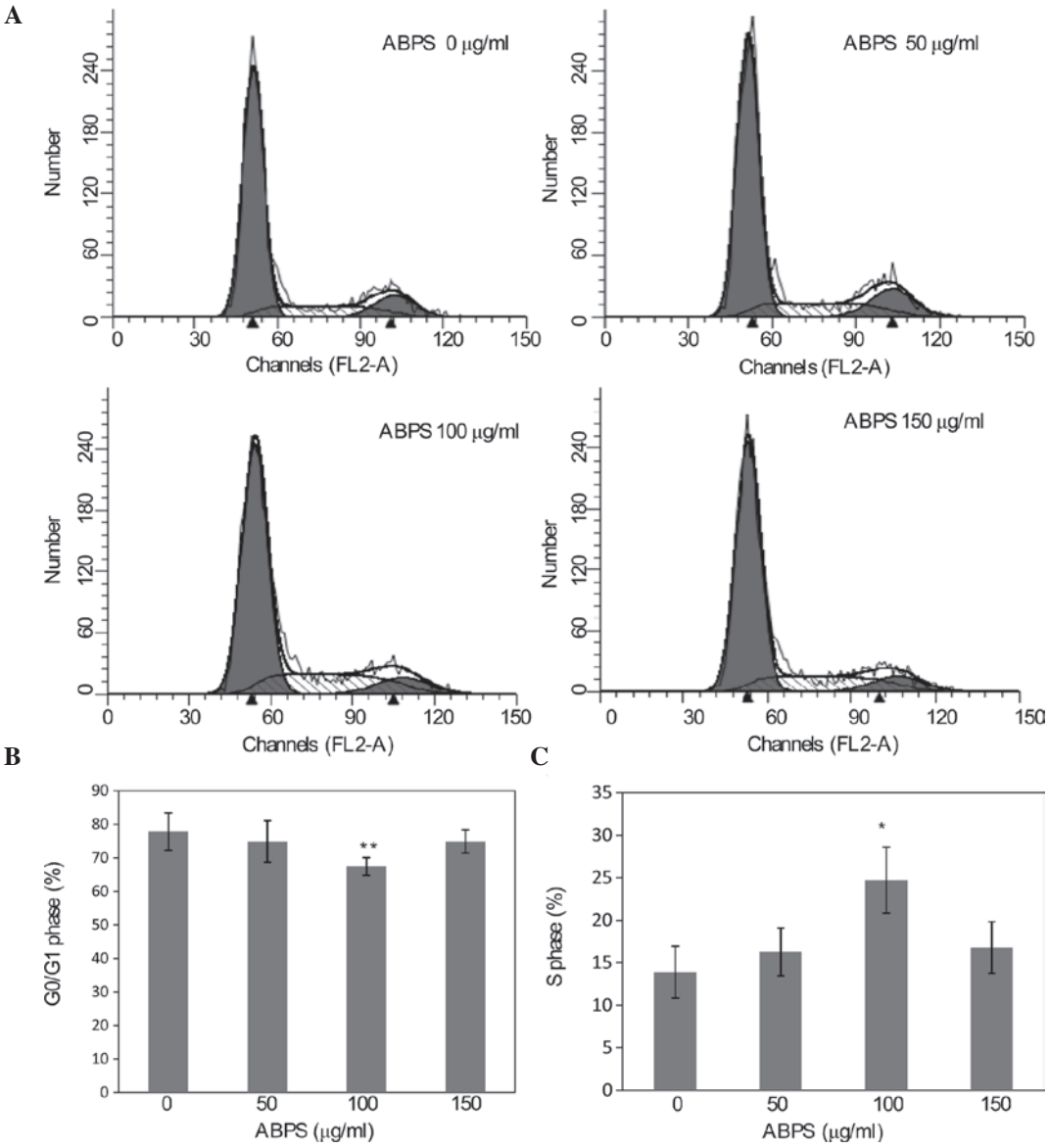

Figure 4. Effect of *Achyranthes bidentata* polysaccharide (ABPS) treatment on the cell cycle of the chondrocytes. (A) Subsequent to ABPS treatment, the chondrocytes were collected and stained using PI staining followed by FACS analysis. (B) Percentage of chondrocytes in the G_0/G_1 phase subsequent to ABPS treatment. (C) Percentage of chondrocytes in the S phase subsequent to ABPS treatment. The data are presented as mean ± SD. *P<0.01, **P<0.05, vs. the untreated group. PI, propidium iodide.

