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The promotion of osteochondral repair by combined intra-articular injection of parathyroid hormone-related protein and implantation of a bi-layer collagen-silk scaffold

Wei Zhang [a,b,1], Jialin Chen [a,b,1], Jiadong Tao [a,b], Changchang Hu [a,b], Longkun Chen [c], Hongshi Zhao [a,b], Guowei Xu [a,b], Boon C. Heng [d], Hong Wei Ouyang [a,b,*]

[a] Center for Stem Cell and Tissue Engineering, School of Medicine, Zhejiang University, Hangzhou, China
[b] Zhejiang Provincial Key Laboratory of Tissue Engineering and Regenerative Medicine, Hangzhou, China
[c] Zhejiang Xingyue Biotechnology Co., Ltd., China
[d] Department of Biosystems Science & Engineering (D-BSSE), ETH-Zurich, Mattenstrasse 26, Basel 4058, Switzerland

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ABSTRACT

The repair of osteochondral defects can be enhanced with scaffolds but is often accompanied with undesirable terminal differentiation of bone marrow-derived mesenchymal stem cells (BMSCs). Parathyroid hormone-related protein (PTHrP) has been shown to inhibit aberrant differentiation, but administration at inappropriate time points would have adverse effects on chondrogenesis. This study aims to develop an effective tissue engineering strategy by combining PTHrP and collagen-silk scaffold for osteochondral defect repair. The underlying mechanisms of the synergistic effect of combining PTHrP administration with collagen-silk scaffold implantation for rabbit knee joint osteochondral defect repair were investigated. *In vitro* studies showed that PTHrP treatment significantly reduced Alizarin Red staining and expression of terminal differentiation-related markers. This is achieved in part through blocking activation of the canonical Wnt/β-catenin signaling pathway. For the *in vivo* repair study, intra-articular injection of PTHrP was carried out at three different time windows (4–6, 7–9 and 10–12 weeks) together with implantation of a bi-layer collagen-silk scaffold. Defects treated with PTHrP at the 4–6 weeks time window exhibited better regeneration (reconstitution of cartilage and subchondral bone) with minimal terminal differentiation (hypertrophy, ossification and matrix degradation), as well as enhanced chondrogenesis (cell shape, Col2 and GAG accumulation) compared with treatment at other time windows. Furthermore, the timing of PTHrP administration also influenced PTHrP receptor expression, thus affecting the treatment outcome. Our results demonstrated that intra-articular injection of PTHrP at 4–6 weeks post-injury together with collagen-silk scaffold implantation is an effective strategy for inhibiting terminal differentiation and enhancing chondrogenesis, thus improving cartilage repair and regeneration in a rabbit model.

1. Introduction

Articular cartilage plays an important role in dissipating mechanical stress and enabling smooth, frictionless limb movement. Due to its biomechanical function, cartilage tissue is highly susceptible to injuries arising from sports activities. In the USA alone, more than 400,000 cartilage related-procedures were performed annually [1]. The repair of cartilage defects remains a major challenge due to the limited regenerative capacity of native cartilage. Cartilage tissue engineering is emerging as a promising alternative treatment approach.

It is well documented that the use of tissue engineering strategies could promote cartilage repair [2–4]. However, some studies reported that healing of osteochondral defects was often accompanied with chondrocyte terminal differentiation, usually followed by inadvertent endochondral ossification, a process that generally occurs in the growth plate during endochondral bone formation [5–7]. Bone marrow-derived mesenchymal stem cells (BMSCs), which are recruited from the marrow cavity to the defect site

* Corresponding author. Mailbox #39, Center for Stem Cell and Tissue Engineering, School of Medicine, Zhejiang University, #866 Yu Hang Tang Road, Hangzhou 310058, China. Tel./fax: +86 571 88208262.
E-mail addresses: hwoy@zju.edu.cn, hongweiouyang@yahoo.com.cn (H.W. Ouyang).
[1] These co-authors contributed equally to this study.

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during the healing process [8,9], often undergo uncontrolled terminal differentiation [4,10,11]. The biochemical markers of terminal differentiation and matrix enzymes are up-regulated, resulting in progressive hypertrophy, calcification, ossification and degradation of the cartilage matrix [5,12,13].

We have previously used a bi-layer collagen type 1 (col1) – silk incorporating hydroxyapatite (HAp) scaffold (collagen-silk scaffold) to treat osteochondral defects, the materials of which are extensively used in cartilage (collagen) and bone (silk & HAp) tissue engineering [3,14–17]. Although this scaffold promoted the repair of osteochondral defects, the regenerated tissue exhibited obvious chondrocyte hypertrophy, which made the repaired cartilage unstable and negated the benefits of scaffold implantation [12]. Similar observations have been widely reported in the clinic [7,18–20]. Excessive osseous tissue would alter the biomechanical properties of the joint surface and impair the function of the repaired cartilage. Therefore, to further improve cartilage tissue engineering, it is imperative to inhibit chondrocyte terminal differentiation during the repair process.

Parathyroid hormone-related protein (PTHrP) has long been well known to be an anti-hypertrophy factor in the growth plate [21–24]. It is secreted by periarticular proliferating chondrocytes, and acts in conjunction with Indian hedgehog (Ihh) through a negative feedback loop to regulate endochondral ossification by inhibiting chondrocyte maturation and hypertrophy [25]. Recently, PTHrP was also found to be expressed on the surface layer of articular cartilage [26], where it plays a key role in articular cartilage maintenance and protection [27,28]. Previous studies demonstrated that it inhibits terminal differentiation of cultured articular chondrocytes and MSCs [29–32], as well as prevent osteoarthritis (OA) progression [28,32]. However, this inhibitory function has not yet been observed in the cartilage repair model *in vivo*. Recent genome-wide expression analysis revealed that the expression of PTHrP is markedly decreased in repaired cartilage tissue [13]. Hence, supplementation with exogenous PTHrP may have the potential of enhancing cartilage repair by inhibiting terminal differentiation.

