Isopsoralen Induces Differentiation of Prechondrogenic ATDC5 Cells via Activation of MAP Kinases and BMP-2 Signaling Pathways

Liang Li[1,2], Jae Soon Eun[2], Manoj Nepal[1], Jae-Ha Ryu[3], Hyoung Kwon Cho[4], Bo-Yun Choi[1] and Yunjo Soh[1,*]

[1]Department of Dental Pharmacology, School of Dentistry and Institute of Oral Bioscience, Brain Korea 21 Project, Chonbuk National University, Jeonju 561-756, [2]College of Pharmacy, Woosuk University, Wonju 565-701,
[3]College of Pharmacy, Research Center for Cell Fate Control, Sookmyung Women's University, Seoul 140-742,
[4]Hanpoong Pharm & Foods Co. Ltd, Jeonju 561-841, Republic of Korea

Abstract

Endochondral bone formation is the process by which mesenchymal cells condense to become chondrocytes, which ultimately form new bone. The process of chondrogenic differentiation and hypertrophy is critical for bone formation and as such is regulated by many factors. In this study, we aimed to indentify novel factors that regulate chondrogenesis. We investigated the possible role of isopsoralen in induction of chondrogenic differentiation in clonal mouse chondrogenic ATDC5 cells. Isopsoralen treatment stimulated the accumulation of cartilage nodules in a dose-dependent manner. Further, ATDC5 cells treated with isopsoralen were stained more intensely with Alcian blue than control cells, suggesting that isopsoralen increases the synthesis of matrix proteoglycans. Similarly, isopsoralen markedly induced the activation of alkaline phosphatase activity compared with control cells. Isopsoralen enhanced the expressions of chondrogenic marker genes such as collagen II, collagen X, OCN, Smad4 and Sox9 in a time-dependent manner. Furthermore, isopsoralen induced the activation of extracellular signal-regulated kinase (ERK) and p38 MAP kinase, but not that of c-jun N-terminal kinase (JNK). Isopsoralen significantly enhanced the protein expression of BMP-2 in a time-dependent manner. PD98059 and SB 203580, inhibitors of ERK and p38 MAPK, respectively, decreased the number of stained cells treated with isopsoralen. Taken together, these results suggest that isopsoralen mediates a chondromodulating effect by BMP-2 or MAPK signaling pathways, and is therefore a possible therapeutic agent for bone growth disorders.

Key Words: Isopsoralen, ATDC5 cells, Chondrogenesis, MAP kinases, BMP-2, Growth disorders

INTRODUCTION

During the initial stages of bone formation, generation of cartilage is followed by ossification, the last step in creating bone tissue. Cartilage formation during embryogenesis is initiated by the condensation and differentiation of mesenchymal stem cells (MSCs) into chondrocytes, which form transient hyaline cartilage that is degraded and replaced by bone via a process known as endochondral ossification (Kronenberg, 2003). Endochondral ossification is an essential process during the rudimentary formation of long bones (Netter, 1988), the lengthening of long bones (Brighton *et al.*, 1973), and the natural healing of bone fractures (Brighton *et al.*, 1986). Chondrogenesis and ossification are regulated by a multitude of genetic and hormonal, growth, environment, and nutrition factors (Nakajima *et al.*, 2009).

Psoralea corylifolia L. is an important medicinal plant found in tropical and subtropical regions that is used as a tonic or aphrodisiac and for the prevention and treatment of several disorders including skin disease, inflammatory disease, and tumorigenesis (Sun *et al.*, 1998; Pae *et al.*, 2001; Sultan *et al.*, 2011). Furthermore, extracts of the seeds of *P. corylifolia* L. are also used as remedies for bone fractures, osteomalacia, and osteoporosis (Zhang *et al.*, 2005). Isopsoralen (Fig. 1) is the main active ingredient extracted from the seeds of *P. corylifolia* L, and has both antitumor (Guo *et al.*, 2003) and anti-depressant properties (Kong *et al.*, 2001). In addition, isopsoralen regulates chondroprogenitor cell differentiation; however, the mechanism of regulation remains elusive.

In this study, we found that isopsoralen had stimulatory effects in ATDC5 cells, leading to increased chondrogenesis. In addition, isopsoralen induced the expressions of chondro-

genic marker genes such as type II collagen, osteocalcin, and bone sialoprotein, as well as chondrogenic signaling molecules including Smad4, Sox9, and β-catenin. Isopsoralen also up-regulated the expression of BMP2, a well-known family of proteins that serve as growth factors regulating the proliferation and differentiation of chondrocytes.

MATERIALS AND METHODS

Materials

Isopsoralen was isolated from the extracts of the seeds of *Psoralea corylifolia*. Cell culture medium and fetal bovine serum (FBS) were obtained from (Gaithersburg, MD, USA). PCR primers were purchased from Bioneer (Daejeon, Korea). SB203580 and PD 98059 were purchased from Calbiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless specified otherwise, as described previously (Choi *et al.*, 2011).

