

Original Article

Dioscin stimulates differentiation of mesenchymal stem cells towards hypertrophic chondrocytes *in vitro* and endochondral ossification *in vivo*

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Abstract: Dioscin has been shown to play important roles in suppression of osteoclast maturation. It is proposed as a potential natural product for the treatment of osteoclast-related diseases. We hypothesized in this study that treatment of dioscin on bone marrow mesenchymal stem cells (BMSCs) could increase the osteo-chondrogenic differentiation of BMSCs and promote endochondral ossification of BMSCs in bone fracture environment. BMSCs were extracted from femur and tibia of male C57b mice. Stemness of BMSCs was studied by performing proliferation assay and multilineage differentiation. Glycosaminoglycans (GAG) and collagen contents were assessed to examine the chondrogenesis of BMSCs. Real time quantitative PCR was carried out to examine the expression of hypertrophic marker collagen type X. Efficacy of Dioscin was then tested in mouse bone fracture model on the distal side of femur. Results showed treatment of dioscin on BMSCs increased chondrogenic differentiation of BMSCs as well as the expression of collagen type X. Local delivery of dioscin promoted endochondral ossification at bone fractured site, as shown by histological examination. Results of immunohistochemistry showed that dioscin increased collagen type X expression in bone fracture model of mice. In conclusion, our results demonstrated that treatment of dioscin promote the hypertrophic differentiation of BMSCs derived chondrocytes. Dioscin could be a useful drug to promote bone regeneration after fracture.

Keywords: Dioscin, bone marrow mesenchymal stem cells, osteogenic differentiation, endochondral ossification

Introduction

Traditional Chinese medicine has been used in China and some other oriental countries for thousands of years. In the past few decades, its high efficiency and low toxicity have given us great hope of finding hidden therapeutic chemicals [1]. It has been reported that the differentiation, proliferation and migration of MSCs can be regulated by traditional Chinese medicine treatment [2]. Proliferation and chondrogenic differentiation were shown to be promoted by treating Bone Marrow-Derived Mesenchymal Stem Cells (BMSCs) with some Traditional Chinese Herbs known to be "Kidney-Tonifying" [3]. Osteogenic differentiation of BMSCs was enhanced by quercetin via mitogen-activated protein kinase signaling [4]. Some traditional decoction was reported to promote the expressions of angiogenic miRNA and VEGF in exo-

somes secreted by BMSCs and was believed to improve angiogenesis of brain tissue after stroke in rat models [5]. All these recent findings indicated that herbs used in traditional Chinese medicine was a good source of signaling molecules that could regulate proliferation, differentiation and apoptosis of BMSCs. More specifically, these chemicals may be very useful in muscle-skeletal regeneration.

Dioscin (diosgenyl 2,4-di-O- α -l-rhamnopyranosyl- β -D-glucopyranoside) is one of the most studied bioactive steroidal saponins, that is originally isolated from natural *Dioscorea* herbs, such as *D. nipponica* Makino and *D. zingiberensis* Wright [6, 7]. Recent studies had revealed that dioscin increased superoxide dismutase expression, reduced intra-cellular reactive oxygen species and malondialdehyde levels, decreased Bax expression, increased Bcl-2

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expression and protect cardiac cells from ischemia/reperfusion injury by regulating the mitochondrial apoptotic pathway through delay of oxidative stress [8]. It is also one of the best known complementary and alternative clinical medicines to treat coronary disease and angina pectoris in China [9]. Results from cell biology suggested that Dioscin possesses anti-inflammatory, lipid-lowering and anti-cancer activities [10, 11]. It's also reported that Dioscin has potent effects against alcohol-induced liver injury and nonalcoholic fatty liver disease [12]. More recent studies indicated that Dioscin suppressed RANKL-induced osteoclast differentiation via Akt signaling pathway [13]. However, the effect of this compound on the function of osteoblast and differentiation of stem cells towards osteogenic lineage has not been investigated so far.

In this study, we treated murine BMSCs with Dioscin to examine the effects of this chemical compound on the osteogenic differentiation of stem cells. A sub-chondral bone fracture and growth plate injury model was used to study the effects of Dioscin *in vivo*. Our data showed that dioscin could be a potential natural drug for the treatment of bone fractures.

Materials and methods

Cell culture and laboratory expansion

The use of experimental animals in this study has been approved by a local Medical Ethical Committee. BMSCs were isolated from bone marrow of femur from 8 week old C57 black mice according to procedures in previous publications [14, 15]. Three male mice were used for bone marrow extraction under sterile condition. BMSCs were then seeded in culture flasks with MSC proliferation medium (α -MEM supplemented with 10% fetal bovine serum, 1% L-glutamin, 0.2 mM ascorbic acid, 100 U/ml penicillin, 10 mg/ml streptomycin). Media were refreshed every 2 days to get rid of non-attached cells. When cells were confluent, trypsin was used to detached and sub-culture cells. The cells were expanded to passage 2 before experimentation. Bone marrow from three male animal were pooled for *in vitro* studies. All reagents used for cell culture were purchased from Gibco, Invitrogen (Carlsbad, CA), unless otherwise stated. Routinely used chemicals were purchased from Sigma-Aldrich (St. Luis, MI).

