 Contribution of PTHrP to mechanical strain-induced fibrochondrogenic differentiation in entheses of Achilles tendon of miniature pigs

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ARTICLE INFO

Article history:
Accepted 11 April 2014

Keywords:
Entheses
Mechanical strain
Cyclic tensile strain
Ihh
PTHrP
Fibrochondrocytes

ABSTRACT

Background: Fibrochondrocytes are involved in entheses repair, but their response to mechanical strain (MS) is ill known.

Objective: To determine if parathyroid hormone-related protein (PTHrP) expression in fibrochondrocytes from fibrocartilaginous entheses is modulated by MS, and to further observe the regulatory effects of human (h) PTHrP on fibrochondrocyte differentiation.

Methods: Fibrochondrocytes from fibrocartilaginous entheses of Guizhou miniature pig’s Achilles tendon were submitted or not to MS (4%, 8% or 12% cyclic tensile strain; 1 Hz). Fibrochondrocytes were also exposed to: cyclopamine (Indian hedgehog (Ihh) inhibitor) (10 μM), hPTHrP (10 nM) or cyclopamine/ hPTHrP (cyclopamine 10uM + hPTHrP 10 nM). Types I, II and X collagen and PTHrP expressions were measured by real-time RT-PCR and Western blot.

Result: Under 4% strain load for 12 h, types I and II collagen mRNA expressions were increased (p = 324% and +659%, P < 0.001), while type X collagen was decreased (−89%, P < 0.001). At 12%, types I and II collagen mRNA expressions were decreased (−62% and −62%, P < 0.001), while type X collagen was increased (+375%, P < 0.05). Under 4% strain load, PTHrP mRNA expression was increased in relation with strain duration (from 3 to 12 h: +168%, P < 0.001), while at 12%, PTHrP expression decreased with time (from 3 to 12 h: −81%, P < 0.001). Using cyclopamine for 24 h, PTHrP mRNA expression was significantly decreased (−88%, P < 0.05), types I and II collagen were decreased (−90% and −82%, P < 0.001), and type X collagen was increased (+261%, P < 0.001).

Conclusions: Dynamic MS modulate PTHrP expressions. Thus, PTHrP might play an important role in fibrochondrocyte differentiation, indirectly revealing a role in entheses’ formation and repair.

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1. Introduction

Injuries of insertion sites of ligament or tendon (entheses) are common (Benjamin et al., 2002), and medical treatment or surgical reconstruction does not always allow the complete recovery of their anatomy and physiological functions (Koike et al., 2005; Benjamin and Ralphs, 1998). Surgical repair of tendons is associated with a variable efficacy and with some complications. Indeed, even if surgery decreases the risk of rerupture, complications such as infection, nerve injury, adhesions and deep vein thrombosis are common (Khan and Carey Smith, 2010; Wilkins and Bisson, 2012; Willits et al., 2010; Wallace et al., 2011). Previous studies indicate that a non-operative tendon repair protocol might improve the outcomes by decreasing the risk of complications (Suchak et al., 2008).

Entheses are classified into two major subgroups: fibrocartilagenous and fibrous. In fibrous entheses, tendons are directly attached to the bone. On the other hand, fibrocartilagenous entheses are composed of four layers (fibrous connective tissue, fibrocartilage, calcified fibrocartilage and bone) that provide a graded distribution of load from tendons and muscles to bone due to variations in the extracellular matrix composition (Benjamin et al., 2002; Claudepierre and Voisin, 2005). Tendons are rich in type I collagen, while bones are composed of heavily mineralized type X collagen. Fibrocartilagenous entheses are rich in type II collagen secreted by fibrochondrocytes. In addition, a mineralization gradient is observed from the tendon to the bone (Wopenka et al., 2008). These properties provide an efficient transfer of muscle force load from tendon to bones (Genin et al., 2009). Yet, these

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http://dx.doi.org/10.1016/j.jbiomech.2014.04.022
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tissues are poorly understood, especially the regulation of growth and development of the four layers.

