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Inhibitory effect of *YQHYRJ recipe* on osteoblast differentiation induced by BMP-2 in fibroblasts from posterior longitudinal ligament of mice

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Received January 28, 2011, accepted April 8, 2011

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Pharmazie 66: 784–790 (2011) *doi: 10.1691/ph.2011.1515*

Ossification of posterior longitudinal ligament (OPLL) is a common disease in Asian countries. Osteoblast differentiation in posterior longitudinal ligamentous fibroblast is a pathologic basis of OPLL. Nowadays, an effective pharmacotherapy for OPLL is still hunted for. *YQHYRJ Recipe* (YQHYRJ) is designed based on traditional Chinese medicine (TCM) theories, and previous clinic trials reported its effect on relieving syndromes of cervical spondylopathy. To clarify the YQHYRJ effect of OPLL on a cellular level, we induced mice fibroblasts from posterior longitudinal ligaments to differentiate into osteoblasts by human recombinant BMP-2, and treated them with YQHYRJ and its three sub-compounds: YQ, HY and RJ. YQHYRJ and the sub-compounds reduced the increase of fibroblast proliferation, mineralization, type I collagen secretion induced by BMP-2 via MTT, alizarin red staining and immunochemical examination. Moreover, these agents inhibited BMP-2 induced upregulation of ossification-related genes ALP, Col I and OC as well as BMP signal molecules Smad1, Smad 5 and Runx2 mRNA expression. These results suggested YQHYRJ to be effective in inhibiting osteoblast differentiation induced by BMP-2 in fibroblasts from posterior longitudinal ligament. YQHYRJ might be a promising medicine for preventing OPLL disease.

1. Introduction

Ossification of posterior longitudinal ligament (OPLL) refers to progressive heterotopic bone formation, which inserts into vertebral lumens, intervertebral foramen, and extrudes dural sac, causing severe spinal injury and nerve root irritation. It is a common disease in Asian countries with an incidence of 2-4%, and is reported to be associated with cervical spondylosis (Tsukimoto 1960; Epstein 1996; Ogata and Kawaguchi 2004).

In practice, anticonvulsants, antidepressants, opioids, topical agents, local anesthetics, and anti-inflammatory drugs such as neurotropin, mecobalamin are primarily used to relieve neuropathic pain in OPLL patients (Jackson 2006; Murakawa 2000; Hata et al. 1988; Sun et al. 2005; Furukawa 2008). On another hand, for treatment of retrogression of OPLL after surgery, bisphosphonates are chosen as inhibitors of mineralization. However, this agents may result in osteonecrosis and oral complications as potential adverse effects (Yamamoto et al. 2004). Thus, safer and more effective drugs are required for the therapy of OPLL.

YQHYRJ Recipe (YQHYRJ) was designed for pharmacotherapy of OPLL based on traditional Chinese medicine (TCM) theories (Wang et al. 2003). Our previous clinic trials revealed that YQHYRJ could relieve symptoms of cervical spondylopathy in 64 cases (Mo et al. 2003). It consists of seven components involving three hands of effects: 1. *YQ sub-Recipe* (YQ) includes *Radix Astragali* (in Chinese named Huangqi) and *Codonopsis pilosula* (in Chinese named Dangshen). 2. *HY sub-Recipe* (HY) is composed of *Rhizoma Chanxiong* (Chuanxiong), *Salvia miltorrhiza* (Danshen), and *Moschus artificialis* (Shexiang). 3. *RJ sub-Recipe* (RJ) uses *Seaweed* (Haizao) and *Kelp* (Kunbu). Clinical trials revealed that YPHYRJ may be effective in both pain relief and reduction of heterotopic ossification.

Several studies demonstrated that bone morphogenetic proteins (BMPs) play an important role in ossification of spinal ligaments (Ducy et al. 1999; Sato et al. 1998). For example, BMPs were highly expressed at the calcified ligamentum flavum, moreover, implanting BMP-2 in the lumbar extradural space would cause ligamentum flavum hypertrophied and ossified (Miyamoto et al. 1992; Hayashi et al. 1997), indicating that fibroblasts are target cells of BMP-2, which might induce ligamentous fibroblasts to differentiate into osteoblasts or chondrocytes involved in ossification of spinal ligaments (Hoshi et al. 1997). Interestingly, OPLL derived cells are easy to show bone characteristics stimulated by BMP-2, whereas no change was observed in non-OPLL cells (Koyanagi et al. 2003). However, further experiments gave the evidence that the normal spinal ligament cells could also respond to BMP-2 by an increase of ALP and osteocalcin (OC) (Kon et al. 1997; Moon et al. 2004). So, fibroblasts with BMP-2 stimuli were chosen for observing the medicinal effect on the cell level.