Growth curves of the BMSCs

The growth of BMSCs was examined by direct quantification of cell numbers using counting chambers. Initially, 100,000 BMSCs were plated in a well of 12-well plate. Cells were detached from plastic surface at day 3, day 6, day 9 and day 12. Cell numbers in each well were then counted. 3 wells per time point were counted.

Osteogenesis evaluation and alizarin red S staining

Osteogenic differentiation of BMSCs was induced in osteogenic medium (OS) containing DMEM supplemented with 10% FBS, 0.1 nM dexamethasone, 10 mM β -glycerophosphate (β -GP), 0.01 μ M 1,25-dihydroxy vitamin D₃, and 50 μ M ascorbic acid in α -MEM [16]. After 3 weeks induction, BMSCs were fixed with 10% formalin. Then, mineralized nodules were visualized by alizarin red S staining. Briefly, phosphate-buffered saline (PBS) was used to rinse the plate, before cells were incubated with 40 mM of alizarin red S (pH 4.2) for 10 min. Cells were rinsed 5 times with water followed by 15-min washing with PBS to reduce nonspecific staining of alizarin red S. The nodules were photographed by a phase contrast microscope (Olympus, Janpan).

Adipogenic differentiation and Oil red O staining

For adipogenic differentiation, BMSCs were seeded at density of 12,000 cells/cm² and cultured in adipogenic medium (α -MEM supplemented with 10% FBS, 10⁻⁶ M of dexamethasone, 10 μ M of Insulin, 0.5 mM of IBMX (isobutylmethylxanthine), 200 μ M of Indomethacin, 100 U penicillin/ml and 100 μ g/ml streptomycin) for 3 weeks. Fat droplets were shown by Oil Red O staining.

Chondrogenic differentiation and toluidine blue staining

For chondrogenic differentiation, 200,000 of BMSCs were seeded in one well of a 96-well plate with round bottom. Cell pellets were made by centrifuge at 500 g for 3 min. Then pellets were cultured in chondrogenic differentiation medium (DMEM supplemented with 40 μ g/mL of proline, 50 μ g/mL ITS-premix, 50 μ g/mL of Ascorbic Acid, 100 μ g/mL of Sodium Pyruvate, 10 ng/mL of Trans Growth Factor- β 3, 100 mM

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of dexamethasone, 100 U penicillin/ml and 100 µg/ml streptomycin) for 3 weeks. Pellets were applied to histological examination as described. To test the effects of Dioscin on chondrogenic differentiation, 1 µM of Dioscin (AbCam, Cambridge, MA) was added to the chondrogenic differentiation medium.

GAG and DNA assay

Cell aggregates were washed with PBS and digested in proteinase K solution for more than 16 hours at 56°C. GAG content was determined with 1,9-dimethylmethylene blue chloride (DMMB) staining by using an Multiskan GO Microplate Spectrophotometer (Waltham, MA) at a wavelength of 520 nm. Standard curve was generated by using series dilutions of chondroitin sulfate. Quantification of total DNA using a CyQuant DNA Kit (Molecular Probes, Eugene, OR) was performed for normalization purpose.

Collagen quantification

Total collagen content of cell aggregate was measured by using the hydroxyproline assay [17]. Briefly, cell aggregates were first dissociated in papain buffer (0.5 mg/ml of papain in 0.1 M Na₂HPO₄, 5 mM EDTA, 5 mM L-Cysteine HCl) for overnight. The digested solutes were hydrolyzed in 6N HCl at 110°C for overnight. Hydroxyproline was then measured spectrophotometrically at absorbance of 560 nm after reaction with 0.05 M of chloramine-T and 10% (w/v in 2-methoxyethanol) p-dimethylamino-benzaldehyde, using a Multiskan GO Microplate Spectrophotometer. A standard curve was generated with L-hydroxyproline for calculating the Hydroxyproline concentration.

RNA isolation and quantitative RT-PCR

Total RNA of cells were isolated with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The cDNA samples were amplified with a Pfu PCR kit (Tiangen, Beijing, China). Real time PCR was performed on cDNA samples by using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). PCR Reactions were performed on MyiQ2 Real-Time PCR machine (Bio-Rad, Hercules, CA) under the following conditions: reaction mixture was preheated for 15 min at 95°C for acti-

vation of Taq enzyme, denatured for 5 min at 95°C, followed by 45 cycles, consisting 15 s at 95°C, 15 s 60°C and 30 s at 72°C. For each reaction a melting curve was generated to test primer dimer formation and non-specific priming. The primers for real-time PCR are as follows: Col2a1 Forward: 5'-GGGTCACAGAGGT-TACCCAG-3', Reverse: 5'-ACCAGGGGAACCACT-CTCAC-3'; Col10a1 Forward: 5'-TTCTGCTGCTA-ATGTTCTTGACC-3', Reverse: 5'-GGGATGAAGTA-TTGTGTCTTGGG-3'; GAPDH Forward: 5'-AATG-GATTTGGACGCATTGGT-3', Reverse: 5'-TTTGCA-CTGGTACGTGTTGAT-3'. Calculation of Relative Expression was based on the double delta Ct method [18]. GAPDH was used for normalization.