Cell culture and differentiation

Pre-chondrogenic ATDC5 cells were purchased from the RIKEN Cell Bank (Ibaraki, Japan). ATDC5 cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37℃. For chondrogenic induction, the medium was replaced with medium containing 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite, and 10 μg/ml bovine insulin (differentiation medium). Cells were cultured in the differentiation medium for 14 days; media were replaced every two days. We used isopsoralen instead of bovine insulin to induce chondrogenic differentiation of ATDC5 cells in the present study.
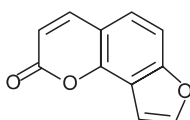
Fig. 1. Chemical structure of Isopsoralen.

MTT assay

ATDC5 cells were seeded in 96-well plate at a density of 5×10^3 cells/well and incubated overnight in media supplemented with 10% FBS followed by treatment with various concentrations of isopsoralen. After 24h of incubation, cells were washed with phosphate-buffered saline (PBS) and then treated with media containing 100 μg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 2 h at 37℃. The cells were then washed with PBS, and dissolved in 200 μl of DMSO. The resulting solubilized purple formazan was quantified by measuring the absorbance at a wavelength of 540 nm using a spectrophotometer.

Alcian blue staining

Cells were washed twice with PBS and fixed with methanol at -20°C for 5min, after which cells were stained with 0.1% Alcian blue 8GX in 0.1 M HCl overnight. After staining, cells were washed with 3% acetic acid three times for 30 s and then photographed. Stained cells were dissolved in 10% acetic acid for subsequent quantification of the absorbance at 650 nm (Shukunami *et al.*, 1996).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from ATDC5 cells was extracted at the indicated times using Trizol (Invitrogen). Next, cDNAs were prepared from 4 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The primer sequences and conditions used for PCR reactions are listed in Table 1. After an initial denaturation step at 95°C for 1 min, PCR was performed for a varying number of cycles (30 s at 94℃, 1 min at the primer-pair specific annealing temperature, and 2 min at 72℃) using Taq polymerase (Promega, Madison, WI, USA). PCR products were electrophoresed on 1% agarose gels in Tris–acetic acid–EDTA buffer and visualized using ethidium bromide. Band intensities of digitally imaged gels obtained were determined using a phosphoimager and Quantity One software (Bio-Rad, Hercules, CA, USA).

Alkaline phosphatase (ALP) activity

After exposing cells to differentiation stimuli for several different time periods, cells were harvested and homogenized in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA). Next, the lysed suspension was centrifuged for 15

Table 1. Primer sequences and conditions for RT-PCR

Target genes (Accession number)	Primers		PCR condition		
	Forward	Reverse	Tm (℃)	Cycles	Size (bp)
Collagen II (NM_031163)	5'-ctgtaagaacagcatcgcctacctg-3'	5'-caggaatttggtgtggacatagg-3'	60	27	271
SOX9 (NM_011448)	5'-cacaagaaagaccaccccgatac-3'	5'-ggcaaagttgatctgaagcgaga-3'	60	32	272
Collagen 1 (NM_007742)	5'-ttctcctggtaaagatggtgc-3'	5'-tgttaaaggtgatgctggtcc-3'	50	35	254
Collagen X (NM_009925)	5'-cgtctctgcttttactgtca-3'	5'-ctcacagaaaatgaccaggt-3'	48	35	300
Runx2 (NM_009820)	5'-actttctccaggaagactgc-3'	5'-acagcaacagcaacaacagc-3'	50	35	366
β-catenin (NM_007614)	5'-ggtggactgcagaaaatggt-3'	5'-ccttgggactctagtgcagc-3'	52	27	394
OCN (NM_001032298)	5'-cagcttggtgcacacctagc-3'	5'-ggagcagtgtcacgttaacct-3'	58	30	242
BSP (NM_008318)	5'-gagccaggactgccgaaaggaa-3'	5'-gcagcagcggaggagaaacgg-3'	60	27	653
GAPDH (NM_008084)	5'-accacagtccatgccatcac-3'	5'-tacagcaacagggtggtgga-3'	56	25	452
Smad4 (AY493561)	5'-atctatgcccgtctgtggag-3'	5'-aacattcctgtggcttccac-3'	54	30	383