In this study, we isolated fibroblasts from posterior longitudinal ligaments in mice, and added human recombinant BMP-2 into the culture medium to induce ossification. The effect of

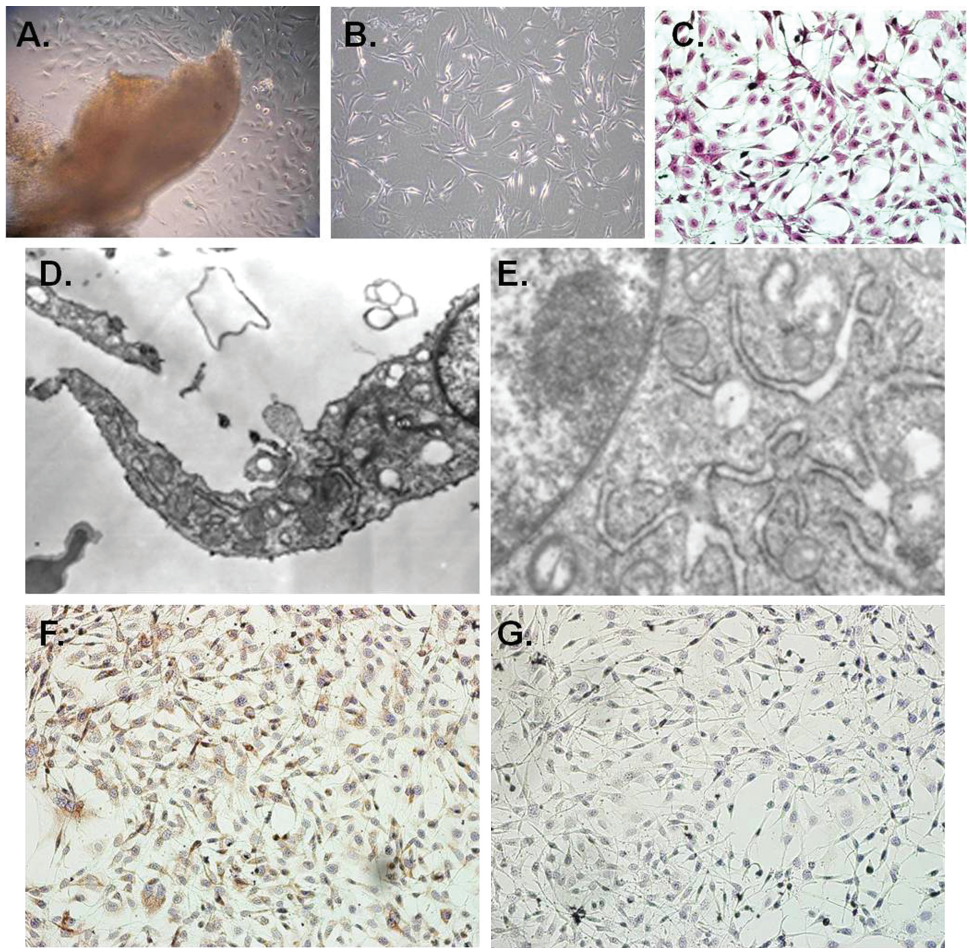

Fig. 1: Identification of mice fibroblast. Cells from mice cervical posterior longitudinal ligament tissue ex vivo (A). P3 fibroblasts general appearance (B) and morphological examination by H & E staining (C). Ultra-structure of P3 fibroblasts under electron microscope (D, E). Immunochemical staining for vimentin (F) and keratin (G)

YQHYRJ and its three sub-compounds: YQ, HY and RJ on inhibiting fibroblast ossification was evaluated using alizarin red staining, immunochemistry for Col I, real time RT-PCR examination of ALP, Col1, OC, Runx2, Smad1, Smad5 mRNAs level, and cell proliferation by MTT method. Some molecular characteristics of the herbal drugs were determined by HPLC.

2. Investigations and results

2.1. Identification of mice fibroblast by morphology examination and immunocytochemical detections for vimentin and keratin

Cells from posterior longitudinal ligament tissue in mice *ex vivo* showed cube, polygon and spindle sharps, indicating non-fibroblast contamination (Fig. 1A). P3 cells seemed to be uniform in spindle sharp (Fig. 1B, C) with abundant rough endoplasmic reticulum and golgi apparatus (Fig. 1D), oval mitochondria, plenty of chromatin, heterochromatin closing to karyolemma, in which nucleolus existed (Fig. 1E). Immunochemical findings demonstrated positive staining for vimentin, a key marker of fibroblast (Schwab et al. 2001) (Fig. 1F) while negtive staining for a marker of epithelial, keratin (Fig. 1G).