ABPS upregulate the expression of cyclin D1, CDK4 and CDK6. In order to further explore the mechanism of the ABPS-induced promotion of chondrocyte proliferation, RT-PCR and western blot analysis were used to examine the mRNA and protein expression levels of cyclin D1, CDK4 and CDK6. As shown in Fig. 5, compared with the 0-μg/ml group, the cells that underwent ABPS treatment had significantly increased levels of cyclin D1 and CDK6 mRNA expression (P<0.01), while the mRNA expression of CDK4 in the 100 μg/ml group was also significantly increased (P<0.05). The protein expression of cyclin D1, CDK4 and CDK6 was similar to their respective mRNA levels (Fig. 6).

Discussion

Polysaccharides are high molecular weight compounds formed from repeating sub-units of sugars and are widely distributed in Chinese herbs. Polysaccharides are one of the four basic materials that compose life, with the ability to store bioenergy and support the role of the structure components (16). To further investigate the underlying mechanisms behind the effects of ABPS on chondrocytic functions, chondrocytes with varying concentrations of ABPS using differing times *in vitro* were observed. According to the results from the MTT assay, cell viability was enhanced when using 100 μg/ml ABPS treatment for 48 h, therefore 0, 50, 100 and 150 μg /ml ABPS and 48 h were set as variables for the further experimental program. The present study demonstrated that ABPS treatment promotes chondrocyte proliferation via promotion of the transition from G_1 to S phase.

As the principle function of cartilage is to provide a low friction load-bearing surface that facilities free movement of the joints, the generation of cartilage is a significant cause of OA (17). Chondrocytes form the essential composition of