min at 12,000 g, the supernatant was collected, and the cellular protein concentration was determined by Bradford assay. ALP activity was measured using a spectrophotometer with para-nitrophenylphosphate (Sigma) as the substrate. Optical density was measured at 405 nm using an enzyme-linked im-
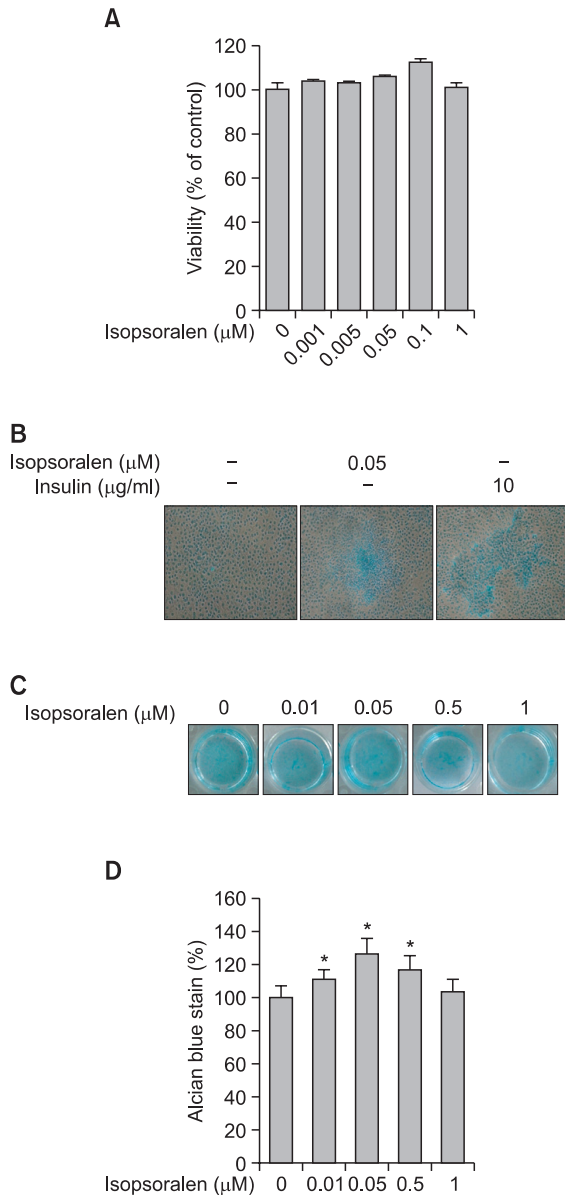
munosorbent assay (ELISA) reader.

Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 200 μl lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml aprotinin, and 20 mM NaF). Total cell lysates were then incubated for 20 min and centrifuged at 12,000 g for 20 min at 4°C. Harvested proteins were separated by 8-10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Next, membranes were blocked with 5% nonfat skim milk in Tris-buffered saline (TBS) containing 0.25% Tween-20 (TTBS) at 16°C for 1 h and incubated at 4°C for 16 h with rabbit anti-phospho ERK (Cell Signaling Technology Inc., Beverly, MA, USA), anti-phospho p38 (Cell Signaling Technology), anti-phospho JNK (Cell Signaling Technology), anti-ERK (Cell Signaling Technology), anti-JNK (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), or anti-BMP-2 (Abcam) antibodies diluted at 1:500-1:1000 in 5% nonfat skim milk in TTBS. Horseradish peroxidase-conjugated anti-rabbit or mouse antibodies (Santa Cruz Biotechnology) were used as secondary antibodies (1:5000-1:10,000 dilution
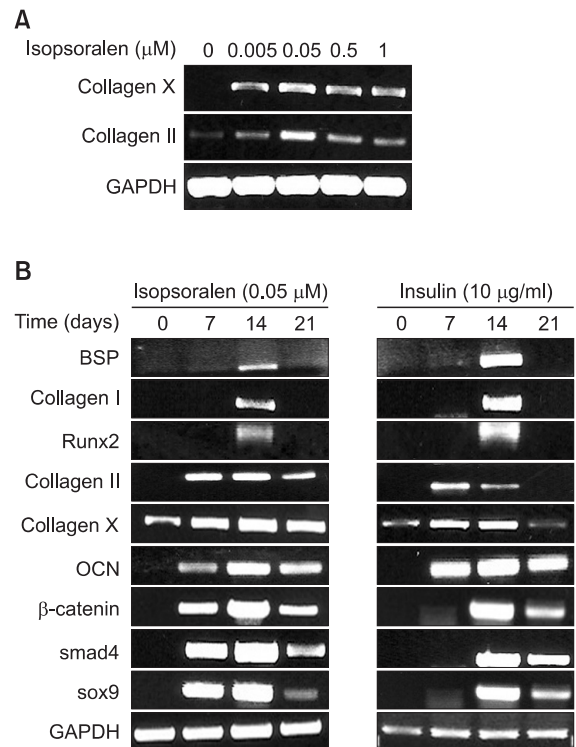

Fig. 2. Effects of Isopsoralen on proliferation and differentiation of ATDC5 cells. Cell Viability was measured with MTT assay (A). Untreated cells or cells treated with 0.05 μg/ml Isopsoralen or 10 μg/ml insulin were incubated for 14 days, rinsed with phosphate buffered saline twice, fixed with 95% methanol, and then stained for 16 h with 1% Alcian blue. Cells were washed with 3% acetic acid for 30 s three times, and then photographed (×100) (B). Cells were plated (5×10⁴) cells per well 24-multiwell plates and treated with 0, 0.01, 0.05, 0.5, and 1 μM Isopsoralen for 14 days. Stained cells were dissolved in 10% acetic acid for subsequent quantification of the absorbance at 650 nm (C). The histogram represents the levels of Alcian blue staining compared to the control group (D). The asterisk (*) indicates a significant difference (p<0.05) compared to the control. Each histogram represents the mean ± S.E.M. (n=3).