2.2. The effect of YQHYRJ and subcompounds on fibroblast proliferation by MTT assay

BMP-2 promoted fibroblast proliferation at four time points compared with control. All the rat phamoco-serum combined could reduce the increase of fibroblast growth induced by BMP-2, in which, YQHYRJ and RJ serum showed relatively significant effect (Fig. 2).

2.3. Effect of YQHYRJ and subcompounds on reduction of fibroblast ossification by alizarin red staining and immunochemical examination for type I collagen

More calcified tubercles (red staining) could be seen in BMP-2 induced cells (Fig. 3A, B). PS-serum had no significant changes in quantity of calcified tubercles (Fig. 3C). Both YQHYRJ and three subcompounds decreased calcified tubercles, especially YQHYRJ (Fig. 3D-F).

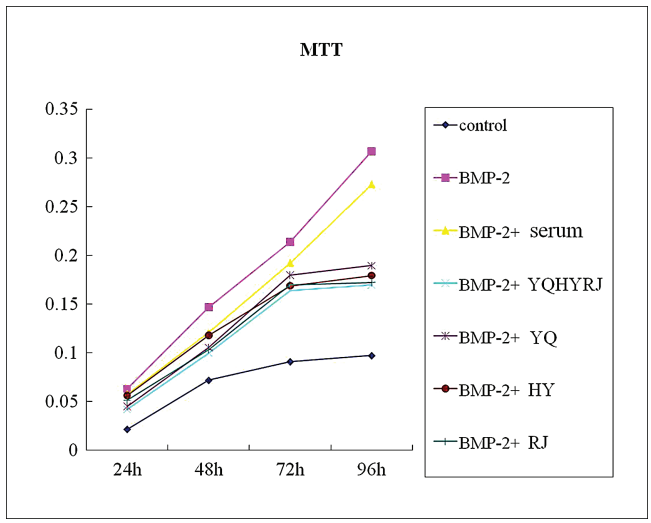

Fig. 2: MTT assay for cell proliferation. BMP-2 promoted fibroblast proliferation at 24 h, 48 h, 72 h, 96 h. All the rat phamoco-serum and PS-serum reduced the proliferation increased by BMP-2

