

Bone morphogenetic protein 2 improves patellar tendon healing by promoting migration and proliferation of tenocytes

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The repair of injured tendons remains a great challenge because of the poor intrinsic healing capacity of tendons. In this study, we examined the spatiotemporal expression pattern of the bone morphogenetic protein 2 (*bmp-2*) gene in normal and experimentally injured rat patellar tendons. We also investigated the ability of exogenously applied BMP-2 to promote patellar tendon healing. *In situ* hybridization with *bmp-2* and *alk-6* (*bmp-2* receptor) antisense riboprobes revealed that both genes were normally expressed at low levels in intact rat tendons. However, *bmp-2* expression was significantly upregulated in tenocytes found in the wound site at 7 d and later following tendon injury. In addition, it was found that *bmp-2* was expressed in cultured patellar tenocytes. Application of exogenous BMP-2 to the tendon wound site significantly enhanced tendon repair. Moreover, *in vitro* and *in vivo* studies further demonstrated that BMP-2 enhanced tenocyte proliferation and migration to the wound site. The detectable amount of BMP-2 in normal tendons suggests that a basal level of *bmp-2* expression was likely present to maintain the normal functions of the patellar tendon. Injury to the tendon induced increased *bmp-2* expression intrinsically, but the expression level was insufficient for proper tendon repair. Our findings indicate that it is possible to significantly improve tendon healing by applying exogenous BMP-2 to the wound site, inferring that this protein could be developed as a potential therapeutic reagent for the treatment of damaged tendons.

BMP-2, tendon healing, tenocyte proliferation and migration

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Torn ligaments and tendons are among the most commonly encountered injuries. The standard method of treatment is to use the patellar tendon as an autograft for reconstruction of the injured ligament [1–4]. However, clinical studies have revealed that the patellar tendon donor site heals very slowly following the extraction of a strip of tendon to create the autograft. Many other complications have also been identified, including patellofemoral pain, rupture of the

patellar tendon and weakness in the quadriceps skeletal muscles [5–7]. In animal studies, it has been reported that scar formation in the patellar tendon extract/wounded site was the main feature, and all new collagen fibers produced at the wounded site were rudimentary in size and randomly aligned. Furthermore, the mechanical load that these experimental tendons were able to sustain before tearing was significantly reduced when compared with control tendons [8]. Effective healing of the patellar tendon is most likely related to the following 2 key events that must occur at the

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cellular and molecular levels: (1) Tenocytes from intact regions of the injured patellar tendon must be able to proliferate and migrate into the wounded site; (2) once these cells have fully occupied the wounded site, they must be able to produce collagen fibers that structurally complement the rest of the fibers in the intact tendon. Therefore, we would like to establish a method that can enhance these key events for effective tendon healing. In the present study, we investigated the possibility of enhancing tendon repair by intervening in the natural healing process of the damaged tendon with exogenous bone morphogenetic protein 2 (BMP-2) in the rat model. We established the spatiotemporal expression pattern of *bmp-2* and its receptor, *alk-6*, in normal and experimentally injured patellar tendons. We also determined the effects of exogenous BMP-2 on tenocyte proliferation and migration during the tendon healing process.

1 Materials and method

1.1 Animal surgery

Eight-week-old male Sprague-Dawley (SD) rats (Guangdong Medical Laboratory Animal Center, China) were anesthetized with 2.5% pentobarbital solution injected into the peritoneum. The hair on the lower limbs was shaved off at the level of the stifle, and the skin was sterilized with chlorhexidine (Hibitane). An incision was then made in the skin to expose the patellar tendon. A fragment of tendon measuring 1 mm × 4 mm was surgically removed from the central region of the patellar tendon. The tendon sheath and skin were then sutured. As a sham control, the skin and tendon sheath of the contralateral limbs were exposed and then immediately sutured. The experimental and sham control patellar tendons were harvested 2, 7, 14, 40, and 60 d postoperatively for analysis. All of the surgical procedures performed were approved by the animal ethics committees of Jinan University and the Chinese University of Hong Kong.

1.2 Histology

All of the tendons recovered were fixed in 4% paraformaldehyde at 4°C overnight. The specimens were then dehydrated in a graded series of alcohol, cleared in xylene, and embedded in paraffin wax. The wax blocks were sectioned at 6 μm and mounted onto TESPA-coated glass slides. The sections were used for routine histology and *in situ* hybridization studies. Hoechst 33342 (Sigma-Aldrich, USA) fluorescent stains were used to stain the nuclei to confirm the presence of cells in the wounded site.