The fibrocartilagenous layer contributes the most to the biomechanical and biological functions of enthesis by absorbing force load and by biologically responding to strain stimulation (Mau et al., 2007). The regulation of fibrochondrocyte differentiation is important for repair and regeneration of enthesis. Proliferation and retention of cells in the premature stage could enlarge the fibrochondrocyte cell pool and prevent entheses degeneration (Kim et al., 2008). Force load is regarded as the initial factor inducing proliferation and regeneration of fibrochondrocyte (Chen et al., 2007; Rabie et al., 2003), but the regulating signaling pathways are still poorly understood.

Recently, studies showed that tendon strain stimuli is associated with the parathyroid hormone-related protein (PTHrP), an autocrine/paracrine regulatory factor with a number of functions in development (Simmonds and Kovacs, 2010). PTHrP was first identified in fibrocartilagenous insertions (Chen et al., 2007), and it seems to be induced by mechanical strain. PTHrP expression is correlated to strain strength, and may regulate proliferation and differentiation of fibrochondrocytes (Mau et al., 2007; Kim et al., 2008). PTHrP may also play an important role in mediating fibrochondrocytes’ response to mechanical stimulation, and in stimulating the development of the fibrocartilagenous layer. Indirect evidences for this possibility are provided by studies showing that mechanical stimulation enhances PTHrP expression both in vivo and in vitro (Rabie et al., 2003). Furthermore, mechanical strain exerts anabolic action in the growth phase, with increased fibrocartilage proliferation and matrix synthesis (Wang and Mao, 2002).

Thus, the development of new non-surgical recovery protocols might provide more beneficial outcomes. However, the development of these protocols requires a better understanding of the fibrocartilagenous layer biology. Therefore, the objective of the present study was to investigate the effects of cyclic tensile strain (CTS) on the expression of PTHrP in an in vitro model of cultured fibrochondrocytes from fibrocartilagenous entheses of Guizhou miniature pig’s Achilles tendon. Specifically, we hypothesized that CTS induces matrix protein secretion by fibrochondrocytes, and that the extent of this response is dependent on regulation by PTHrP.

2. Materials and methods

2.1. Animals

We used ten Guizhou miniature pigs (Experimental Animal Centre of the Third Military Medical University, Chongqing, China), weighing 25.6 ± 3.6 kg and aged 5.5 ± 2.4 months. Animals were treated according to the NIH Guide for the Care and Use of Laboratory Animals (NIH 2011). The experiments were approved by the Third Military Medical University Committee for Animal Experimentation.

2.2. Fibrochondrocyte isolation, culture and identification

Pigs were sacrificed by an overdose of pentobarbital sodium. Entheses of Achilles tendons were dissected under sterile conditions, in a size of about 1.5 × 1.5 × 2 cm3, according to the previous studies (Zhang et al., 2012; Lyons et al., 2006). Using a SZX7 stereomicroscope (Olympus Corporation, Tokyo, Japan), the Achilles tendon fibers were first removed (Fig. 1, F) to expose the cartilage fibers, and the fibrocartilage (Fig. 1, FC) was then carefully separated. Due to the fact that the calcified fibrocartilagenous layer (Fig. 1, CF) and the subchondral bone (Fig. 1, B) were harder than the fibrocartilage layer, we stopped cutting when the bone tissue began or when it became hard to cut. Fibrocartilage tissue was chopped into pieces of about 0.2–0.5 mm3.

Fibrocartilage pieces were placed in a digestion chamber. After incubation with 0.2% trypsin for 2 h and 0.2% collagenase for 18 h (GIBCO, Invitrogen Inc., Carlsbad, CA, USA), fibrocartilagenous cells were centrifuged, and the pellet was resuspended in Dulbecco’s modified eagle serum (DMEM) (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen Inc., Carlsbad, CA, USA), and 1% penicillin/streptomycin (GIBCO, Invitrogen Inc., Carlsbad, CA, USA), and 1% penicillin/streptomycin (GIBCO, Invitrogen Inc., Carlsbad, CA, USA), and 1% penicillin/streptomycin (GIBCO, Invitrogen Inc., Carlsbad, CA, USA).