Immunofluorescence and immunohistochemistry

Cell aggregates were washed briefly with PBS, fixed in 4% paraformaldehyde overnight. 10 µm cryo-sections of cell aggregates were cut with a cryotome (Cryotome™ FSE Cryostats, Thermo Scientific, Waltham, MA). Sections were permeabilized and blocked in a mixture of 1% Triton-X 100 and 1% bovine serum albumin (BSA) for 15 min at room temperature. Slices were incubated with rabbit polyclonal antibodies against collagen type II or collagen type X (AbCam, Cambridge, UK) at 4°C overnight. Sequentially, slides were washed and incubated with secondary antibodies conjugated to Alexa 488 (Invitrogen, Carlsbad, CA), and nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR). After rinsing with TBS, sections were examined and imaged with DMi 6000 B fluorescent microscope (Leica, Bensheim, Germany).

For immunohistochemistry, paraffin sections of distal femur were used. Briefly, sections were deparaffinized and rehydrated with routine procedure. After antigen retrieval, sections were incubated overnight at 4°C with rabbit polyclonal antibodies against collagen type II or collagen type X (AbCam, Cambridge, MA). Sections were incubated with secondary biotinylated antibodies and horseradish peroxidase-conjugated streptavidin to detect the primary antibodies. The peroxidase reaction was developed using 3,3-diaminobenzidine tetrahydrochloride as chromogens. After rinsing in distilled water, sections were dehydrated in ethanol series, cleared in xylene and mounted with cover slips for microscopic photograph.

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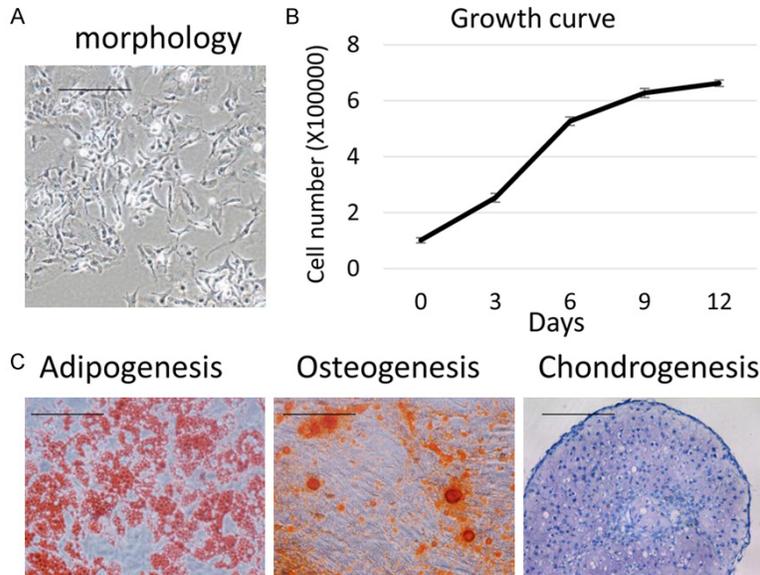


Figure 1. Growth curve and multilineage differentiation of bone marrow mesenchymal stem cells. A: Morphology of BMSCs at passage 2 in culture flasks was illustrated by phase contrast image. Bar = 100 μ m. B: Growth curve of BMSCs at passage 2. C: Adipogenic, osteogenic and chondrogenic differentiation of BMSCs were examined by Oil Red O, Alizarin Red S, and Toluidine Blue staining. Bar = 100 μ m.

Subchondral bone fracture on distal femur

Twelve mice were operated to create a drill-hole injury in subchondral bone on the distal end of femur following an established protocol [19]. Briefly, under isoflurane inhalation, a 5-mm longitudinal incision was made on the skin to expose the anterior-medial aspect of the distal femur and growth plate of the left hind limb. A cortical window in the metaphyseal bone on the medial side was firstly made with a Kirschner wire (2 mm in diameter). Then the Kirschner wire passed through the epiphyseal growth plate region perpendicularly via the cortical window. The articular surface of the femur was kept intact. After surgery, six mice were fed with normal water, while the other six were administrated with 40 mg/kg/day of Dioscin. On week 4 post-operation, mice were euthanized for examination. Distal left femur specimens were collected, containing the articular cartilage, epiphysis, growth plate and surrounding metaphysis to a distance of 5 mm, fixed in 10% buffered formalin for 48 h, decalcified with Richard-Allan Scientific™ Decalcifying Solution (Richard-Allan Scientific, Kalamazoo, MI) at room temperature for >16 h. The specimens were processed routinely for paraffin embedding.

Statistical analysis

All statistical analysis was made by using the Student's t test for paired samples. *P* values of <0.05 were considered as statistically significant.