1.3 *In situ* hybridization

Digoxigenin-labeled sense and antisense riboprobes were synthesized from a psP72 plasmid containing a 1550-bp

insert encoding *bmp-2* (kindly provided by B. Hogan) and a pcDNA3 plasmid containing 1200 bp encoding *alk-6* (kindly provided by P. ten Dijke) using T3 and T7 RNA polymerases (Boehringer Mannheim Biochemica, USA). The size of the probes was reduced by hydrolysis in 40 mmol L⁻¹ sodium bicarbonate and 60 mmol L⁻¹ sodium carbonate at 60°C for 15 min. The probes were precipitated with 4 mol L⁻¹ LiCl and ethanol, then resuspended in hybridization buffer. For *in situ* hybridization, paraffin sections were dewaxed, hydrated, and equilibrated in PBS. The sections were then treated with 10 μg/mL proteinase K for 10 min and postfixed in 4% paraformaldehyde. The treated specimens were washed twice with PBS for 10 min and then incubated in prehybridization buffer for 3 h. Afterward, 1 μg/mL digoxigenin-labeled sense or antisense riboprobes were diluted in hybridization buffer and added to the treated specimens. The sense probes were used as the control. The hybridization temperature for *bmp-2* and *alk-6* sense and antisense riboprobes was 50°C, and the incubation time was 18 h. After hybridization, all unbound cDNA probes were stringently washed off in 2×SSC at 42°C for 10 min (3 changes), followed by 60% formamide in 0.2×SSC buffer for 15 min and 0.2×SSC buffer for 10 min. The location of hybridized probes was determined using alkaline phosphatase-conjugated digoxigenin antibodies. The antibodies were added to the specimens for 1 h, washed in PBS, and developed in NBT/BICP.

1.4 Culture of tenocytes

Patellar tendon explant cultures were prepared as previously described [9]. Briefly, 3-month-old male SD rats were sacrificed using a chloroform overdose. The patellar tendon was excised and immediately transferred to prewarmed Dulbecco's phosphate buffered saline (Sigma-Aldrich, USA) containing 0.4% bovine serum albumin. The excised tendon was divided into small pieces of approximately 1 mm³ each, and each piece was implanted into 5 μL of 10% rat tail collagen gel containing Minimal Essential Medium (Sigma-Aldrich, USA). The explants were incubated at 37°C for 20 min. Finally, the culture dishes were flooded with Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco, USA) containing 10% heat inactivated fetal bovine serum (FBS) (Gibco, USA). The cultures were maintained at 37°C in a humidified incubator containing a mixture of 5% CO₂ in air. The medium was changed every 3 d until a confluent monolayer was established. These primary cultures were subsequently passaged by incubation with 0.25% trypsin (Invitrogen, USA). Trypsin activity was inhibited by addition of 10% FBS in DMEM, and the cells were pelleted by centrifugation at 459×g for 5 min at room temperature. The pellet was resuspended in DMEM containing 10% FBS and subcultured. The subcultured tenocytes were used in the RT-PCR, "scratch wound" assay, transwell motility assay, collagen matrix contract assay, and MTT assay.

1.5 RT-PCR

For the RT-PCR, total RNAs were isolated from the isolated tenocytes using TRIzol Reagent (Invitrogen, USA). The first-strand cDNA was synthesized by using the First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. PCR was performed by using *bmp-2* primers (Sense: 5'-AGCGGAAGCGTCTTAAGTC-CAG-3'; antisense: 5'-AGGTGATCAGCCAGGGGAAAA-G-3'), and the program was: denaturation at 94°C (5 min), 35 cycles of denaturation at 95°C (15 s), annealing at 55°C (30 s), and extension at 72°C (60 s). The PCR products were visualized following electrophoresis on ethidium-bromide-stained 1.5% agarose gel.

1.6 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT) assay

MTT assay was performed to measure the extent of tenocyte proliferation in the presence and absence of human recombinant BMP-2 (0.6 µg/mL) (R&D Systems, USA) using the method described by Mosmann in 1983 [10]. Briefly, 50 µL of MTT solution was added to each of the 96 wells containing tenocytes and incubated for 4 h at 37°C. DMSO (150 µL) (Sigma-Aldrich, USA) was then added into each of the wells for 10 min to solubilize the blue MTT-tetrazolium dye. The dye was measured on an ELISA plate reader (Molecular Device SpectraMax 340, USA) with the absorbance set at 595 nm. The data were collected from 3 independent experiments.

1.7 Cell cycle analysis by flow cytometry

BMP-2 (6 µg/mL)-treated and untreated patellar tenocytes were collected 48 h after culture. The cells were fixed in 4% paraformaldehyde and resuspended in 1 mL of PBS and 0.1% Triton X-100. Twenty microliters of RNAase (10 mg/mL) (Fermentas, USA) and 20 µL of propidium iodide solution (MP biomedical, USA) were then added to the cell suspension. The suspension was incubated at 37°C for 30 min in the dark. The stained cells were resuspended and passed through a 40-µm mesh filter. A total of 10⁶ cells/mL were used for flow cytometry analysis (BD-FACSAria, USA). An argon laser (488-nm wavelength) was used to excite the propidium-iodide-stained cells. The emitted fluorescent signals were collected in a photomultiplier tube with a red pass filter (620-nm wavelength). The results obtained were analyzed using ModFit LT™ Version 3.2 software (Verity Software House, USA) to determine the percentage of cells in the G₀/G₁, S, and G₂/M phases of the cell cycle.