2.3. Application of cyclic tensile strain

Fibrochondrocytes from the third passage (5 × 105 cells/well) were grown on collagen 1-coated BioFlex 6-well culture plates (Flexcell, McKeesport, PA, USA) to 80% confluence (6–7 days) in 5% CO2 at 37 °C. Cells were subjected to a cyclic biaxial tensile strain (CTS) (Wong et al., 2003; Saminathan et al., 2013; Saminathan et al., 2013; Chen et al., 2013) at 45, 85, and 12% of tension, using a tensile frequency of 1 Hz, in a Flexcell-4000™ flexcell System (Flexcell, McKeesport, PA, USA). Fibrochondrocytes not subjected to CTS were used as negative control. Experimental fibrochondrocytes were collected after 3, 6 or 12 h for real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) and Western blot detection of types I, II and X collagen and PTHrP mRNA and protein expression. Types I and II collagen are secreted by fibrochondrocytes, and type X collagen is secreted from chondrocytes during endochondral ossification (Leitinger and Kwan, 2006; Dai et al., 2013). These three types were then assessed to determine fibrochondrogenic differentiation.

Before being used for Western blot and PCR assays, culture medium was discarded and membranes were washed with PBS to remove any unattached cell that was not submitted to strain. Attached strain-submitting cells were obtained by scraping the membrane. Using commercial BioFlex dishes, only a few cells were found to be detached (<1%).

2.4. Treatments of fibrochondrocytes

Cyclopamine is an inhibitor of the Indian hedgehog (Ihh) pathway, which is involved in chondrocytes hypertrophy and in bone formation, creating a negative feedback loop with PTHrP (St-Jacques et al., 1999). Cyclopamine (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 100% DMSO, then diluted in complete medium (DMEM + 10%FBS). DMSO concentration was less than 0.1% in each well. Ihh/PTHrP (PeproTech, Rocky Hill, NJ, USA) was dissolved in complete medium.

Thus, four experimental groups were established: control (DMSO (~0.1%) + medium, n = 3) as negative control, Ihh- (Cyclopamine 10 μM, n = 3), human (h) PTHrP (hPTHrP 10 nM, n = 3), and Ihh-/PTHrP (Cyclopamine 10 μM + PTHrP 10 nM, n = 3) (Chen et al., 2002; Kosher et al., 1998). Fibrochondrocytes were collected for analysis after 3, 6, 12 or 24 h of treatments. Types I, II and X collagen and PTHrP expressions were determined by real-time RT-PCR and Western-blot.

2.5. Real-time RT-PCR

mRNA expression of PTHrP, and types I, II and X collagen were analyzed by real-time RT-PCR. Total RNA was extracted using TRIzol (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer’s instructions. RNA purity was determined using absorbance at 260 and 280 nm (A260/A280), and RNA integrity was verified by electrophoresis in formaldehyde gels. cDNA synthesis was performed using ReverTra Ace-a-First Strand cDNA synthesis kit (TOYOBO Co., Ltd., Tokyo, Japan), according to the manufacturer’s instructions. Specific mRNA quantification was performed by real-time PCR using the SYBR® Green Realtime
PCR Master Mix (TOYOBO Co., Ltd., Tokyo, Japan) in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Amplification was carried out for 40 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C. The Ct value was defined as the number of PCR cycles in which the fluorescence signal exceeded the detection threshold value. The relative mRNA expression of target genes was calculated using the $2^{-\Delta \Delta Ct}$ method (Livak and Schmittgen, 2001). β-Actin was used as the internal control. Primers are shown in Table 1. The experimental cells were compared relatively to the negative controls, and all negative controls were attributed a value of 1.

2.6 Western blot

PTHrP, and types I, II and X collagen were analyzed from total protein extracts obtained using the RIPA lysis buffer (Genemine, Chongqing, China). Proteins were quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein (18 μg) were separated by 6–10% SDS-PAGE. After electrophoresis, proteins were electrotransferred onto Immun-Blot™ PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). Non-specific sites were blocked with 5% non-fat milk for 2 h. Proteins were detected using the following primary antibodies: goat polyclonal antibody against type I collagen, mouse polyclonal antibody against type II collagen, mouse polyclonal antibody against type X collagen, mouse polyclonal antibody against β-actin, rabbit polyclonal antibody against PTHrP, and rabbit polyclonal antibody against Ihh (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After an overnight incubation with the primary antibody, each blot was washed four times with Tris-buffered saline and Tween 20 (TBST) buffer. Blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, and HRP-conjugated rabbit anti-goat IgG (Zhongshan Goldenbridge Biotechnology Co. Ltd., Beijing, China). The western Lighting Chemiluminescence Reagent + (Perkin-Elmer Life Sciences, Waltham, MA, USA) was used as HRP substrate. Protein bands were semi-quantitatively assessed by densitometry analysis using the Kodak Image Station 1000 (Kodak, Rochester, NY, USA) and the KODAK 1D image analysis software. The experimental cells were compared relatively to the negative controls, and all negative controls were attributed a value of 1.