Results

Dioscin increases chondrogenic differentiation of BMSCs

Mesenchymal stem cells isolated from mouse bone marrow were characterized for basic stem cells properties. As shown in **Figure 1A**, BMSCs displayed spindle shape with some feeler spreading out. BMSCs cultured *in vitro* showed exponential growth rate after a few days seeding in culture plates (**Figure 1B**). Triple-lineage differentiation of BMSCs was also tested. Oil red O staining, Alizarin red S staining and Toluidine blue staining confirmed the adipogenic, osteogenic and chondrogenic potentials of BMSCs respectively (**Figure 1C**).

Then, Dioscin was added to culture medium (1 μ M) to test its effect on chondrogenic differentiation of BMSCs. After 3 weeks culture in differentiation medium, BMSCs pellets deposited significant amount of glycosaminoglycans (GAG), as shown by toluidine blue staining (**Figure 2A**). With 1 μ M of Dioscin in the medium, BMSCs deposit more GAGs. Notably, the number and size of lacunae formed by BMSCs-derived chondrocytes is bigger in pellets treated with Dioscin than pellets without Dioscin. Total GAGs in pellets were measured spectrophotometrically. Total collagens were quantified as well by measuring concentration of hydroxyproline. Dioscin treatment significantly increased the contents of GAGs and collagens in BMSCs pellets (**Figure 2B, 2C**).

BMSC-derived chondrocytes become hypertrophic after dioscin treatment

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Since the size of lacunae is an indication for hypertrophic differentiation and terminal matu-

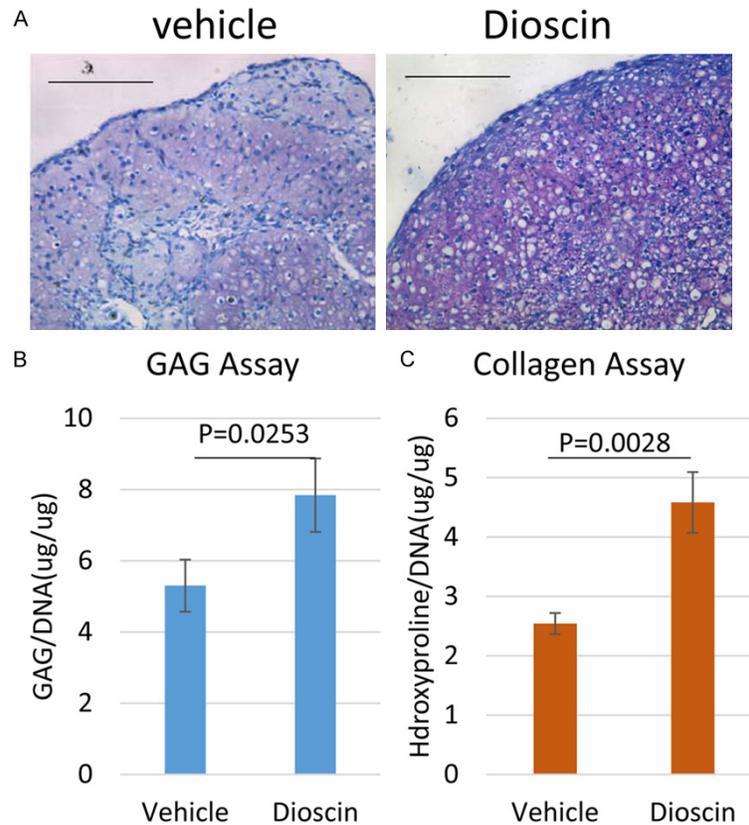


Figure 2. Dioscin increases chondrogenic differentiation of BMSCs. A: Toluidine blue staining showed the deposition of GAGs in BMSCs pellets. Bar = 50 μ m. B: Total GAGs were measured by spectrophotometry. C: Total collagens were quantified by hydroxyproline assay. Statistical analysis was performed by student's t-test.

ration of chondrocytes [20], we speculated that Dioscin treatment may increase the hypertrophy of BMSCs derived chondrocytes. Collagen type X was considered as the most exclusive marker for hypertrophic differentiation of chondrocytes [21]. Collagen type II and type X expressions were examined by immunofluorescent staining and qRT-PCR. As shown in **Figure 3A** and **3B**, Dioscin treatment clearly increased the expression of collagen type II and type X at protein level. This expression was confirmed at the mRNA level by real time PCR (**Figure 3C, 3D**).

Dioscin stimulate endochondral ossification after sub-chondral bone fracture and growth plate injury

To test if Dioscin increase endochondral ossification, mice carried subchondral bone fracture were give 40 mg/kg/day of Dioscin by oral administration. Four weeks after injury, femoral

samples of the mice were collected for histological examination. Dioscin treated mice formed large piece of cartilaginous tissue at the site of injury, while control mice produced some fibrotic tissue in the middle of injury site (**Figure 4**). Results of immunohistochemistry indicated that the cartilaginous tissues formed in Dioscin treated mice were positive for collagen type II and type X, but negative for collagen type I staining (**Figure 5**). Meanwhile, fibrotic tissues in control mice expressed a mixture of collagen type I and type II. Weak expression of collagen type X was found on these tissues. At the boundary between original tissue and injury site, we observed transitioning tissues between GAG-rich cartilage and calcified bone in both Dioscin treated mice and control mice (**Figure 4**). However, the transitioning tissue was much more abundant in Dioscin treated mice than in control mice.