1.8 Collagen matrix contraction assay

The patellar tenocytes (1×10⁵) were suspended in 2 mL of 2× DMEM containing 0.5 mL 100 mmol L⁻¹ NaOH and 0.5

mL gelatin-A sepharose-pretreated FCS. Collagen lattices were prepared in 30-mm dishes mixing 2 mL of the tenocytes in 0.7 mL of rat tail tendon collagen solution (1 mg/mL) [11]. The tenocyte-populated collagen lattices (TPCLs) were allowed to set for 60 min at 37°C. Two milliliters of 2% FCS/DMEM with BMP-2 (0.6 µg/mL) and without BMP-2 was added into individual TPCLs for further culture at 37°C and 5% CO₂. The extent to which the collagen lattice was contracted by the tenocytes was determined by measuring the diameter of the lattice. The average diameter of the lattice was determined 2, 3, and 7 d post-culture and from 3 independent experiments.

1.9 Scratch wound assay

A scratch wound assay was used to determine the effects of BMP-2 on cell migration and proliferation at the "wounded" site *in vitro*. The patellar tenocytes were cultured in culture dishes with DMEM + 10% FBS until they were confluent and monolayered. A sterile pipette tip was then used to scrape the cells off the midline of the culture to create an artificial wound. The width of the "wound" (5 mm) was verified to be consistent under dissection in all experiments. The cultures were then washed in PBS and cultured in the presence of (1) DMEM + 2% FBS or (2) DMEM+2% FBS + BMP-2 (0.6 µg/mL). The cultures were photographed at various time points. The experiment was repeated independently 3 times.

1.10 Transwell motility assay

Migration assays were performed using Nucleopore Blindwell chemotaxis chambers (Costar Corporation, USA) and Nucleopore polycarbonate filters (8-µm pore size, Costar) [12]. The filters were precoated with 100 µg/mL human plasma fibronectin (Gibco, USA) overnight and then air-dried. DMEM+BMP-2 (0.6 µg/mL) + 2% FBS were introduced into the bottom well of each Blindwell chamber. A Nucleopore filter was placed over the well and securely held in a retainer, forming the upper chamber. A suspension of patellar tenocytes (10⁵ cells) suspended in DMEM+2% FBS was added to the upper chamber. The cells were cultured in the Blindwell chamber at 37°C and 5% CO₂ for 16 h. After culturing, the tenocytes were wiped off the upper surface of the filter with a cotton swab. The cells that had migrated to the underside of the filter were fixed, stained with eosin, and counted under a microscope. Each experiment was repeated 3 times.

1.11 Preparation of BMP-2/alginate bead

BMP-2 was loaded into alginate beads as follows. A 1.2% alginate solution was prepared by dissolving sodium alginate (Sigma-Aldrich, USA) in 2× DMEM. The mixture was then exposed to UV light for 12 h with stirring. A suitable

amount of BMP-2 was added into the sterilized alginate mixture to obtain a final concentration of 2 μg BMP-2/mL. Ten microliters of the BMP-2/alginate mixture was then introduced into 102 mmol L⁻¹ sterilized CaCl₂ to generate the BMP-2/alginate gel bead. All BMP-2/alginate beads were balanced in DMEM (pH 7.4) for 30 min before transplantation *in vivo*.

1.12 Statistical analysis

All values are expressed as means \pm SD. The unpaired Student's *t*-test with the use of a 2-tailed distribution was used to calculate the statistical significance between the means of the 2 groups. A *P*-value of <0.05 was considered to be significant.

2 Results

2.1 *bmp-2* expression profile during tendon healing

The spatiotemporal expression patterns of *bmp-2* and its receptor, *alk-6*, were established during the tendon healing process by *in situ* hybridization. It was found that *bmp-2* was normally expressed at a relatively low level in the nonoperated patellar tendons (Figure 1(b)). There was no staining in sections hybridized with the sense control riboprobe (Figure 1(a)). In 2-d postinjured tendons, fibroblast-like cells were found to infiltrate the wounded site (Figure 1(c) and (g)). These cells and the tenocytes in the intact regions of the tendon also expressed the gene, but in low levels (Figure 1(c)). In contrast, when the injured

tendons were examined after 7 d, the fibroblast-like cells in the wounded site strongly expressed *bmp-2*. Furthermore, the intensity of the expression was greater than that in the tenocytes of the unoperated tendon (Figure 1(d)). Two weeks postinjury, the cells in the wounded site still strongly expressed *bmp-2*, but this expression was weaker than that seen at 7 d postinjury (Figure 1(e) and (f)). Correspondingly, *alk6* was expressed in exactly the same manner as that of *bmp-2* at all time points examined (Figure 1(h); day 2 and 7 results not shown).