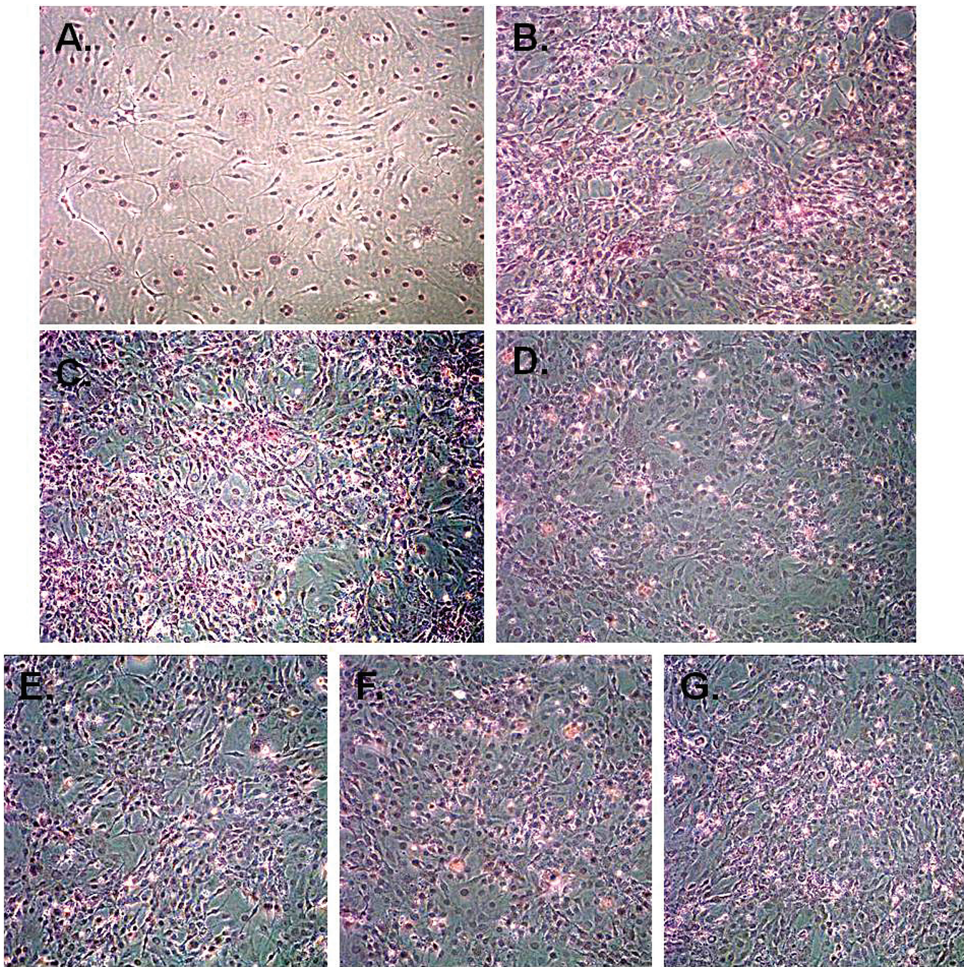

Fig. 3: Alizarin red staining for mineralization (red staining). Control group (A) BMP-2 group (B) BMP-2 + PS-serum (C) BMP-2 + YQHYRJ-serum group (D) BMP-2 + YQ-serum (E) BMP-2 + HY-serum (F) BMP-2 + RJ-serum (G)

The immunochemical examination for type I collagen showed similar results. That was, BMP-2 and BMP-2 + PS induced a significant increase of Col I protein secretion (Fig. 4A-C). YQHYRJ and subcompounds decreased Col I protein level compared with BMP-2 group. Least positive staining appeared in YQHYRJ group except the control (Fig. 4D-F).

2.4. YQHYRJ and subcompounds inhibited BMP-2 induced up-regulation of ossification-related genes ALP, Col I and OC mRNA expression

Real time RT-PCR showed BMP-2 induced upregulation of ossification-related genes ALP, Col I and OC mRNA expression ($p < 0.01$). YQHYRJ and subcompounds inhibited mRNA level of these three genes upregulated by BMP-2. BMP-2 + YQHYRJ seemed to be more closing to the expression level in the control (Fig. 5).

2.5. YQHYRJ and subcompounds inhibited BMP-2 induced up-regulation of BMP signal molecules Smad1, Smad 5 and Runx2 mRNA expression

We also examined the expression of BMP signal molecules Smad1, Smad 5 and Runx2, and found BMP-2 increased these three genes expression while YQHURJ, YQ, HY and RJ compounds in serum could decrease their mRNA level compared with BMP-2 group. Moreover, the gene expression level in YQHYRJ group was as low as that in the control group (Fig. 6).

2.6. Six effective molecules contained in four compounds

The main components of YQHURJ were polysaccharides (1.91 g), salvianolic acid B (174.88 mg), tanshinone IIA (281 μg), ferulic (2.9690 mg), ligustrazine hydrochloride (2.48 mg). YQ was mainly composed of polysaccharides (1.37 g), and astragaloside (2.32 g). HY contained polysaccharides (0.15 g), salvianolic acid B (130.35 mg), tanshinone IIA (319 μg), ferulic acid (3.0379 mg), ligustrazine hydrochloride (1.60 mg). Only polysaccharides (0.91 g) could be detected in RJ.

3. Discussion

Cell proliferation is one of the mainly pathological characteristics of OPLL (Ducy et al. 2000). It has been reported that cells in the posterior longitudinal ligament accelerated to proliferate under the culture with 250 ng/ml BMP-2 for 3 days (Song et al. 2006). Our study showed the according result. However, proliferation activity could be partly weakened by YQHYRJ and subcompounds.

Type I collagen (Col I) is reported to be expressed both in the committed preosteoblast and the mature osteoblast (Robling et al. 2006). Alkaline phosphatase (ALP) is a key enzyme in the mineralization process. Its activity is an early indicator of bone formation. Osteocalcin (OC), one of bone extracellular matrix proteins, is considered as a candidate of mature osteoblasts (Lian et al. 2001). These three molecules play a key role in physiological and pathological ossification. Our study found that BMP-2 facilitated the expression of Col I protein and Col I, ALP, OC