Fig. 3. Effects of Isopsoralen on mRNA levels of chondrogenic maker genes in ATDC5 cells. (A) ATDC5 cells were treated with various concentrations of Isopsoralen for 21 days. The mRNA levels of chondrogenic marker molecules including type II collagen, aggrecan, and type X collagen were determined by RT-PCR analysis and compared to the levels of GAPDH (A). ATDC5 cells were treated with 0.05 μM Isopsoralen or 10 μg/ml insulin for 0, 7, 14, 21 days (B). Relative expression of BSP, collagen I, collagen II, collagen X, Runx2, OCN, β-catenin, smad4 and sox9 were observed by PT-PCR analysis and compared to GAPDH.

in 5% nonfat skim milk in TTBS, 1 h incubation at room temperature). Blots were developed with ECL western blot detection reagent (Amersham Biosciences, Piscataway, NJ, USA) and exposed to X-ray film.

Statistical analysis

Dunnett's multiple comparison test was performed using SPSS ver. 13.0 software. All experiments were performed three times and the data were expressed as mean ± S.E.M. Values of $p < 0.05$ were considered significant.

RESULTS

Effects of isopsoralen on proliferation and differentiation of ATDC5 cells

We evaluated the effect of isopsoralen on ATDC5 proliferation and differentiation *in vitro* by light microscopy and assessments of matrix proteoglycan formation. In addition, cell growth was evaluated with the treatment of various concentrations of isopsoralen for 24 h, and by MTT assay. Isopsoralen did not affect the growth rates of ATDC5 cells, as shown in Fig. 2A.

The formation of cartilage nodules is important for chondrogenic differentiation. In order to characterize whether isopsoralen has effects on chondrogenesis, we used 0.05 μM isopsoralen instead of 10 μg/ml insulin to induce chondrogenic differentiation of ATDC5 cells. Treatment with isopsoralen induced differentiation similar to insulin with respect to the development of cartilage nodules, as indicated by Alcian blue staining (Fig. 2B). Matrix proteoglycan production was verified by staining cells with Alcian blue dye, as shown in Fig. 2C and 2D. In control cultures, little Alcian blue staining was observed on day 14. The sizes and numbers of stained nodules were assessed in ATDC5 cells cultured with isopsoralen, which increased in a concentration-dependent manner compared to control cultures. However, the number of stained nodules was reduced when the concentration of isopsoralen was greater than 0.05 μM, suggesting that isopsoralen can stimulate the differentiation of ATDC5 cells.

Effects of isopsoralen on mRNA levels of chondrogenic maker genes in ATDC5 cells

Isopsoralen significantly increased the expressions of chondrogenic marker genes such as Type II and Type X collagen (Fig. 3A). To further investigate the influence of isopsoralen on chondrogenesis, we also assessed various chondrogenesis related genes associated with bone formation signaling molecules by real-time PCR in ATDC5 cells cultured with 0.05 μM isopsoralen at different time points. As shown in Fig. 3B, type I collagen, bone sialoprotein (BSP), and Runx2 were significantly expressed at 14 days. The expression pattern was similar to that of insulin, which was used as a positive control. Likewise, type II collagen, osteocalcin (OCN), type X collagen, Smad, Sox9, and β-catenin gradually increased for 14 days and thereafter remained at a steady state as observed at 21 days. Expressions of chondrocyte-specific markers suggested that isopsoralen induces chondrogenic differentiation in ATDC5 cells.
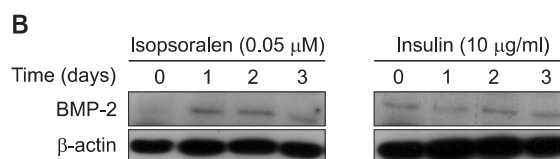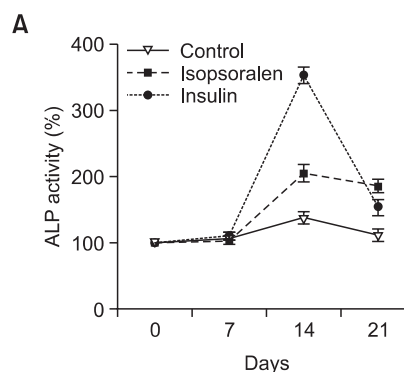