2.2 *bmp-2* expression in cultured tenocytes

The patellar tenocytes isolated from patellar tendons were cultured, and RT-PCR was applied to analyze the expression of the *bmp-2* gene. It was found that cultured tenocytes expressed *bmp-2* moderately (Figure 1(i)).

2.3 Effect of BMP-2 on tenocyte proliferation *in vitro*

Because the *in situ* hybridization results demonstrated that *bmp-2* expression was increased in the healing patellar tendons, we tested whether BMP-2 could influence the proliferation of tenocytes *in vitro*. We isolated patellar tenocytes from the patellar tendon and cultured them in the presence of BMP-2. An MTT assay was used to determine the extent of cellular proliferation. The results showed that BMP-2 significantly enhanced tenocyte proliferation *in vitro* when compared with the untreated control cultures (*P* < 0.05) (Figure 2(a)). Furthermore, our flow cytometry analysis demonstrated that the number of S-phase tenocytes was

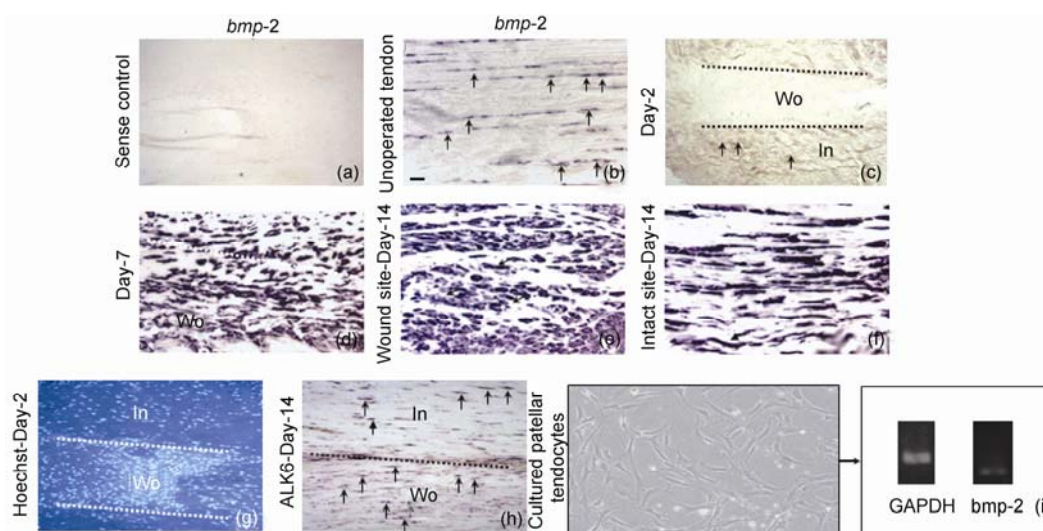


Figure 1 *In situ* hybridization showing *bmp-2* (a–f) and *alk-6* receptor (h) expression during tendon healing. (a) Sense control. (b) *bmp-2* was normally expressed at low levels by tenocytes in unoperated patellar tendons (arrows). (c) In operated patellar tendons examined 2 d postoperatively, *bmp-2* was found to be expressed by tenocytes (arrows) in the intact regions (In) and near the wounded site (Wo). In contrast, the cells that occupied the wounded site (Wo) did not express *bmp-2*. (d) In 7-d-old operated tendons, the tenocytes within the wounded site began to strongly express *bmp-2* (arrows). Similarly, in 14-d-old operated specimens, the tenocytes continued to express *bmp-2* in the wounded site (e) and the intact regions (f). (g) Nuclear Hoechst stain demonstrating the presence of cells within the wounded site of 2-day-old operated specimens. (h) *alk-6* was coexpressed with *bmp-2* in tenocytes within the wounded site and intact regions of 14-d-old operated specimens (arrows). The results were determined from 3 independent experiments. (i) *bmp-2* was expressed in cultured patellar tenocytes. In, intact region; Wo, wounded site.