Discussion

In recent years, the role of Dioscin on inhibiting osteoclast maturation has drawn attention. As a natural product, it may provide new therapeutics for bone related diseases. In this study, we tested the effects of Dioscin on bone regeneration using both *in vitro* and *in vivo* models. Our data indicated that that chondrogenic differentiation of BMSCs was increased by Dioscin. Hypertrophic differentiation of BMSCs derived chondrocytes was accelerated by treatment of Dioscin. In a mouse subchondral bone fracture model, Dioscin was shown to improve endochondral bone formation.

Herbs were widely used in Traditional Chinese Medicine (CHM) to treat bone disease for thousands of years [22]. They are normally low-cost with very few side effects comparing to synthesized chemical-based drugs. Since been tested for many years, Chinese herbal formula is gen-

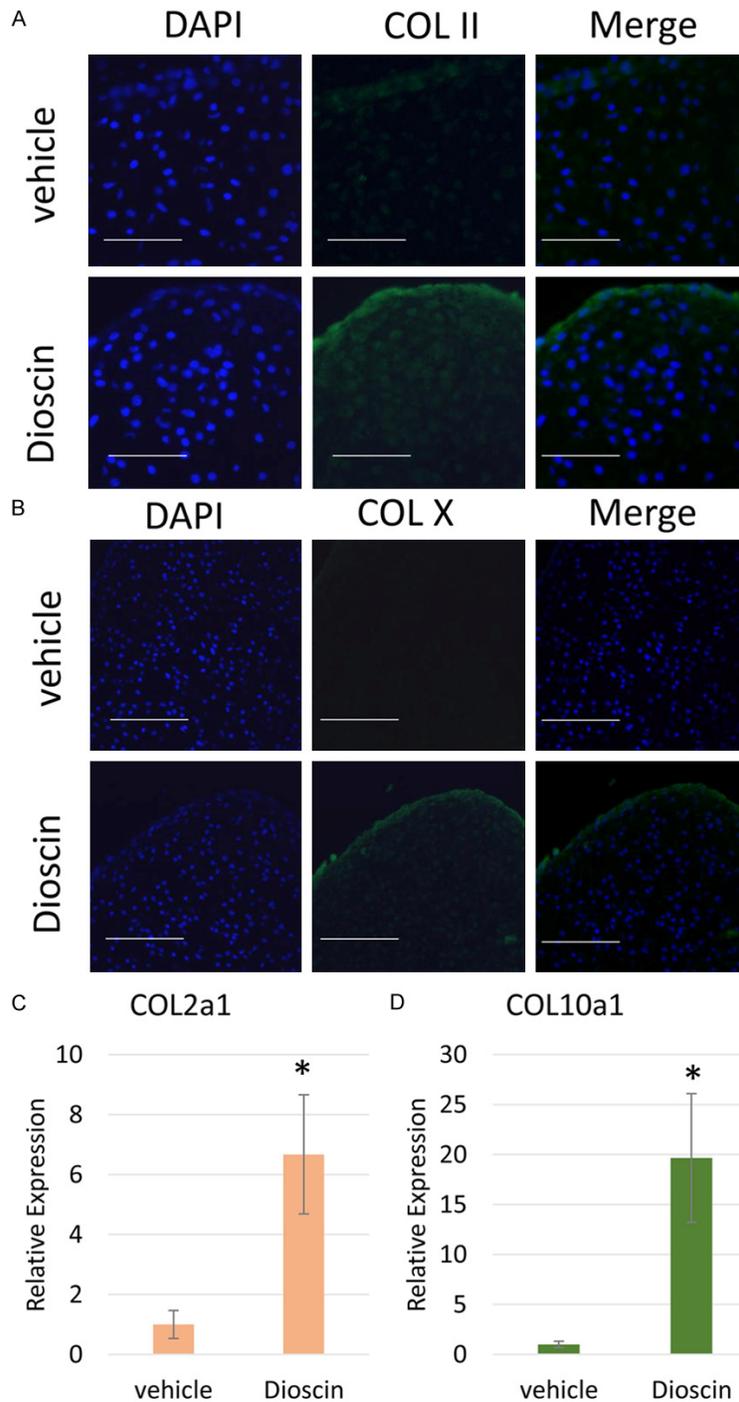


Figure 3. Dioscin promotes expression of collagen type II and type X. A, B: Immunofluorescent staining showed the expression of collagen type II and type X in pellets. Bar = 50 μ m. C, D: Expressions of collagen type II and type X at mRNA level were measured by real time PCR. Asterisks indicated that $P < 0.01$.

erally considered as a safe alternative medicine for bone-related diseases such as osteoporosis [23]. It was believed that natural prod-

ucts from traditional Chinese herbs were alternative sources for bone growth factor that could be used in bone regeneration [24]. Recently, a few compounds purified from the leaf or stem of plants which were used in Traditional Chinese Medicine had been shown to improve bone healing in animal models and human patients [25, 26]. Total extracts from *Dioscorea nipponica Makino* synergistically attenuated the loss of bone mass after ovariectomy, suggesting natural products from *Dioscorea nipponica Makino* could potentially regulate the balance of mineral deposition and absorption [27]. It's also reported that extracts from roots of *Dioscorea batatas* stimulated maturation of osteoblasts by increasing extracellular matrix synthesis, collagen production, Alkaline phosphatase activity, and calcium deposition [28]. As the main bioactive steroidal saponins that isolated from the plants belong to *Dioscorea Genus*, Dioscin was never been reported to regulate the maturation and hypertrophy of chondrocytes. Data from the current study showed that Dioscin increased the chondrogenic differentiation of BMSCs as well as the hypertrophy of BMSCs derived chondrocytes.