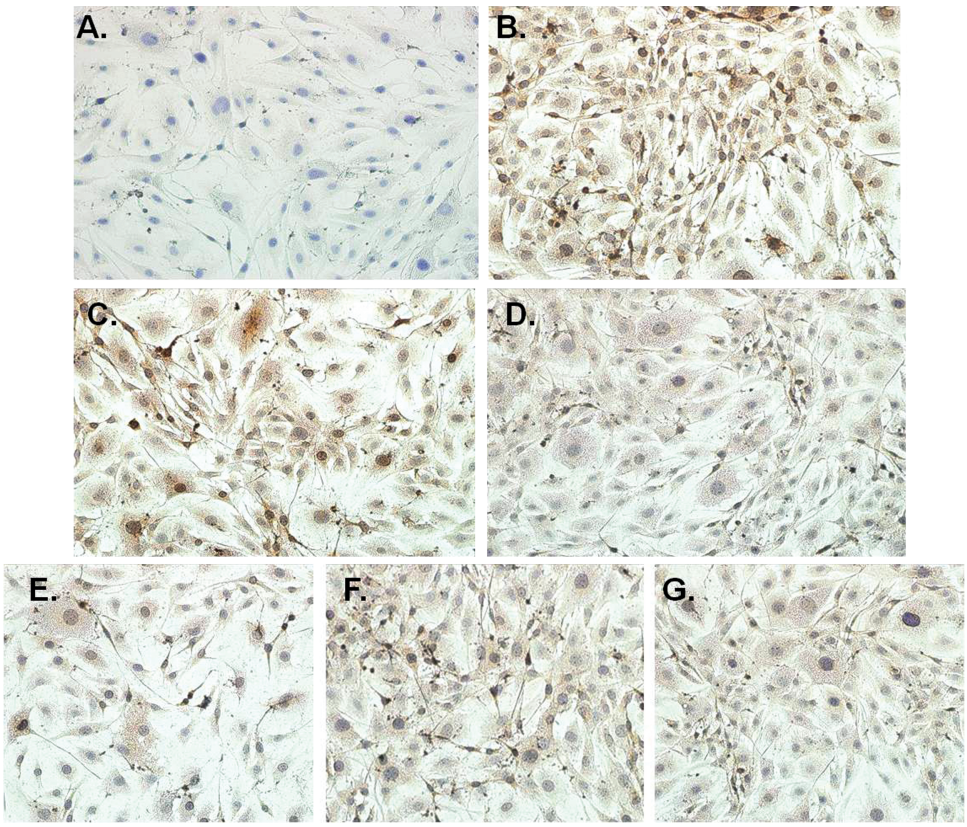

Fig. 4: Immunochemical examination for type I collagen. Control group (A), BMP-2 group (B), BMP-2 + PS-serum (C), BMP-2 + YQHYRJ-serum group (D), BMP-2 + YQ-serum (E), BMP-2 + HY-serum (F), BMP-2 + RJ-serum (G)

mRNA in mice fibroblasts, indicating the potentiality to differentiate into osteoblasts. YQHYRJ and its three subcompounds effectively reduced the up-regulation trend, demonstrating the inhibitive effect of YQHYRJ and subcompounds on ossification caused by BMP-2.

The transcription factors runt-related transcription factor-2(Runx2) is considered a "master gene" that plays a critical role in ossification (Komori et al. 1997). In detail, expression of Runx2 is required to push the proliferating precursor cells toward the osteoblast lineage and away from other lineages. Further differentiation of the preosteoblast into a mature, bone forming osteoblast phenotype also requires the expression of Runx2 (Ducy et al. 1997). So, this transcription factor plays a role in two phases of osteoblast differentiation. Moreover, its downstream includes several bone formation related markers such as ALP, Col I and OC (Thies et al. 1992). It was found that BMP-2 enabled Runx2 transcription in C2C12 cells, and upregulated Runx2 mRNA in the differentiation process of non-hematopoietic stem cells to osteoblasts in human bone marrow (Zhao et al. 2002; Gori et al. 1999). Runx2 regulated by BMP-2 also requires for interaction of Smad1 and Smad5, which act downstream of intracellular signaling of BMP-2 that induces osteoblast differentiation (Yamamoto et al. 1997). Therefore, we further investigated Runx2, Smad1 and Smad5 gene expression in BMP-2 induced mice fibroblasts. Our results revealed that BMP-2 elevated these three genes level while YQHYRJ and subcompounds significantly inhibited this effect.

In the present study, YQHURJ showed a more significant inhibitive effect than three simple subcompounds: YQ, HU and RJ, on osteoblast differentiation in fibroblast induced by BMP-2. It prompted the importance of a synergistic effect of three subcompounds in YQHURJ. On another hand, we also found rat serum itself with drug free partly reduced gene expression, which would be due to some molecules such as growth factors in the serum.