2.7 Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as means ± SEM from three independent experiments performed in triplicates. All quantitative parameters were subjected to statistical analysis by one-way analysis of variance (ANOVA) with Tukey’s test for post-hoc analysis. The level of significance was set to $P < 0.05$.

3. Results

3.1 Fibrochondrocyte identification

Approximately 2 g of fibrocartilage layer tissue was harvested from entheses of pig’s Achilles tendons. After digestion, separation and culture, cells were subcultured and the third generation was used for these experiments. When observed under inverted microscopy, cells were highly homogeneous, spindle-shaped or oval, with 2–3 short protuberances, rounded nucleus, adherent growth and a good refraction (Fig. 2A). Using HE staining (Fig. 2B), cytoplasm was pink. Using Alcian blue staining (Fig. 2C), cytoplasm was blue, showing that their cytoplasm was rich in proteoglycans. Fig. 2D and E shows types I and II collagen immunohistochemical

<table>
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<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product length (bp)</th>
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<tr>
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<td>Sense</td>
<td>5'-GGG CCT CGG GTC CCA TG-3'</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-GGG CCA TCT GAC GAG CT-3'</td>
</tr>
<tr>
<td>Collagen II</td>
<td>Sense</td>
<td>5'-TTC GGT GTC ACG GGC AGG ATG T-3'</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-GGG GCA GCA CTC TCC GAA GGG-3'</td>
</tr>
<tr>
<td>Collagen X</td>
<td>Sense</td>
<td>5'-GGG CGG GCT GGA ATT TCT GTT AC-3'</td>
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<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-GGG GGC CAG GAG CAC CAT AT-3'</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Sense</td>
<td>5'-CTC CCT CAC CGT GGC TGT CCT C-3'</td>
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<td>5'-GGG AAT GGC CCG CAG CTC TCC-3'</td>
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<tr>
<td>β-Actin</td>
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<td>Anti-sense</td>
<td>5'-CCCAAGACGAGCTGTTACATA-3'</td>
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Fig. 2. Micrographs of the third generation fibrochondrocytes (magnification, × 100). (a) Cell morphology, (b) hematoxylin–eosin staining, (c) alcian blue staining, (d) type I collagen immunohistochemistry, and (e) type II collagen immunohistochemistry.
staining. These staining confirmed that the experimental cells were fibrochondrocytes.

3.2. Effects of CTS on collagen expression

In CTS experiments, using a 4% strain load at 1 Hz, types I and II collagen mRNA expression and protein secretion increased with increased exposure time \((P < 0.05)\) (Fig. 3A and D). From 3 to 12 h, mRNA expression increased from \(1.12 \pm 0.12\) to \(4.24 \pm 0.13\), and from \(1.69 \pm 0.28\) to \(6.59 \pm 0.15\), respectively. On the other hand, type X collagen expression decreased from \(0.34 \pm 0.08\) to \(0.11 \pm 0.03\) \((P < 0.05)\).

Using an 8% strain load at 1 Hz, types I and II collagen mRNA expression and protein secretion increased with increased exposure time \((P < 0.05)\) (Fig. 3B and E). From 3 to 12 h, mRNA expression increased from \(1.04 \pm 0.08\) to \(2.61 \pm 0.22\), and from \(1.39 \pm 0.19\) to \(2.62 \pm 0.31\), respectively. This time, type X collagen expression increased from \(1.03 \pm 0.08\) to \(1.53 \pm 0.21\) \((P < 0.05)\).