Endochondral ossification is the key process for long bone formation and growth during the skeleton development and maturation [29]. This process involves replacement of the primordial cartilaginous template by bone tissue through temporally and spatially coordinated events of chondrocyte proliferation, elongation, differentiation,

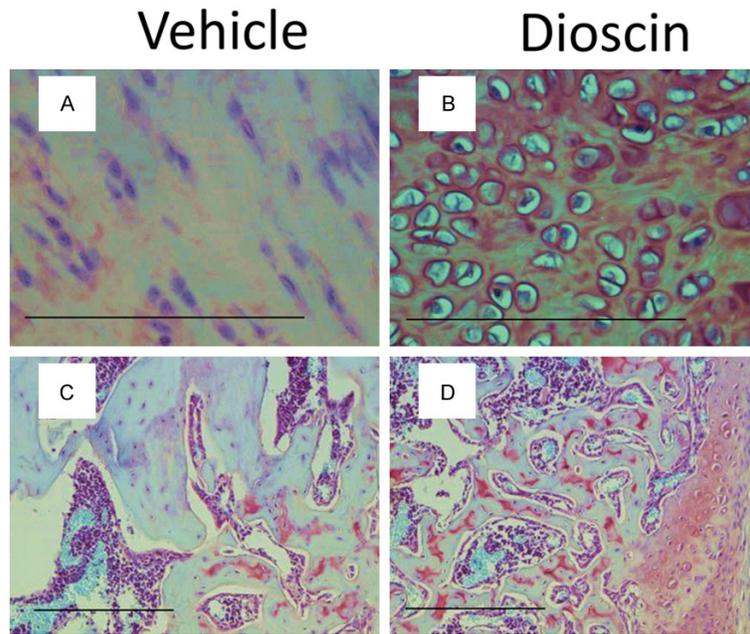


Figure 4. Dioscin enhances endochondral ossification in bone fracture. Safarin O/Fast green/Hematoxylin staining was performed at week 4 after surgery. Bar = 100 μ m. A, C: Showed the cartilage templates at the injury site at different magnification. B, D: Showed ossification zone at the injury site.

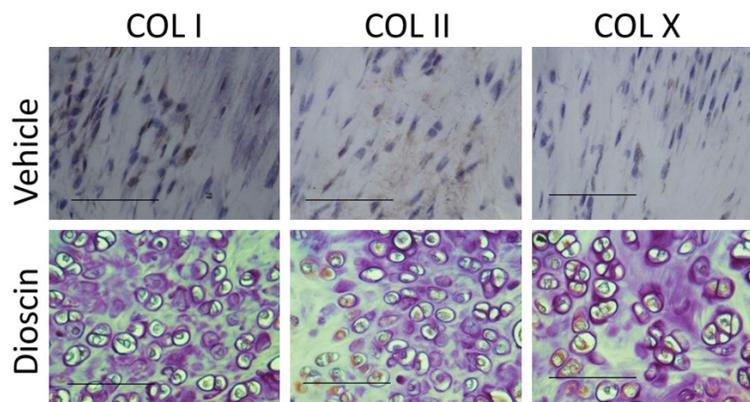


Figure 5. Dioscin increase collagen type X expression at the injury site. Immunohistochemistry was carried out on mice 4 weeks after surgery. Bar = 50 μ m.

and hypertrophy. As the final step of the process, hypertrophic cartilage calcified, blood vessels invaded, and bone matrix formed to replace the cartilage template [30]. For treatment of critical bone defects, autograft is currently considered the best in the medical applications, with all the characteristics indispensable for bone regeneration: osteogenesis, osteoconductivity, and osteoinductivity [31, 32]. Availability and donor site morbidity is the

major concern for autografts. Another option for bone regeneration is to use suitable bioactivity and appropriate degradation of biomaterials to engineer large piece of bone tissue [33]. Large piece of *in vitro* engineered bone tissue contained living cells which required significant amount of oxygen and nutrients after implantation [34]. Unlike autograft which is highly vascularized, engineered bone tissue normally experienced ischemia right after implantation due to lack of vasculature [35]. However, engineering bone tissue through endochondral ossification might a good strategy to tackle this problem, since vascularization is the natural step prior to endochondral bone formation. In the current study, endochondral ossification was promoted in mice with bone fracture by oral administration of Dioscin. Dioscin could be used as a bioactive molecule in engineering bone tissue through endochondral ossification.