In conclusion, YQHYRJ inhibits osteoblast differentiation induced by BMP-2 in fibroblasts from posterior longitudinal ligament of mice. Its protective effects are associated with interfering in cell proliferation, downregulating ossification related markers ColI, ALP and OC expressions and BMPs signaling related molecules Smad1, 5 and Runx2 levels. YQHYRJ may be a promising medicine for preventing OPLL disease in the future.

4. Experimental

4.1. Preparation of drug

YQHYRJ, YQ, HY, RJ were purchased from Longhua Hospital, Shanghai University of TCM (Shanghai, China). We followed the Guiding Principles for the Care and Use of Laboratory Animals Approved by Animal Regulation of National Science and Technology Committee. Serum obtaining drugs were prepared as followed: 1. Twenty-five 3-month SD rats were randomly divided into five groups: physiological saline (PS), YQHYRJ, YQ, HY and RJ. 2. Every rat was intragastric administrated at 8:00 and 16:00 every day for 3 days (1.5 ml/100 g). Blood was collected 1 h after the last drug administration from the abdominal aorta. 3. Blood samples were centrifuged, inactivated (56 °C, 30 min), filtered and stored at −20 °C.

4.2. HPLC

The four compounds were heated three times with a 20-fold volume of water at 100 °C, 20 min per time, and the supernatant was concentrated as A solutions. The A solutions were added with a 4-fold volume of alcohol, and ultrasonically extracted for 20 min, centrifuged (8000 rev/min, 5 min) and the filtrate was discarded.

To the residue a 20-fold amount of water was added, ultrasonically extracted for 20 min, and centrifuged (8000 rev/min, 5 min). The supernatant was added 3-fold of alcohol overnight and centrifuged (8000 rev/min, 5 min). The residue was dissolved with water to 100 mL, then stored at 4 °C as B solutions. B stock solutions 100 μl were filled up with water to 2 ml, then added 5% phenol 1 ml, slowly added sulfuric acid 5 ml and placed in boiling water for 15 min. The mixture was cooled to room temperature and detected by an UV spectrophotometer (330 nm, 480 nm wavelength). Six standard samples were determined in these four compounds, including polysaccharides,

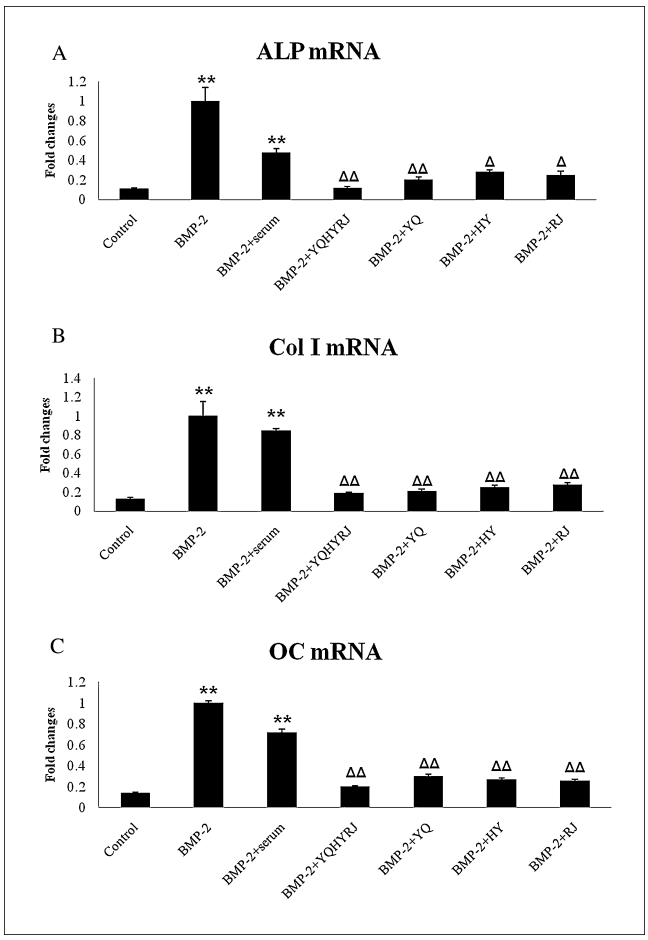

Fig. 5: The mRNA expression of ALP, Col I and OC. The columns represent the mean ± SE of three independent experiments. ** $p < 0.01$ vs the control group. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ vs the BMP-2 group