Using a 12% strain load at 1 Hz, types I and II collagen mRNA expression and protein secretion decreased with increased exposure time \((P < 0.05)\) (Fig. 3C and F). From 3 to 12 h, mRNA expression decreased from \(0.89 \pm 0.17\) and \(0.38 \pm 0.11\), and from \(0.91 \pm 0.15\) to \(0.38 \pm 0.11\), respectively. Type X collagen expression increased from \(1.70 \pm 0.09\) to \(4.75 \pm 0.22\) \((P < 0.05)\).

Thus, results show that appropriate mechanical stimulation promoted tendon development by the secretion of types I and II collagen, while excessive mechanical stimulation increased the degeneration of tendon tissue into mineralized tissue.

3.3. Effect of CTS on PTHrP expression

In CTS experiments, using a 4% strain load at 1 Hz, PTHrP mRNA expression and protein secretion increased with increased duration of strain load. The mRNA expression relative values were \(1.39 \pm 0.24, 2.71 \pm 0.16\) and \(3.73 \pm 0.65\) at 3, 6 and 12 h, respectively (Fig. 4A).

Using an 8% strain load at 1 Hz, PTHrP mRNA expression and protein secretion increased with increased time of strain load. The mRNA expression relative value was \(0.99 \pm 0.16, 1.44 \pm 0.26, 2.35 \pm 0.34\) at 3, 6 and 12 h, respectively (Fig. 4B).

Using a 12% strain load at 1 Hz, PTHrP mRNA expression and protein secretion increased with increased time of strain load. The mRNA expression relative value was \(0.89 \pm 0.12, 0.59 \pm 0.15, 0.25 \pm 0.08\) at 3, 6 and 12 h, respectively (Fig. 4C).

Protein expression followed the same trend as gene expression (Fig. 4D–F).

These results suggest that mechanical stimulation affected PTHrP secretion.

3.4. Effects of cyclopamine and hPTHrP on collagen expression

Using cyclopamine to block the upstream Ihh signal, PTHrP mRNA expression was significantly decreased \((P < 0.05)\): \(0.75 \pm 0.08, 0.54 \pm 0.10, 0.32 \pm 0.12\) and \(0.12 \pm 0.06\) at 3, 6, 12 and 24 h, respectively (Fig. 5A). At the same time, types I and II collagen decreased from 3 h to 24 h (Fig. 6A): from \(0.83 \pm 0.17\) to \(0.10 \pm 0.04\), and from \(0.95 \pm 0.15\) to \(0.18 \pm 0.10\), respectively.

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Fig. 3. Types I, II and X collagen mRNA expression under 4% (A), 8% (B) and 12% (C) CTS load for 3, 6 and 12 h. Types I, II and X collagen protein expression under 4% (D), 8% (E) and 12% (F) CTS load for 3, 6 and 12 h. Values are presented relative to the negative control group (without CTS, value of 1) as means ± SEM of three independent experiments, each performed in triplicates. ***P < 0.001.
On the other hand, type X collagen expression increased \((P < 0.05)\) (Fig. 6A); gene expression increased from 1.39 ± 0.21 to 3.61 ± 0.29.

Using hPTHrP, PTHrP mRNA expression was significantly decreased \((P < 0.05)\): 0.67 ± 0.06, 0.42 ± 0.04, 0.28 ± 0.05 and 0.11 ± 0.07 at 3, 6, 12 and 24 h, respectively (Fig. 5B). Types I and II collagen mRNA expression significantly improved \((P < 0.05)\) (Fig. 6B): from 1.52 ± 0.22 to 3.51 ± 0.25, and from 1.45 ± 0.22 to 4.18 ± 0.26, respectively. On the contrary, type X collagen mRNA expression decreased from 0.90 ± 0.15 to 0.06 ± 0.04 \((P < 0.05)\) (Fig. 6B).