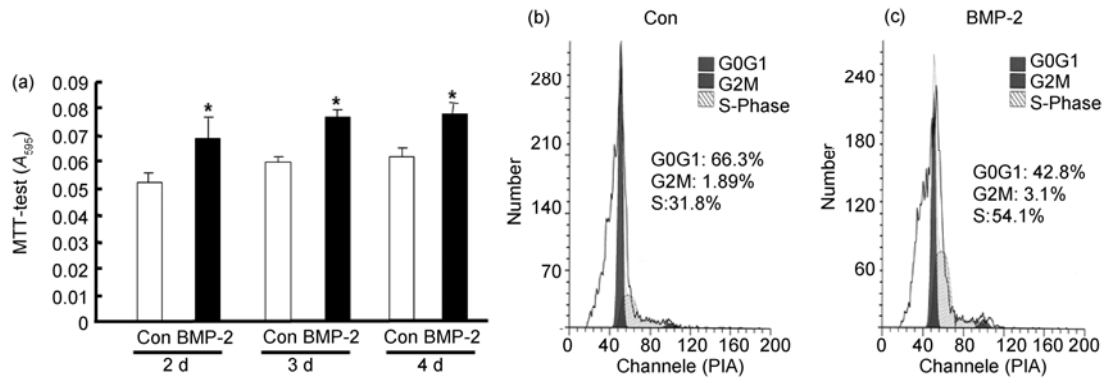


Figure 2 (a) MTT assay showing the effects of BMP-2 (0.6 $\mu\text{g}/\text{mL}$) on patellar tenocyte proliferation. *, $P < 0.05$ (BMP-2 vs. Con). The results were determined from 3 independent experiments ($n = 3$). (b) Flow cytometry analysis revealed that the number of S phase in BMP-2 (0.6 $\mu\text{g}/\text{mL}$)-treated patellar tenocytes was 54.1% after treatment with BMP-2 for 48 h, while it was 31.8% in untreated controls. Con, control.

54.1% after BMP-2 treatment for 48 h, while it was 31.8% in the untreated controls (Figure 2(b)).

2.4 Effects of BMP-2 on tenocyte-mediated contraction of collagen gel

Collagen remodeling is one of the key steps in the tendon healing process. Hence, we tested whether BMP-2-treated patellar tenocytes inserted into a collagen lattice could enhance the contraction of the lattice—a representative phenomenon observed during extracellular matrix remodeling [13]. The patellar tenocytes were loaded into a drop of collagen gel, which was induced to solidify and allowed to free-float on the culture medium. The contractile kinetics of the tenocyte-collagen gels were recorded over a 7-d period. The results revealed that BMP-2 could not promote a significant increase in tenocyte-

mediated contraction of collagen lattices ($P > 0.05$) (Figure 3).

2.5 Effects of BMP-2 on tenocyte migration *in vitro*

Because cell migration is a critical step in the tendon healing process, we investigated whether BMP-2 is capable of influencing tenocyte movement. This was determined using a transwell assay and an artificial wound closure assay. In the transwell assay, BMP-2 was introduced into the bottom of the transwell chamber and tenocytes were placed in the upper chamber. A Millipore filter with pores that allowed cell migration was inserted between the 2 chambers. The results of the study showed that there were significantly more migrating tenocytes on the undersides of the Millipore filters in the BMP-2-treated samples than in the control samples ($P < 0.05$) (Figure 4). We also used an artificial wound closure

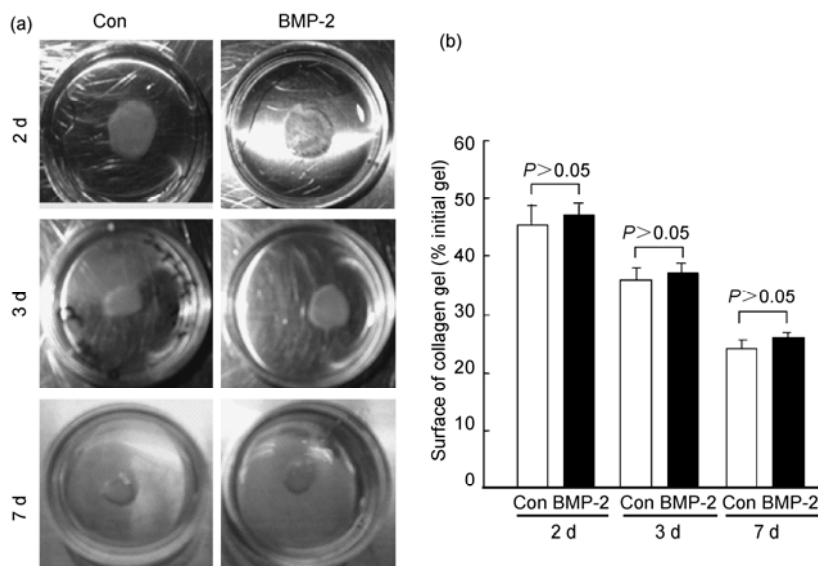


Figure 3 BMP-2 did not enhance the contraction of free-floating collagen lattices. Tenocytes were seeded into collagen lattices and allowed to float in the culture media (control) or medium containing BMP-2 (0.6 $\mu\text{g}/\text{mL}$) for 2 to 7 d. (a) Photographs of a representative experiment; (b) graphic representation of the semi-quantification of the experiment shown in (a). Under such experimental conditions, the lattices contracted to the same extent between the BMP-2-treated group and the control group ($P > 0.05$). The results were established from 3 independent experiments ($n = 3$).