Hypertrophic differentiation of chondrocyte is one of the major physiological events happened in the process of endochondral ossification. Collagen type X expression is only detected in deep zone cartilage and growth plate cartilage, where hypertrophic chondrocytes were found [21]. The exclusive distribution of collagen type X in hypertrophic cartilage implies an important role in endochondral ossification that this particular type of collagen must play. It's reported that in an experimental fractures model of chicken humerus, collagen type X synthesis only occurred in the areas of cartilaginous callus composed of hypertrophic and degenerative chondrocytes, but not in either skeletal muscle or bone [36]. Another piece of evidence linked collagen type X with

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endochondral ossification laid in osteoarthritic cartilage. Gene expression of collagen type X was detected in areas where there appeared to be a re-initiation of the endochondral bone formation [37, 38]. Collagen type X was used as the molecular marker in this study to indicate hypertrophy of BMSCs derived chondrocytes. We also found a strong relationship between collagen type X expression and endochondral ossification. Dioscin treatment *in vitro* and *in vivo* significantly increased the expression of collagen type X at both mRNA and protein level.

In conclusion, our data obtained from the current study indicated that hypertrophic differentiation of BMSCs derived chondrocytes was accelerated Dioscin in pellet cultures. Results from subchondral bone fracture models demonstrated that Dioscin increased the endochondral ossification after bone fracture.

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Disclosure of conflict of interest

None.

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References

- [1] Xu J, Yang Y. Traditional Chinese medicine in the Chinese health care system. *Health Policy* 2009; 90: 133-139.
- [2] Dong S, Su SB. Advances in mesenchymal stem cells combined with traditional Chinese medicine therapy for liver fibrosis. *J Integr Med* 2004; 12: 147-155.
- [3] Cai B, Zhang AG, Zhang X, Ge WJ, Dai GD, Tan XL, Roodrajeetsing G, Cai JP. Promoting Effects on Proliferation and Chondrogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells by Four "Kidney-Tonifying" Traditional Chinese Herbs. *Biomed Res Int* 2015; 2015: 792161.
- [4] Li Y, Wang J, Chen G, Feng S, Wang P, Zhu X, Zhang R. Quercetin promotes the osteogenic differentiation of rat mesenchymal stem cells via mitogen-activated protein kinase signaling. *Exp Ther Med* 2015; 9: 2072-2080.
- [5] Yang J, Gao F, Zhang Y, Liu Y, Zhang D. Buyang Huanwu Decoction (BYHWD) Enhances Angiogenic Effect of Mesenchymal Stem Cell by Upregulating VEGF Expression After Focal Cerebral Ischemia. *J Mol Neurosci* 2015; 56: 898-906.
- [6] Liu CZ, Zhou HY, Yan Q. Fingerprint analysis of *Dioscorea nipponica* by high-performance liquid chromatography with evaporative light scattering detection. *Anal Chim Acta* 2007; 582: 61-68.
- [7] Li H, Huang W, Wen Y, Gong G, Zhao Q, Yu G. Anti-thrombotic activity and chemical characterization of steroidal saponins from *Dioscorea zingiberensis* C.H. Wright. *Fitoterapia* 2010; 81: 1147-1156.
- [8] Qin J, Kang Y, Xu Z, Zang C, Fang B, Liu X. Dioscin prevents the mitochondrial apoptosis and attenuates oxidative stress in cardiac H9c2 cells. *Drug Res (Stuttg)* 2014; 64: 47-52.
- [9] Zhu H, Xu JD, Mao Q, Shen H, Kong M, Chen JP, Li SL. Metabolic profiles of dioscin in rats revealed by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. *Biomed Chromatogr* 2015; 29: 1415-1421.
- [10] Kaskiw MJ, Tassotto ML, Mok M, Tokar SL, Pycko R, Th'ng J, Jiang ZH. Structural analogues of diosgenyl saponins: synthesis and anticancer activity. *Bioorg Med Chem* 2009; 17: 7670-7679.
- [11] Sautour M, Mitaine-Offer AC, Miyamoto T, Dongmo A, Lacaille-Dubois MA. A new steroidal saponin from *Dioscorea cayenensis*. *Chem Pharm Bull (Tokyo)* 2004; 52: 1353-1355.
- [12] Xu T, Zheng L, Xu L, Yin L, Qi Y, Xu Y, Han X, Peng J. Protective effects of dioscin against alcohol-induced liver injury. *Arch Toxicol* 2014; 88: 739-753.
- [13] Qu X, Zhai Z, Liu X, Li H, Ouyang Z, Wu C, Liu G, Fan Q, Tang T, Qin A, Dai K. Dioscin inhibits osteoclast differentiation and bone resorption through down-regulating the Akt signaling cascades. *Biochem Biophys Res Commun* 2014; 443: 658-665.
- [14] Jurgens WJ, Oedayrajsingh-Varma MJ, Helder MN, Zandiehoulabi B, Schouten TE, Kuik DJ, Ritt MJ, van Milligen FJ. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. *Cell Tissue Res* 2008; 332: 415-426.
- [15] Lee SY, Nakagawa T, Reddi AH. Mesenchymal progenitor cells derived from synovium and infrapatellar fat pad as a source for superficial zone cartilage tissue engineering: analysis of superficial zone protein/lubricin expression. *Tissue Eng Part A* 2010; 16: 317-325.
- [16] Wu L, Wu Y, Lin Y, Jing W, Nie X, Qiao J, Liu L, Tang W, Tian W. Osteogenic differentiation of