Using cyclopamine + hPTHrP, PTHrP mRNA expression was significantly decreased \((P < 0.05)\): 0.55 ± 0.07, 0.39 ± 0.11, 0.17 ± 0.11 and 0.06 ± 0.03 at 3, 6, 12 and 24 h, respectively (Fig. 5C). Types I and II collagen mRNA expression significantly improved \((P < 0.05)\) (Fig. 6C) from 2.35 ± 0.29 to 4.45 ± 0.28, and from 2.50 ± 0.32 to 5.66 ± 0.18, respectively. On the contrary, type X collagen mRNA expression decreased from 0.74 ± 0.12 to 0.10 ± 0.02 \((P < 0.05)\) (Fig. 6C).

PTHrP, types I, II and X collagen protein expression followed the same trend as mRNA expression (Figs. 5D–F and 6D–F).

4. Discussion

The objective of the present study was to investigate the effects of CTS on the expression of PTHrP in an in vitro model of cultured fibrochondrocytes. We showed that a low mechanical strain stimulated the fibrochondrocytes to secrete types I and II collagen, two major tendon constituents, while a high mechanical strain stimulated the secretion of type X collagen, which is involved in tendon mineralization. We also showed that CTS increased PTHrP expression. In addition, cyclopamine, an Ihh inhibitor, decreased PTHrP secretion, as well as decreasing types I and II collagen expression, while increasing type X collagen expression. Treating fibrochondrocytes with hPTHrP increased types I and II collagen expression, and decreased type X collagen expression. Thus, we showed that the differentiation of fibrocartilage was regulated by CTS via PTHrP signaling. We also demonstrated that the stage of chondrocyte differentiation was dependent upon the magnitude of CTS.

Isolation and purification of fibrochondrocytes was performed according to Zhang et al. (2012). Chondrocytes from the fibrocartilagenous layer lack specific phenotype markers (Diaz-Romero et al., 2005). The identification of fibrochondrocytes from cells with similar phenotype depends on multiple immunohistochemistry staining, and on the affinity for specific stains. Types I and II collagen and glycosaminoglycan are characteristically secreted by fibrochondrocytes. Immunohistochemistry staining for types I and II collagen was positive in our isolated fibrochondrocytes. Furthermore, HE staining and Alcian blue staining showed that these cells had a compatible morphology, and that they secreted glycosaminoglycan, in accordance with the previous studies (Alini et al., 2003).

A number of previous studies assessed the effects of mechanical strain stimuli using the Flexcell device (Wong et al., 2003; Saminathan et al., 2013, 2012; Chen et al., 2013; Banes et al., 1995; Xu et al., 2012). Banes et al. (1995) showed that tendon cells reacted within milliseconds to mechanical strain by activating a number of ion pumps and a number of signaling pathways. These results were confirmed and expanded by a number of studies (Wong et al., 2003; Saminathan et al., 2013, 2012; Chen et al., 2013; Banes et al., 1995; Xu et al., 2012).
Indeed, a previous study in chondrocytes used the Flexcell tension unit using a single 10% tension strain at 0.5 Hz, and showed that mechanical strain induced the calcification of end-plate chondrocytes, as shown by type X collagen secretion (Xu et al., 2012). In the present study, the aim was to examine the effect of different tension strains on different types of collagen, and we therefore used 4%, 8% and 12%. Different cell types may react differently to these conditions. For fibrochondrocytes, 4% and 8% strain may be not exactly consistent with their physiological conditions, even if previous studies used similar strain load (Chen et al., 2013; Deschner et al., 2006), and 10–12% may be more representative of physiological conditions (Wong et al., 2003; Saminathan et al., 2013, 2012; Xu et al., 2012; Deschner et al., 2006). However, there is no evidence indicating the exact strain load experienced by entheses. Indeed, previous studies, using ultrasound, showed that the strain load experienced by entheses is less than 4% (Magnusson et al., 2008; Maganaris et al., 2008). However, this strain is distributed along the whole length of the tendon, while it is concentrated at the level of the entheses. In addition, a previous study suggested that the fluid shear stress might be more important in the mechanics of the tendons than stretching (Lavagnino et al., 2008). Therefore, in the present study, we selected to study 4%, 8% and 12% stretching in order to simplify the stress stimulation and to observe possible associations with increasing strain. Nevertheless, more refined studies of the exact strain load specifically experienced by entheses are needed.