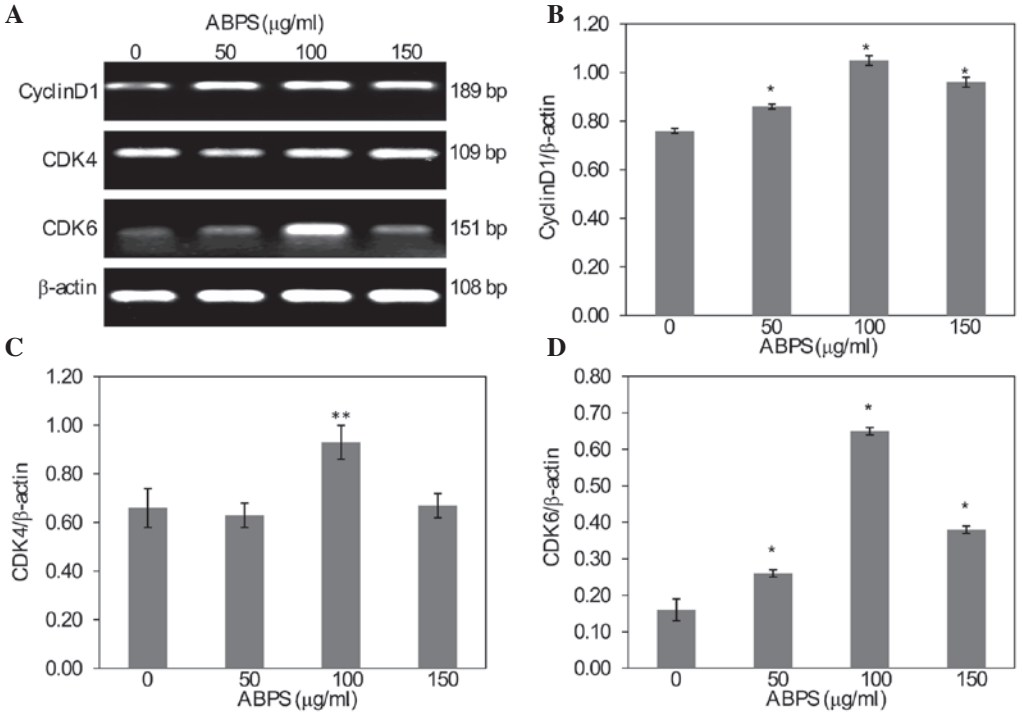

Figure 5. Effect of *Achyranthes bidentata* polysaccharides (ABPS) on the mRNA expression of cyclin D1, CDK4 and CDK6 in the chondrocytes. (A) mRNA expression of cyclin D1 in ABPS-treated and -untreated groups. (B) mRNA expression of CDK4. (C) mRNA expression of CDK6 in ABPS-treated and -untreated groups. (D) Quantification of RT-PCR analysis, the data are presented as mean ± SD from ≥3 independent experiments. *P<0.01, **P<0.05, vs. the untreated group. CDK, cyclin dependent kinase.

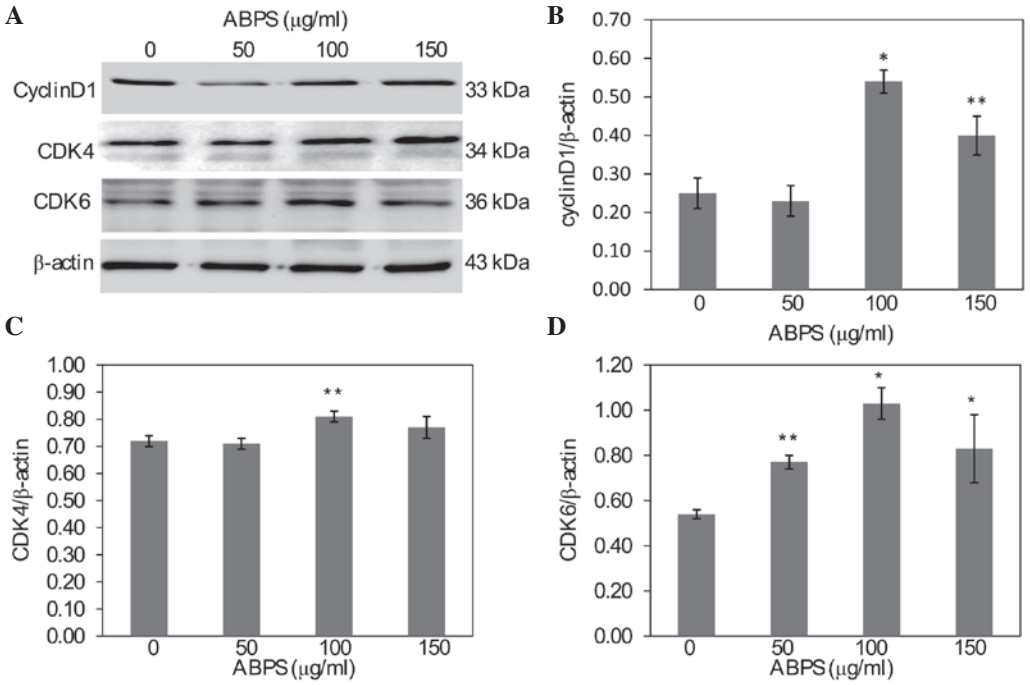

Figure 6. Effect of *Achyranthes bidentata* polysaccharides (ABPS) on the expression of cyclin D1, CDK4 and CDK6 in the chondrocytes. (A) Protein expression levels of cyclin D1, CDK4 and CDK6 were analyzed by western blotting, β-actin was used as the internal control. (B) Protein expression levels of cyclin D1. (C) Protein expression levels of CDK4. (D) Protein expression levels of CDK6. *P<0.01, **P<0.05, compared with the untreated groups. CDK, cyclin dependent kinase.

articular cartilage, therefore their functional changes play an extremely significant role in the damage of cartilage, and the proliferation of chondrocytes is necessary in maintaining cellular functions (18).