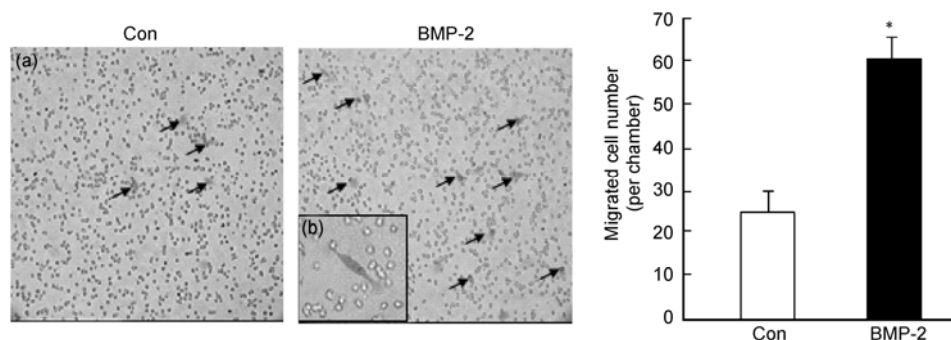


Figure 4 Transwell motility assay revealing that BMP-2 chemotactically attracted patellar tenocytes. The assay was performed in the absent (control) and presence of BMP-2 (0.6 $\mu\text{g}/\text{mL}$). (a) Representative results showing that there were significantly more tenocytes (arrows) present in the BMP-2 assay than in the control assay; (b) high-power image of the tenocytes that had migrated through the Millipore filters interposed between the 2 chambers making up the transwell apparatus; (c) bar chart of the semi-quantification of the experiments shown in (a). *, $P < 0.05$ (BMP-2 vs. Con). Results established from 3 independent experiments ($n = 3$). Con, control.

assay, in which tenocytes were grown on culture dishes to confirm the findings. A 5-mm-wide scratch was created in the culture to mimic an artificial wound. The results of the experiment revealed that BMP-2-treated tenocytes migrated into the “wounded” site significantly faster than did untreated tenocytes. The BMP-2-treated tenocytes invaded approximately 70% of the “wound” within 72 h. In contrast, only approximately 40% of the “wound” site was filled with tenocytes in the untreated control cultures (Figure 5(a), (b), (e) and (f)).

2.6 BMP-2 promotes tendon healing *in vivo*

Our *in vitro* studies demonstrated that BMP-2 not only sig-

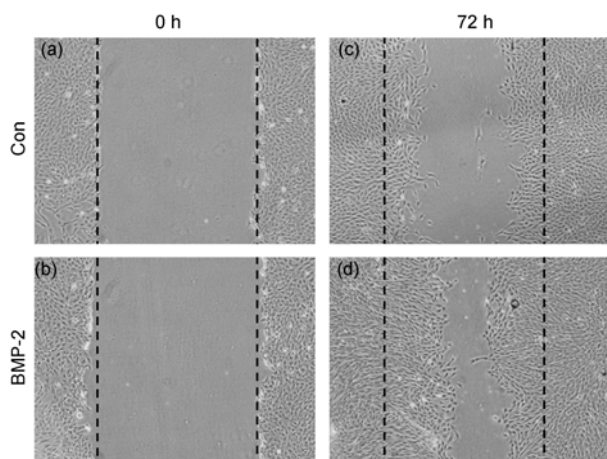


Figure 5 BMP-2 enhanced patellar tenocyte migration in the scratch wound assay. A 5-mm gap was scratched in a confluent tenocyte culture as a model to mimic a wound in an injured tendon. The culture was then maintained in DMEM + 2% FBS or DMEM + 2% FBS + BMP-2. The gap/wound closure was monitored by microscopy. A representative micrograph for each treatment is shown, immediately (0 h) or 72 h after wounding. In the BMP-2-treated group, the tenocytes migrated into the gap/wound area and filled approximately 70% of the gap within 72 h (d). In contrast, only approximately 40% of the wound area was filled with tenocytes in control specimens (c). All of the experiments were repeated 3 times ($n = 3$). Con, Control.

nificantly enhanced patellar tenocyte proliferation, but also migration. In addition, the *in situ* hybridization revealed that during the tendon healing process, *bmp-2* and *alk-6* expressions were upregulated in the cells that infiltrated the wounded site of the tendon. These results infer that BMP-2 may be indispensable during tendon repair and warranted further investigation *in vivo*, in a tendon injury animal model. We surgically created a wound in rat patellas and implanted alginate gel loaded with BMP-2 into the wounded site. As a control, only the medium used for suspending the BMP-2 was loaded into the alginate gel. The results of the study showed that the size of the wound in BMP-2-treated tendons was significantly smaller than that in the control tendons when examined 40 to 60 d after experimentation (Figure 6(a), (c)–(e)).