Fig. 4. Effects of Isopsoralen on alkaline phosphatase activity and BMP-2 in ATDC5 cells. ATDC5 cells were treated with 0.05 μM Isopsoralen and 10 μg/ml insulin for 21days, then extracted for alkaline phosphatase activity assay as described in the "Materials and methods". The cells were harvested and homogenized in lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA), and 50 μg of the cell lysates was used to measure the alkaline phosphatase activity at 405 nm (A). The ATDC5 cells were treated with 0.05 μM Isopsoralen or 10 μg/ml insulin for 1, 2, 3 days and cells lysates were immunoblotted with an antibody against BMP-2 (B). The asterisk (*) indicates a significant difference ($p < 0.05$) compared to the control. Each graph represents the mean ± S.E.M. (n=3).
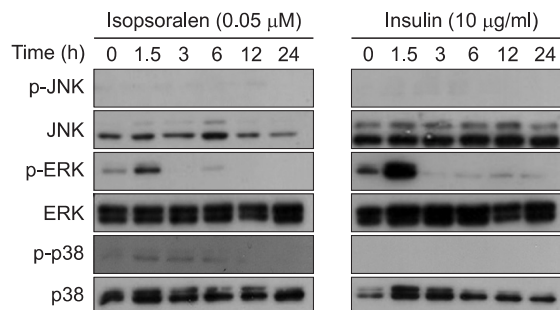

Fig. 5. Effects of Isopsoralen on MAP kinase activation in ATDC5 cells. ATDC5 Cells were incubated serum-starved for 16 h, and then treated with 0.05 μM Isopsoralen or 10 μg/ml insulin for the indicated times. The cells extracts were prepared and analyzed by Western blot using the antibodies specific to the phosphorylated forms of the enzymes and the data were compared to data obtained with antibodies directed against the unphosphorylated states of the kinases.

Effects of isopsoralen on alkaline phosphatase activity and BMP-2 in ATDC5 cells

The enzymatic activity of ALP was determined in cells treated with 0.05 μM isopsoralen and compared to that in insulin-treated cells. In our study, ALP activity was increased 1.5-fold in cells treated with 0.05 μM isopsoralen compared to control cells, while ALP activity also significantly increased 3.7-fold following treatment with 10 μg/ml insulin compared to control cells after 14 days (Fig. 4A). To determine whether isopso-
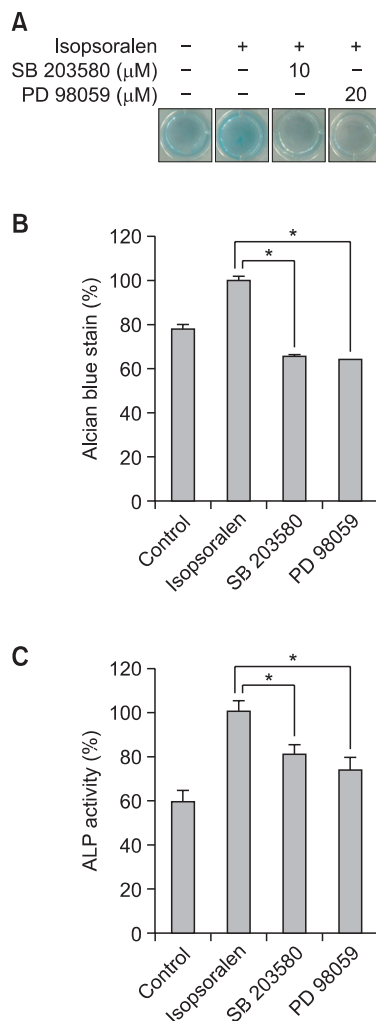
A

Isopsoralen	−	+	+	+
SB 203580 (μM)	−	−	10	−
PD 98059 (μM)	−	−	−	20

B

C

Fig. 6. Effect of signaling inhibitors on the synthesis of matrix proteoglycans and ALP activity in ATDC5 cells treated with Isopsoralen. Cells were seeded at a density of ($5×10^4$) cells per well and treated with Isopsoralen for 14 days in the presence or absence of 20 μM PD 98059 and 10 μM SB203580 (A). The cells were stained by Alcian blue as described in the legend to Fig. 2B and then photographed (×100). Stained cells were quantitated as described in the legend to Fig. 2C. The lower histogram indicates the levels of Alcian blue staining compared to the control group (B). Cell extracts were assayed for ALP activity as described in the "Materials and methods" (C). The asterisk (*) indicates a significant difference ($p<0.05$) compared to the control. Each histogram represents the mean ± S.E.M. (n=3).

ralen activates chondrogenesis in ATDC5 cells via BMP-2 signaling pathways, we exposed ATDC5 cells to isopsoralen in a time-dependent manner. Interestingly, isopsoralen efficiently activated BMP-2 expression in ATDC5 cells. As shown in Fig. 4B, treatment with 0.05 μM isopsoralen significantly increased the expression of BMP-2 after 24 h, while insulin did not alter expression of BMP-2. Based on these results, isopsoralen activated chondrogenesis through BMP-2 signaling pathways.