The cell cycle, a set of events that are responsible for the duplication of the cell, is composed of 4 stages: G_1, the preparation for DNA synthesis; S, DNA synthesis; G_2, the preparation for mitosis; and M, mitosis. The S and M phases are the two

most important processes. Between these phases, there are two gaps, G_1 prior to the S phase and G_2 prior to the M phase. The G_1/S and G_2/M transitions are the two checkpoints regulating stage transition and cell cycle progression (7,19). MTT data from the present study showed that ABPS treatment promoted chondrocyte viability within certain doses and times. To further explore the mechanism of ABPS activity, flow cytometry was used to examine the changes in the chondrocyte cell cycle brought about by treatment with ABPS; the results showed that the percentage of chondrocytes in the G_0/G_1 phase was reduced and that the percentage of chondrocytes in the S phase was significantly increased, demonstrating that ABPS treatment promotes chondrocyte proliferation via the promotion of cell cycle progression.

The CDKs and the cyclins are two basic protein families of the cell cycle control system that associate with each other as CDK/cyclin complexes to regulate the progress of the cell cycle. The complex that regulates the progression of each phase of the cell cycle varies, for example, cyclin D associates with CDK4 and CDK6 during early G_1 phase, cyclin E binds to CDK2 during G_1 to S phase transition and cyclin A activates CDK2 during the S phase and the S to M phase transition. CDKs, which allow progression through the phases of the cell cycle by phosphorylating substrates, have kinase activity which is dependent on the presence of their activating subunits, the cyclins. Only when the specific CDK/cyclin complexes are activated does their phosphorylation of particular proteins permit cell cycle progression to continue (20-23). In the present study, the results showed that ABPS treatment enhances the mRNA and protein expression of cyclin D1, CDK4 and CDK6, suggesting that ABPS treatment promotes the progression of chondrocytes from the G_1 to the S phase by regulating cyclin D1, CDK4 and CDK6.

In conclusion, the data demonstrated that ABPS effectively promote proliferation via the promotion of the G_1/S cell cycle transition and upregulation of the expression of cyclin D1, CDK4 and CDK6. This suggests that ABPS may be potential novel therapeutic agents for the treatment of OA.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81102609), the Key Project of Fujian Provincial Department of Science and Technology (grant no. 2012Y0046), the Natural Science Foundation of Fujian Province (grant no. 2011J05074) and the Developmental Fund of Chen Keji Integrative Medicine (grant no. CKJ20110003).