During the development of fibrochondrocyte hypertrophy and differentiation, types I and II collagen and glycosaminoglycan are gradually replaced by an increased secretion of type X collagen (Kosher et al., 1986; Swalla et al., 1988). Type X collagen can be monitored as an index of the differentiation extent of fibrochondrocytes (Xu et al., 2012). Expression and secretion of PTHrP in fibrochondrocytes were elevated after CTS, according to the time and extent of the strain load. After proper mechanical strain (4%, 1 Hz), PTHrP mRNA and protein expression increased with strain duration. At the same time, types I and II collagen mRNA and protein expression also increased. These results indicated that a proper strain load may stimulate the proliferation and differentiation of fibrochondrocytes, retaining them in the pre-hypertrophy stage. This stage was characterized by a continuous expression and secretion of types I and II collagen. CTS may then enlarge the fibrochondrocyte cell pool, and help to thicken the fibrocartilaginous layer, which may help to enhance the repair and regeneration of entheses after a trauma. However, after an excessive mechanical strain (12%, 1 Hz), PTHrP expression and secretion were decreased, and was associated with a concomitant decrease in the secretion of types I and II collagen and an increased type X collagen secretion, which was previously observed using a mechanical strain of 10%, 0.5 Hz (Xu et al., 2012). This may indicate that mechanical strain is the initiating factor for fibrochondrocyte differentiation, and thus the origin of enthesis development and regeneration.

PTHrP may be an important factor in fibrochondrocyte proliferation and differentiation. Strain load patterns obviously influence the expression pattern of PTHrP. When fibrochondrocytes were stretched at 4%, PTHrP expression increased with strain duration. However, PTHrP expression did not gradually increase with increasing CTS, as we anticipated, but decreased when strain extent was increased from 4% to 8% to 12%. In addition, when the stretching extent was increased to 12%, PTHrP expression decreased with time. These results suggest that mechanical strain load and PTHrP expression may increase the proliferation and differentiation of fibrochondrocytes. To understand whether PTHrP directly influence fibrochondrocyte proliferation and
differentiation, we intervened on PTHrP signaling. Types I and II collagen expression increased when the cells were submitted to higher extracellular PTHrP concentrations. At the same time, expression of type X collagen was inhibited. On the other hand, cyclopamin was used to inhibit Ihh, which also inhibit PTHrP expression (St-Jacques et al., 1999; Cooper et al., 1998). Types I and II collagen expression were found to be decreased by the inhibition of PTHrP.

PTHrP is under the direct control of the Ihh pathway (Chen et al., 2008; St-Jacques et al., 1999; Chau et al., 2011). To rule out the influence of Ihh on fibrochondrocytes, we concomitantly used cyclopamin to inhibit Ihh and extracellular PTHrP. Extracellular PTHrP still upregulated the expression of types I and II collagen without changes by Ihh inhibition, suggesting that Ihh regulates fibrochondrocyte proliferation and differentiation by upregulating PTHrP, which is in accordance with a study in an arthritis model suggesting that PTHrP and Ihh were involved in the maintenance of articular cartilage health (Chau et al., 2011). Mechanical strain may regulate the biological behavior of fibrochondrocytes through the Ihh/PTHrP signaling pathway. However, pilot testing showed that cultures cells were improperly stimulated by uniaxial strain, and we used biaxial strain. There is also the possibility that the strains that are reported by the FlexCell system are different from the strains actually experienced by the cells; indeed, it was found that the stretching strain using the FlexCell system was homogenous and reproducible, but that only about half of the strain strength was transferred to the cells (Bieler et al., 2009). Finally, there is a possibility that the cells may be actually stimulated by the fluid shear stress in the dish rather than by the stretch of the membrane itself, and this effect should be taken into consideration (Thompson et al., 2011). Therefore, we agree that our experimental conditions are imperfect and need to be improved. Nevertheless, we observed significant associations when using these conditions.

In conclusion, the present study suggests that a low mechanical strain increased types I and II collagen secretion by fibrochondrocytes, and that PTHrP signaling may be involved. A high mechanical strain increased type X collagen secretion. However, further studies are required to observe the effects of increased mechanical strain and PTHrP on tendon injury repair, and studies on how the timing and frequency of mechanical strain contribute to the regulation of fibrochondrocyte biology.