Effects of isopsoralen on MAP kinase activation in ATDC5 cells

Therefore, we investigated whether isopsoralen modulates

the activation of MAP kinases such as ERK, JNK and p38. The effect of isosporalen on the phosphorylation of ERK, JNK and p38 in ATDC5 cells were determined treated at various time points by western blot analysis. After treatment for 1.5 h with 0.05 μM isopsoralen, the phosphorylation of ERK and p38 was inctreased, while insulin, the positive control, induced the phosphorylation of ERK only as shown in Fig. 5. Together, these results suggest that isopsoralen can induce the differentiation of ATDC5 cells via ERK and p38 signaling pathways.

Effect of MAP kinase inhibitors on chondrogenesis with isopsoralen in ATDC5 cells

To confirm the induction of ERK and p38 in ATDC5 cells treated with isopsoralen and their effect on differentiation, we pretreated cells with 20 μM PD 98059 (MAPK/ERK kinase inhibitor) and 10 μM SB 203580 (p38 inhibitor) for 30min, followed by treatment with isopsoralen for 14 days. As shown in Fig. 6A, only ATDC5 cells treated with isopsoralen alone increased the size and number of nodules stained by Alcian blue dye, whereas co-treatment with SB 203580 and PD 98059 decreased staining. Subsequently, matrix proteoglycan dye was dissolved and quantified. As shown in Fig. 6B, the production of matrix proteoglycans decreased by 65.9 ± 0.5% and 64.7 ± 0.23% by the pre-treatment with 10 μM SB203580 and 20 μM PD98059, respectively. We also observed the effects of 20 μM PD98059 and 10 μM SB203580 on ALP activity. As shown in Fig. 6C, ALP activity was decreased by 80.8 ± 4.2% and 73.9 ± 5.9% in isopsoralen treated cells cultured with 10 μM SB 203580 and 20 μM PD98059, respectively. Therefore, isopsoralen affects the synthesis of matrix proteoglycans and induces ALP activity to mediate ATDC5 cells differentiation via ERK and p38 signaling pathways.

DISCUSSION

Numerous active components extracted from *P. corylifolia* L. have been widely studied in a variety of contexts, including the stimulation of osteoblast proliferation and differentiation (Wang *et al.*, 2001; Tang *et al.*, 2011), protection against bone loss (Lim *et al.*, 2009), and inhibition of osteoclast differentiation (Park *et al.*, 2008). However, studies evaluating the active ingredients that induce these effects in cartilage are limited. In the present study, we investigated the ability of isopsoralen, an active substance extracted from *P. corylifolia* L., to induce the differentiation of pre-chondrogenic ATDC5 cells.

The ATDC5 cell line is a well established model system for investigating the molecular mechanisms underlying the regulation of growth plate maturation and endochondral bone formation. Cells in condensation areas differentiate into chondrocytes to proliferate and produce large numbers of cartilage-characteristic extracellular matrix molecules such as type II collagen (Wahl *et al.*, 2005). Insulin causes the formation of cartilage nodules through the chondrogenic differentiation of ATDC5 cells (Shukunami *et al.*, 1996; Shukunami *et al.*, 1997). Induction of ALP activity in chondrocytes is associated with differentiation, accumulation of matrix vesicles in the ECM, and mineralization (Shukunami *et al.*, 1998). Therefore, we investigated the ability of isopsoralen to induce differentiation of ATDC5 cells compared with that of insulin. ATDC5 cells treated with isopsoralen exhibited significantly increased numbers of cartilaginous nodules compared to control cells. Alcian

blue staining revealed that cells treated with isopsoralen for 14 days exhibited increased synthesis of matrix proteoglycans (Hinoi *et al.*, 2007). Isopsoralen also induced the expressions of chondrogenic marker genes such as type I collagen, type II collagen, type X collagen, RNX 2, bone sialoprotein, and osteocalcin. Moreover, isopsoralen treated cells exhibited increased ALP activity.

Numerous signaling pathways contribute to chondrogenetic signaling, including MAPK and BMP. It was previously reported that the p38 MAPK pathway mediates transduction of signals that promote chondrocyte differentiation in the ATDC5 chondrogenic cell line (Nakamura *et al.*, 1999). Similarly, the MAPK/ERK activating kinase MEK-ERK pathway plays a role in hypertrophic differentiation in chondrocytes (Stanton *et al.*, 2003). In addition, JNK is known to control cell proliferation, differentiation, and apoptosis (Davis, 2000). ATDC5 cells treated with isopsoralen caused functional changes in ERK and p38 MAP kinase activity, but not that of JNK kinase, suggesting that isopsoralen can induce differentiation of ATDC5 cells via ERK and p38 signaling pathways. In addition, BMP-2 has been shown to play significant roles in chondrocyte differentiation and matrix maturation (Gañan *et al.*, 1996; Gründer *et al.*, 2004). Indeed, the upregulation of BMP-2 causes cells to skip cellular condensation stages in early-phase chondrogenic differentiation and also markedly up-regulates the expression of type X collagen mRNA in late-phase differentiation (Akiyam *et al.*, 2000). Therefore, we hypothesized that chondrogenic differentiation by isopsoralen may be mediated BMP-2 signaling pathways. Consistent with this hypothesis, isopsoralen significantly increased the expression of BMP-2 in ATDC5 cells, suggesting that isopsoralen may also activate chondrogenesis through BMP-2 signaling pathways in addition to ERK and p38 signaling pathways.