References

1. Iliopoulos D, Gkretsi V and Tsezou A: Proteomics of osteoarthritic chondrocytes and cartilage. Expert Rev Proteomics 7: 749-760, 2010.
2. Lotz MK and Caramés B: Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. Nat Rev Rheumatol 7: 579-587, 2011.
3. Clouet J, Vinatier C, Merceron C, Pot-vaucel M, Maugars Y, Weiss P, Grimandi G and Guicheux J: From osteoarthritis treatments to future regenerative therapies for cartilage. Drug Discov Today 14: 913-925, 2009.
4. Schroeppel JP, Crist JD, Anderson HC and Wang J: Molecular regulation of articular chondrocyte function and its significance in osteoarthritis. Histol Histopathol 26: 377-394, 2011.
5. Zhang M, Xie R, Hou W, Wang B, Shen R, Wang X, Wang Q, Zhu T, Jonason JH and Chen D: PTHrP prevents chondrocyte premature hypertrophy by inducing cyclin-D1-dependent Runx2 and Runx3 phosphorylation, ubiquitylation and proteasomal degradation. J Cell Sci 122: 1382-1389, 2009.
6. Golias CH, Charalabopoulos A and Charalabopoulos K: Cell proliferation and cell cycle control: a mini review. Int J Clin Pract 58: 1134-1141, 2004.
7. Blagosklonny MV and Pardee AB: The restriction point of the cell cycle. Cell Cycle 1: 103-110, 2002.
8. Li X, Ye H, Yu F, *et al*: Millimeter wave treatment promotes chondrocytes proliferation via G_1/S cell cycle transition. Int J Mol Med 29: 823-831, 2012.
9. Chen Q, Liu Z and He J: *Achyranthes bidentata* polysaccharide enhances immune response in weaned piglets. Immunopharmacol Immunotoxicol 31: 253-260, 2009.
10. Xue JP and Shi JY: Primary study on suspension cell culture and polysaccharides content of Achyranthes bidentata. Zhongguo Zhong Yao Za Zhi 33: 2467-2469, 2008 (In Chinese).
11. Chen XM, Xu YJ and Tian GY: Physical-chemical properties and structure elucidation of abPS isolated from the root of *Achyranthes bidentata*. Yao Xue Xue Bao 40: 32-35, 2005 (In Chinese).
12. Peng ZG, Chen HS, Guo ZM, Dong B, Tian GY and Wang GQ: Anti-HIV activites of *Achyranthes bidentata* polysaccharide sulfate in vitro and in vivo. Yao Xue Xue Bao 43: 702-706, 2008 (In Chinese).
13. Zhu X, Pan Y, Zheng L, Cui L and Cao Y: Polysaccharides from the Chinese medicine herb *Achyranthes bidentata* enhance antimalarial immunity during *Plasmodium yoelii* 17XL infection in mice. Malar J 11: 49, 2012.
14. Zou Y, Meng J, Chen W, Liu J, Li X, Li W, Lu C and Shan F: Modulation of phenotypic and functional maturation of murine dendritic cells (DCs) by purified *Achyranthes bidentata* polysaccharide (ABP). Int Immunopharmacol 11: 1103-1108, 2011.
15. Lin J, Zhang Z and Shan Y: Effect of *Achyranthes bidentata* polysaccharides on the expression of BCL-2 and bax in hepatic tissues after exhaustive exercise in rats. Afr J Tradit Complement Altern Med 7: 307-314, 2010.
16. Boddohi S and Kipper MJ: Engineering nanoassemblies of polysaccharides. Adv Mater 22: 2998-3016, 2010.
17. Harris JD, Siston RA, Pan X and Flanigan DC: Autologous chondrocyte implantation: a systematic review. J Bone Joint Surg Am 92: 2220-2233, 2010.
18. Chan BY, Fuller ES, Russell AK, *et al*: Increased chondrocyte sclerostin may protect against cartilage degradation in osteoarthritis. Osteoarthritis Cartilage 19: 874-885, 2011.
19. Onumah OE, Jules GE, Zhao Y, Zhou L, Yang H and Guo Z: Overexpression of catalase delays G_0/G_1-to S-phase transition during cell cycle progression in mouse aortic endothelial cells. Free Radic Biol Med 46: 1658-1667, 2009.
20. Pietras EM, Warr MR and Passegué E: Cell cycle regulation in hematopoietic stem cells. J Cell Biol 195: 709-720, 2011.
21. Ogasawara T, Mori Y, Abe M, *et al*: Role of cyclin-dependent kinase (Cdk)6 in osteoblast, osteoclast, and chondrocyte differentiation and its potential as a target of bone regenerative medicine. Oral Science International 8: 2-6, 2011.
22. Echalier A, Endicott JA and Noble ME: Recent developments in cyclin-dependent kinase biochemical and structural studies. Biochim Biophys Acta 1804: 511-519, 2010.
23. Inagaki S and Umeda M: Cell-cycle control and plant development. Int Rev Cell Mol Biol 291: 227-261, 2011.