Histological examination also revealed that the wounds in the BMP-2-treated patellar tendons appeared to be almost repaired 40 d after injury. In contrast, a wide, open gap was still evident in the wounded site of the control tendon (Figure 6(e)). Histological examination of the BMP-2-treated tendons revealed that tenocytes migrated into the central area of the wounded site within 7 d postinjury, while significantly fewer tenocytes were found in the same region in the control tendon (Figure 6(b)). It is well known that BMP-2 can induce cells to differentiate into osteoblasts. However, under our experimental conditions, we found no signs of osteogenesis in the BMP-2-treated tendons when examined 60 d postinjury. This is consistent with our *in vitro* observations, which showed that BMP-2 promoted tenocyte migration and proliferation while showing no indications of osteogenesis.

3 Discussion

The patellar tendon is frequently used as an autograft in the reconstruction of the anterior cruciate ligament [3,4]. However, the drawback of this procedure is that the wounded site created in the donor’s patellar tendon heals very slowly

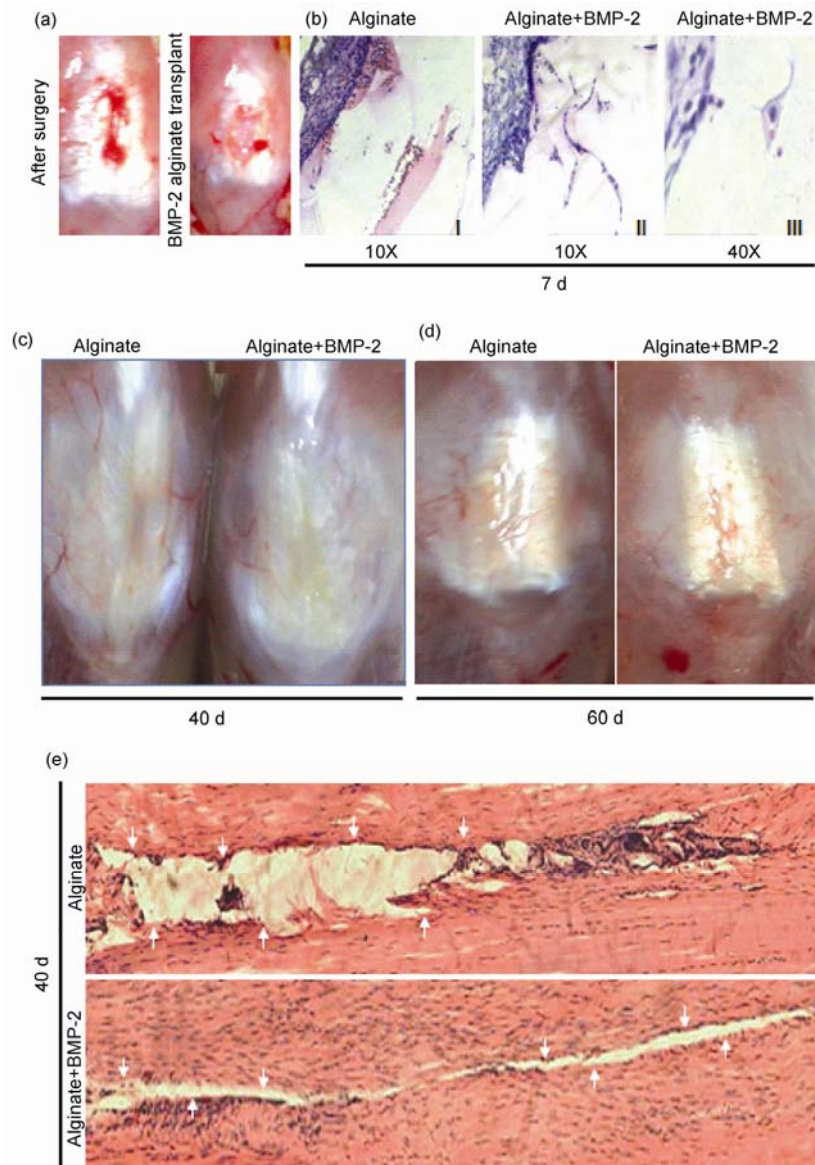


Figure 6 BMP-2 promoted patellar tendon healing *in vivo*. (a) Wound created in the patellar tendon and implanted with alginate gel loaded with BMP-2 protein. (b) H&E staining of the experimental tendon 7 d after implantation of the alginate gel (I; 10 \times) and alginate gel loaded with BMP-2 (II for 10 \times ; III for 40 \times). (c) and (d) The appearance of experimental tendons 40 to 60 d after implantation of alginate gel and alginate gel loaded with BMP-2, respectively. (e) Histology showing the size of the wound in a BMP-2-treated experimental tendon was significantly smaller compared with the alginate control (a, c, d and e). Tenocytes were evident within the wounded site of BMP-2-treated tendons within 7 d after injury, while fewer cells were present in the wounds of control tendons (b). In addition, in the BMP-2-treated tendons, the wounds appeared almost completely healed within 40 d after injury (arrows), while a large gap (arrows) was still evident in the control tendons (e). All of the experiments were repeated 3 times ($n = 3$).

after surgery, resulting in many clinical complications. Therefore, it is important to identify a new therapeutic method that can help promote the healing of the injured tendon and minimize clinical complications.