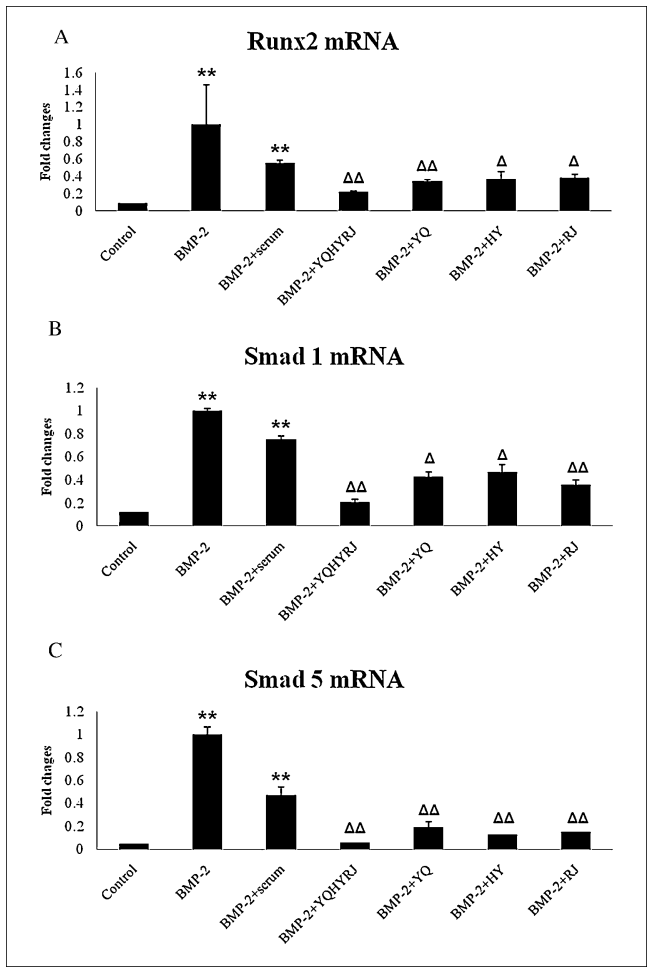

Fig. 6: The mRNA expression of Runx2, Smad1 and Smad5. The columns represent the mean ± SE of three independent experiments. ** $p < 0.01$ vs the control group. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ vs the BMP-2 group

ferulic acid, tanshinone IIA, salvianolic acid B, astragaloside, ligustrazine hydrochloride.

4.3. Cell isolation

Ten 12-week-old C57 mice, provided by Shanghai laboratory animal center (SCXK2007–0005, Science and Technology Commission of Shanghai Municipality, Shanghai Animal Ethics Committee gave approval for the experimental research on animals), were killed by 10% chloral hydrate via intraperitoneal injection. Cervical posterior longitudinal ligaments were obtained aseptically and washed with PBS for three times. A conjunction of ligament and bone was discarded. Small pieces (1 mm^3) were dissected along the tissue for the explant culture (Li et al. 2008). Briefly, the tissue fragments were placed in 60 mm plates with 2 ml of DMEM added with 20% FBS, 0.2 mM ascorbic acid. The expanded cells were trypsinized and cultured in a 25 cm^2 flask. Cells at passages 3-5 were used for the experiment.

4.4. Cell identification

4.4.1. H & E staining

Cells (1×10^6/L) were placed on a sterile small glass sheet in 6-well plate. After a confluence of 80% cells, they were fixed with 40 g/L paraformaldehyde in 4 °C for 20 min, and stained by hematoxylin-eosin staining.

4.4.2. Transmission electron microscopy

Cells were scraped from the flask, and centrifuged at 4000 rpm/min for 10 min, then invested by agar prepared with 2.5% glutaral and phosphate buffer for 2 h. After common rinse, dehydration, drenching, solidifying, ultramicrocutting, double staining by 3% uranyl acetate and lead citrate, the cellular untra-structure was observed under a transmission electron microscope.

4.4.3. Immunochemistry for vimentin, keratin and Col I

After a confluence of 80% cells at passage 3, they were fixed with 40 g/L paraformaldehyde for 30 min, and rinsed by PBS for 3 min × 3. The glass sheet of non-cells surface was pasted onto the glass slide by neutral resin after the cells were dehydrated using 0.85, 0,95, 1.00 alcohol. Endogenous peroxidase activity was blocked by incubation of the sections with freshly prepared 3% H_2O_2 in methanol for 20 min. The slides were incubated with a blocking serum for 5 min, and then incubated overnight with rabbit polyclonal antibodies against vimentin, keratin, Col I (1:200) (Abcam Ltd., Cambridge, UK) at 4 °C in a humidified chamber. After thorough wash, the section was incubated with biotinylated goat anti-rabbit-IgG at 37 °C for 30 min and then with Streptavidin-HRP at room temperature for 10 min. The sections were developed with diaminobenzidine tetrahydrochloride (DAB) until a desirable stain intensity was obtained. Finally, sections were rinsed in tap water and counterstained with hematoxylin.