Sox9 plays a crucial role in mesenchymal condensation and chondrogenesis during musculoskeletal development (Furumatsu *et al.*, 2010). Furthermore, chondrogenic signaling molecules such as Smad4, Sox9, and β-catenin in ATDC5 cells are highly expressed by the treatment with isopsoralen. Similarly, Runx2 plays multiple roles in the regulation of chondrocyte differentiation, maintenance of the chondrocyte phenotype, and induction of chondrocyte maturation (Enomoto *et al.*, 2004), while type II collagen plays a vital role during the early-stage chondrogenesis (Rockel *et al.*, 2009). Consistent with previous reports, in the present study isopsoralen treatment increased the expressions of type II collagen genes as well as type X collagen mRNA, the latter being a marker of late-phase differentiation (Akiyam *et al.*, 2000). Lastly, isopsoralen increased the expression levels of a number of chondrocyte-specific genes after 14 days, but were decreased at 21 days. This expression pattern was similar to that of insulin, which was used as a positive control. Taken together, these results suggest that isopsoralen upregulates both early and late-stage chondrogenic differentiation and has similar effects as insulin with respect to stimulation of chondrogenesis in ATDC5 cells.

In conclusion, our results demonstrate that isopsoralen induces differentiation of ATDC5 cells through ERK, p38, and BMP-2 signaling pathways. Future evaluations of the effects of isopsoralen *in vivo* are needed to determine its potential use as a therapeutic agent for the treatment of growth disorders.

REFERENCES

Akiyama, H., Shukunami, C., Nakamura, T. and Hiraki, Y. (2000) Differential expressions of BMP family genes during chondrogenic differentiation of mouse ATDC5 cells. *Cell Struct. Funct.* **25**, 195-204.

Brighton, C. T. and Hunt, R. M. (1986) Histochemical localization of calcium in the fracture callus with potassium pyroantimonate. Possible role of chondrocyte mitochondrial calcium in callus calcification. *J. Bone Joint Surg. Am.* **68**, 703-715.

Brighton, C. T., Sugioka, Y. and Hunt, R. M. (1973) Cytoplasmic structures of epiphyseal plate chondrocytes. Quantitative evaluation using electron micrographs of rat costochondral junctions with special reference to the fate of hypertrophic cells. *J. Bone Joint Surg. Am.* **55**, 771-784.

Choi, H. J., Nepal, M., Park, Y. R., Lee, H. K., Oh, S. R. and Soh, Y. (2011) Stimulation of chondrogenesis in ATDC5 chondroprogenitor cells and hypertrophy in mouse by Genkwadaphnin. *Eur. J. Pharmacol.* **655**, 9-15.

Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239-252.

Enomoto, H., Furuichi, T., Zanma, A., Yamana, K., Yoshida, C., Sumitani, S., Yamamoto, H., Enomoto-Iwamoto, M., Iwamoto, M. and Komori, T. (2004) Runx2 deficiency in chondrocytes causes adipogenic changes *in vitro*. *J. Cell Sci.* **117**, 417-425.

Furumatsu, T., Shukunami, C., Amemiya-Kudo, M., Shimano, H. and Ozaki, T. (2010) Scleraxis and E47 cooperatively regulate the Sox9-dependent transcription. *Int. J. Biochem. Cell Biol.* **42**, 148-156.

Gañan, Y., Macias, D., Duterque-Coquillaud, M., Ros, M. A. and Hurle, J. M. (1996) Role of TGF beta s and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development* **122**, 2349-2357.

Gründer, T., Gaissmaier, C., Fritz, J., Stoop, R., Hortschansky, P., Mollenhauer, J. and Aicher, W. K. (2004) Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. *Osteoarthritis Cartilage* **12**, 559-567.

Guo, J., Wu, H., Weng, X., Yan, J. and Bi, K. (2003) Studies on extraction and isolation of active constituents from Psoralen corylifolia L. and the antitumor effect of the constituents *in vitro*. *Zhong Yao Cai* **26**, 185-187.

Hinoi, E., Takarada, T., Fujimori, S., Wang, L., Iemata, M., Uno, K. and Yoneda, Y. (2007) Nuclear factor E2 p45-related factor 2 negatively regulates chondrogenesis. *Bone* **40**, 337-344.