BMPs belong to a family of multifunctional secreted proteins. Structurally, they are related to transforming growth factor- β and activin [14–16]. BMPs were originally identified on the basis of their remarkable ability to induce bone and cartilage formation when implanted into the skeletal muscles of adult rats [16,17]. To date, 15 BMPs have been

characterized and cloned [18]. Recent studies have demonstrated that physiologically, BMP-2 plays a pivotal role in many developmental processes, including osteoclast formation [19], chondrogenesis [20,21], myogenesis [22,23], skeletal development [24,25], apoptosis [26] and cardiogenesis [27–29]. *bmp-2* null mutants have defective amnions, chorions, and hearts, and are nonviable [27]. In addition, ectopic expression of *bmp-2* could promote fractured bone healing and tendon-to-bone healing [30,31]. Recently, it was reported that BMP-2 was involved in modu-

lating the fate of tendon stem/progenitor cells [32,33].

In the present study, we examined the expression patterns of the *bmp-2* gene in normal intact and experimentally damaged patellar tendons. Our results showed clear evidence that *bmp-2* is normally expressed at low levels by tenocytes in intact tendons. In addition, we further documented that *bmp-2* was expressed in cultured patellar tenocytes. The detectable amount of BMP-2 in normal tendons suggests that a basal level of *bmp-2* expression was likely present to maintain the normal functions of the patellar tendon. These findings led us to investigate whether BMP-2 plays a role in the proliferation of tenocytes. Indeed, the MTT and flow cytometry results revealed that BMP-2 was able to increase the proliferation of tenocytes.

Furthermore, our *in situ* study also showed that following injury, *bmp-2* expression was strongly upregulated among patellar tenocytes along the borders of the wounded site. It is possible that the expression of BMP-2 and its receptor may increase in the whole tendon after injury rather than just at the wounded site; these alterations could be a compensatory response or a response to inducing factors, which could migrate or present throughout all tissues. Alternatively, it could be a bystander effect, which may affect cells distal from the injured sites. It appears that in cultured tenocytes, the expression of BMP-2 was similar to that of the *in vivo* tenocytes. Previously, we reported that the cells that invaded the wounded area were tenocytes that had mainly originated from the edges of the wound [34]. Therefore, we reasoned that BMP-2 may also act in a chemotactic fashion to stimulate the cells bordering the injury to migrate into the wounded site. In this study, we demonstrated that BMP-2 not only significantly stimulated the tenocytes to migrate in the transwell model, but also significantly promoted the tenocytes to migrate earlier and faster into the artificial wounds that had been created in our "scratch wound" culture assay. In addition, we revealed that BMP-2 could promote the migration of tenocytes into injury sites *in vivo* in an animal model. These *in vitro* and *in vivo* results clearly demonstrated that BMP-2 was able to stimulate the migration of tenocytes.

Interestingly, it was found that tenocytes in the intact sites were expressing *bmp-2*, and once the tenocytes were stimulated to migrate into the injury site *in vivo*, they did not express *bmp-2* as demonstrated in our *in situ* study. However, 7 d after injury, these cells strongly re-expressed *bmp-2* as well as its receptor, *alk-6*. These observations suggest that BMP-2 is an important factor that is required for proliferation and migration of tenocytes. In contrast, BMP-2 appears not to be involved in collagen remodeling; the results of the tenocyte-mediated contraction assay revealed that BMP-2 could not promote a significant increase in tenocyte-mediated contraction of collagen lattices.

To investigate whether exogenous BMP-2 could also promote tendon healing *in vivo*, we transplanted alginate gels loaded with BMP-2 into the wounded sites of experi-

mentally injured tendons. It was demonstrated that the size of the wounds in BMP-2-treated tendons was significantly smaller than that in untreated control specimens when examined at 40 d postinjury. A majority of the BMP-2-treated tendons appeared almost morphologically normal in the 40-d post-injured specimens, while wide, gaping wounds were still evident in the control tendons. These findings further demonstrated that exogenous BMP-2 was able to promote tendon healing. The fact that BMP-2 could increase the proliferation and migration of tenocytes suggests that there are many beneficial effects in the use of BMP-2 to improve tendon healing.

In summary, our data demonstrated that BMP-2 could promote tenocyte migration and proliferation and enhance effective repair of experimentally injured tendons *in vivo*. One of the major concerns of therapeutic application is the fear that BMP-2 could induce osteogenesis when implanted into damaged tendons [35]. On the contrary, we demonstrated that this was not the case, and that BMP-2 in fact promoted tenogenesis. These findings indicate that BMP-2 is a potential candidate for improving the poor healing capacity of damaged tendons.

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