4.5. Induction by BMP-2

Cells of 1×10^6 density were placed in DMEM consisting of 10% FBS. After they are in a confluence, the medium changed without FBS. Twelve hours later, the medium was converted by DMEM supplemented with 250 ng/ml BMP-2, 1% FBS and 0.2 mM ascorbic acid. The cells were cultured in the induction medium for 3 days.

4.6. Intervention by YQHYRJ, YQ, HY, RJ on fibroblasts with BMP-2 stimuli

1×10^4 Cells cultured with serum-free medium for 12 h in 12-well plate after BMP-2 stimulation for 3 days. Then, 10% rat serum, respectively containing PS, YQHYRJ, YQ, HY and RJ was added into the medium. 48 h later, the samples were collected for detection.

Table: Sequences of primers used in the real-time PCR

Genes	Forward primer	Reverse primer
ALP	TGC CTA CTT GTG TGG CGT GAA	TCA CCC GAG TGG TAG TCA CAA TG
Beta-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG
Col I	TGC TGG ACG TCC TGG TGA AG	ACG TTG TCC AGC AAT ACC CTG AG
OC	TGCCCTCCTGGTTCATTTCT	TTCTGTTCCCTCCCTGCTGT
Runx2	GGT TAA TCT CTG CAG GTC ACT ACCA	ACG GTG TCA CTG CGC TG AA
Smad1	GGA ATG CTG TGA GTT CCC ATT TG	TGC TGA GGA TTG TAC TCG CTG TG
Smad5	CTG CCT CTG GAG TTC TGG GAT TAC	CGG ACA AAG ATG GGT TCA GA CA

4.7. Cell proliferation by MTT method

Cells in control group and BMP-2 induction group were cultured in 96-well plate at 2×10^4/ml, and each group had 6 wells for 200 µl each well. OD values were tested after cultured for 24 h, 48 h, 72 h and 96 h. The cellular growth curve was drawn according to the OD values.

4.8. Alizarin red staining

Cells were fixed by 95% alcohol for 10 min after rinsed up by PBS twice, and washed up using distilled water three times. The section was stained with 0.1% alizarin red-Tris-HCL (pH 8.3) at 4 °C for 30 min, then washed up by distilled water and examined under the microscope.

4.9. Real-time RT-PCR analysis

Cells were directly processed following RNAprep pure Cell Kit protocol. One microgram of total RNA was reverse transcribed using the Advantage RT-for-PCR kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Fresh transcribed cDNA (1 µl) was used for quantitative Real-time PCR using SYBR Green (Bio-Bad, Hercules, CA) to monitor DNA synthesis using specific primers (Table) designed by TaKaRa Biotechnology Co. Ltd. The PCR was carried out in RotorGene real-time DNA amplification system (Corbett Research, Sydney, Australia) using the following cycling protocol: a 95 °C denaturation step for 15 min followed by 45 cycles of 95 °C denaturation (20 s), 56–62 °C annealing (30 s), and 72 °C extension (30 s). Gene expression was normalized to the housekeeping gene β-actin. PCR products were subjected to a melting curve analysis, and the data were analyzed and quantified with the RotorGene 6.0 analysis software.

4.10. Statistical analysis

The Data is expressed as mean ± SE and statistical significance was calculated using One-Way ANOVA and analysis of variance by SPSS software (SPSS Inc, Chicago, USA). The significance level was defined as $p < 0.05$.

Acknowledgments: This work was supported by National Basic Research Program in China (973 Plan, 2010CB530400), the Key Grant of National Natural Science Foundation of China (30930111), the International Cooperation Programs of National Natural Science Foundation of China (30710103904), the Project of National Natural Science Foundation of China (30701118, 81001526, 30600829) and the Youth Health Research Project (2009Y092). Shanghai Science Technology Qimingxing Program of China (09QA1405600, 11QA1406600), the Theories and Therapies of Bones Province Key Laboratory of the Ministry of Education, Shanghai University, the Shanghai Education Commission Innovation Team Program Section 6 (2009) and the Ministry of Education senior personnel funding sources: the Professor Changjiang Scholar Program (Teaching People, 2009, 17) and the National Science Fund for Distinguished Young Scholars (30625043).

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