Dioscin stimulates differentiation of mesenchymal stem cells

- adipose derived stem cells promoted by over-expression of osterix. *Mol Cell Biochem* 2007; 301: 83-92.
- [17] Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. *Clin Biochem* 1996; 29: 225-229.
- [18] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-408.
- [19] Lee MA, Nissen TP, Otsuka NY. Utilization of a murine model to investigate the molecular process of transphyseal bone formation. *J Pediatr Orthop* 2000; 20: 802-806.
- [20] Coleman CM, Tuan RS. Growth/differentiation factor 5 enhances chondrocyte maturation. *Dev Dyn* 2003; 228: 208-216.
- [21] Studer D, Millan C, Ozturk E, Maniura-Weber K, Zenobi-Wong M. Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells. *Eur Cell Mater* 2012; 24: 118-135; discussion 135.
- [22] Mukwaya E, Xu F, Wong MS, Zhang Y. Chinese herbal medicine for bone health. *Pharm Biol* 2014; 52: 1223-1228.
- [23] Guo Y, Li Y, Xue L, Severino RP, Gao S, Niu J, Qin LP, Zhang D, Brömme D. *Salvia miltiorrhiza*: an ancient Chinese herbal medicine as a source for anti-osteoporotic drugs. *J Ethnopharmacol* 2014; 155: 1401-1416.
- [24] Dong GC, Chen HM, Yao CH. A novel bone substitute composite composed of tricalcium phosphate, gelatin and *drynaria fortunei* herbal extract. *J Biomed Mater Res A* 2008; 84: 167-177.
- [25] Ji ZL, Zhou H, Wang JF, Han LY, Zheng CJ, Chen YZ. Traditional Chinese medicine information database. *J Ethnopharmacol* 2006; 103: 501.
- [26] Tejedor Garcia N, Garcia Bermejo L, Fernandez Martinez AB, Olmos Centenera G, Kumari R, Xu Q, Cheng X, Watson S, de Lucio Cazaña FJ. MEDLINE-based assessment of animal studies on Chinese herbal medicine. *J Ethnopharmacol* 2012; 140: 545-549.
- [27] Ko BS, Lee HW, Kim da S, Kang S, Ryuk JA, Park S. Supplementing with *Opuntia ficus-indica* Mill and *Dioscorea nipponica* Makino extracts synergistically attenuates menopausal symptoms in estrogen-deficient rats. *J Ethnopharmacol* 2014; 155: 267-276.
- [28] Kim S, Shin MY, Son KH, Sohn HY, Lim JH, Lee JH, Kwun IS. Yam (*Dioscorea batatas*) Root and Bark Extracts Stimulate Osteoblast Mineralization by Increasing Ca and P Accumulation and Alkaline Phosphatase Activity. *Prev Nutr Food Sci* 2014; 19: 194-203.
- [29] Tuan RS. Biology of developmental and regenerative skeletogenesis. *Clin Orthop Relat Res* 2004; S105-117.
- [30] Maes C. Role and regulation of vascularization processes in endochondral bones. *Calcif Tissue Int* 2013; 92: 307-323.
- [31] Vaccaro AR, Chiba K, Heller JG, Patel T, Thalgott JS, Truumees E, Fischgrund JS, Craig MR, Berta SC, Wang JC; North American Spine Society for Contemporary Concepts in Spine Care. Bone grafting alternatives in spinal surgery. *Spine J* 2002; 2: 206-215.
- [32] Neman J, Duenas V, Kowolik CM, Hambrecht AC, Chen MY, Jandial R. Lineage mapping and characterization of the native progenitor population in cellular allograft. *Spine J* 2013; 13: 162-174.
- [33] Siddappa R, Fernandes H, Liu J, van Blitterswijk C, de Boer J. The response of human mesenchymal stem cells to osteogenic signals and its impact on bone tissue engineering. *Curr Stem Cell Res Ther* 2007; 2: 209-220.
- [34] Jukes JM, van Blitterswijk CA, de Boer J. Skeletal tissue engineering using embryonic stem cells. *J Tissue Eng Regen Med* 2010; 4: 165-180.
- [35] Yoshimura K, Eto H, Kato H, Doi K, Aoi N. In vivo manipulation of stem cells for adipose tissue repair/reconstruction. *Regen Med* 2011; 6: 33-41.
- [36] Grant WT, Wang GJ, Balian G. Type X collagen synthesis during endochondral ossification in fracture repair. *J Biol Chem* 1987; 262: 9844-9849.
- [37] Hoyland JA, Thomas JT, Donn R, Marriott A, Ayad S, Boot-Handford RP, Grant ME, Freemont AJ. Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. *Bone Miner* 1991; 15: 151-163.
- [38] Aigner T, Dietz U, Stoss H, von der Mark K. Differential expression of collagen types I, II, III, and X in human osteophytes. *Lab Invest* 1995; 73: 236-243.