Kong, L. D., Tan, R. X., Woo, A. Y. and Cheng, C. H. (2001) Inhibition of rat brain monoamine oxidase activities by psoralen and isopsoralen: implications for the treatment of affective disorders. *Pharmacol. Toxicol.* **88**, 75-80.

Kronenberg, H. M. (2003) Developmental regulation of the growth plate. *Nature* **423**, 332-336.

Lim, S. H., Ha, T. Y., Kim, S. R., Ahn, J., Park, H. J. and Kim, S. (2009) Ethanol extract of Psoralea corylifolia L. and its main constituent, bakuchiol, reduce bone loss in ovariectomised Sprague-Dawley rats. *Br. J. Nutr.* **101**, 1031-1039.

Nakajima, S., Naruto, T., Miyamae, T., Imagawa, T., Mori, M., Nishimaki, S. and Yokota, S. (2009) Interleukin-6 inhibits early differentiation of ATDC5 chondrogenic progenitor cells. *Cytokine* **47**, 91-97.

Nakamura, K., Shirai, T., Morishita, S., Uchida, S., Saeki-Miura, K. and Makishima, F. (1999) p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. *Exp. Cell Res.* **250**, 351-363.

Netter Frank, H. (1988) Musculoskeletal system: anatomy, physiology, and metabolic disorders. W.B. Saunders Co, Philadelphia.

Pae, H. O., Cho, H., Oh, G. S., Kim, N. Y., Song, E. K., Kim, Y. C., Yun, Y. G., Kang, C. L., Kim, J. D., Kim, J. M. and Chung, H. T. (2001) Bakuchiol from Psoralea corylifolia inhibits the expression of inducible nitric oxide synthase gene via the inactivation of nuclear transcription factor-kappaB in RAW 264.7 macrophages. *Int. Immunopharmacol.* **1**, 1849-1855.

Park, C. K., Lee, Y., Chang, E. J., Lee, M. H., Yoon, J. H., Ryu, J. H. and Kim, H. H. (2008) Bavachalcone inhibits osteoclast differentiation through suppression of NFATc1 induction by RANKL. *Biochem. Pharmacol.* **75**, 2175-2182.

Rockel, J. S., Bernier, S. M. and Leask, A. (2009) Egr-1 inhibits the expression of extracellular matrix genes in chondrocytes by TN-Falpha-induced MEK/ERK signalling. *Arthritis Res. Ther.* **11**, R8.

Shukunami, C., Ishizeki, K., Atsumi, T., Ohta, Y., Suzuki, F. and Hiraki, Y. (1997) Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 *in vitro*. *J. Bone Miner. Res.* **12**, 1174-1188.

Shukunami, C., Ohta, Y., Sakuda, M. and Hiraki, Y. (1998) Sequential progression of the differentiation program by bone morphogenetic protein-2 in chondrogenic cell line ATDC5. *Exp. Cell Res.* **241**, 1-11.

Shukunami, C., Shigeno, C., Atsumi, T., Ishizeki, K., Suzuki, F. and Hiraki, Y. (1996) Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 *in vitro*: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. *J. Cell Biol.* **133**, 457-468.

Stanton, L. A., Underhill, T. M. and Beier, F. (2003) MAP kinases in chondrocyte differentiation. *Dev. Biol.* **263**, 165-175.

Sultan, T. and Ali, S. A. (2011) Psoralea corylifolia extracts stimulate cholinergic-like psoralen receptors of tadpole-tail melanophores, leading to skin darkening. *J. Recept. Signal. Transduct. Res.* **31**, 39-44.

Sun, N. J., Woo, S. H., Cassady, J. M. and Snapka, R. M. (1998) DNA polymerase and topoisomerase II inhibitors from Psoralea corylifolia. *J. Nat. Prod.* **61**, 362-366.

Tang, D. Z., Yang, F., Yang, Z., Huang, J., Shi, Q., Chen, D. and Wang, Y. J. (2011) Psoralen stimulates osteoblast differentiation through activation of BMP signaling. *Biochem. Biophys. Res. Commun.* **405**, 256-261.

Wahl, M. B., Heinzmann, U. and Imai, K. (2005) LongSAGE analysis revealed the presence of a large number of novel antisense genes in the mouse genome. *Bioinformatics* **21**, 1389-1392.

Wang, D., Li, F. and Jiang, Z. (2001) Osteoblastic proliferation stimulating activity of Psoralea corylifolia extracts and two of its flavonoids. *Planta Med.* **67**, 748-749.

Zhang, C. Z., Wang, S. X., Zhang, Y., Chen, J. P. and Liang, X. M. (2005) *In vitro* estrogenic activities of Chinese medicinal plants traditionally used for the management of menopausal symptoms. *J. Ethnopharmacol.* **98**, 295-300.