Although PTHrP exhibits potential in inhibiting chondrocyte terminal differentiation, there are still remaining questions with regards to the timing of its administration. Previous studies have reported that BMSCs displayed incomplete chondrogenesis when treated with PTHrP at inappropriate time points [30,33,34]. Thus, a suitable time window to administer PTHrP seems to be crucial for cartilage repair, ensuring optimal chondrogenesis and inhibition of terminal differentiation. Nevertheless, studies to explore a suitable time window for PTHrP administration in cartilage repair has not yet been reported.

Hence, this study aimed to investigate the appropriate timing of PTHrP administration, so as to develop an effective tissue engineering strategy by combining PTHrP treatment with collagen-silk scaffold implantation for osteochondral defect repair. In addition to improving the repair efficacy, two other aspects were also taken into consideration in the present treatment scheme: 1. Inhibiting chondrocyte terminal differentiation and endochondral ossification. 2. Ensuring optimal chondrogenic differentiation of BMSCs. The function of PTHrP in chondrocyte terminal differentiation was evaluated and the underlying mechanisms were explored *in vitro*. After confirming the inhibitory effect, intra-articular injection of PTHrP together with implantation of a bi-layer collagen-silk scaffold was carried out at different time windows in a rabbit osteochondral defect model.

2. Materials and methods

2.1. Chondrocytes isolation and culture

Human articular cartilage samples were explanted from the normal area of knee joints of 3 patients (mean age 60 years, range 53–66 years) with osteoarthritis who had undergone total knee replacement surgery. The institutional review board of Zhejiang University approved this study. Cell isolation and culture were performed as described previously [35]. The tissue was cut into 1–2 mm^2 pieces, washed 3 times with phosphate-buffered saline (PBS) and then digested with 0.25% collagenase (Sigma) overnight at 37 °C. Cell suspensions were cultured in DMEM/F-12 Media (Xingyue Biotechnology, Zhejiang, China) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% (v/v) penicillin–streptomycin solution (Gibco, Grand Island, NY, USA). The medium was changed every 3 days.

2.2. Terminal differentiation of chondrocytes

Chondrocyte terminal differentiation was induced in culture medium consisting of 10 mM β-glycerol phosphate (Sigma), 0.1 μM dexamethasone (Sigma), and 50 μg/ml ascorbic acid (Sigma) in DMEM-high glucose containing 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin for 2 weeks. Where indicated, 10 nM of recombinant human PTHrP (1–40) (CHINESE PEPTIDE, Zhejiang, China) was added. The medium was changed every 3 days.

2.3. Immunocytochemistry

Monolayer cultures of chondrocytes were fixed in 4% (v/v) paraformaldehyde and subjected to immunostaining with primary antibodies of rabbit anti-β-catenin (Abcam, Shatin, N.T., Hong Kong) and rabbit anti-Collagen type 10 (Col10, Abcam, Shatin, N.T., Hong Kong), followed with a goat anti-rabbit-FITC IgG (Invitrogen) secondary antibody. DAPI staining was used to reveal the nuclei.

Col10 fluorescence intensity was quantified in 5 randomly-selected high-power fields (400×) utilizing Image-Pro Plus software (Media Cybernetics, Silver Spring, USA). The data was expressed as average integrated optical density (IOD) per number of cells (IOD/cell) and normalized to the control group.

2.4. Alizarin red staining

To detect calcium mineralization, monolayer cultures of chondrocytes were fixed with 4% (v/v) paraformaldehyde and then stained with alizarin red (0.5%, pH = 4.2). Stained monolayers were visualized under a light microscope (X71; Olympus, Tokyo, Japan). For quantification of staining, the cells were destained with 0.5 N HCl and 5% sodium dodecyl sulfate (SDS). Light absorbance of the extracted dye was measured at 405 nm (TECAN). The data was expressed as average IOD normalized to the control group.

2.5. RNA isolation and real-time PCR

Total cellular RNA isolation and Real-time PCR analysis of genes including Matrix metalloproteinase 13 (MMP13), Col10, A disintegrin and metalloproteinase with thrombospondin motifs 5 (Adamts5), Col1, Frizzled-6 (FZD-6), Low-density lipoprotein receptor-related protein 5 (LRP-5), T-cell factor-1 (TCF-1), Lymphoid enhancer factor-1 (LEF-1), c-jun, axis inhibition protein 2 (axin2) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed as described previously [36]. All primer sequences (Invitrogen Inc., Carlsbad, CA, USA) are summarized in Table 1. Each real-time PCR run was performed with at least three experimental replicates, and the results are presented as target gene expression/GAPDH normalized to the 3d control group.

2.6. Western blot analysis

Western blot analysis was performed as described previously [37]. The primary antibodies utilized were as follows: Col10 (Abcam, Shatin, N.T., Hong Kong), β-catenin (Abcam, Shatin, N.T., Hong Kong), and GAPDH (Beyotime Institute of Biotechnology Inc., Jiangsu, China). The secondary antibodies utilized were as follows: goat anti-mouse and goat anti-rabbit (Beyotime Institute of Biotechnology Inc., Jiangsu, China).

2.7. Preparation and characterization of scaffold

2.7.1. Scaffold fabrication

Bi-layer collagen-silk scaffold was fabricated, which had a collagen layer for cartilage repair and a composite HAp-silk layer for subchondral bone repair. The HAp-silk scaffold was fabricated as previously described with silk:HAp ratios of 6:4 [14]. Briefly, silk fibroin protein was extracted from *Bombyx mori* cocoons with an aqueous solution containing 0.02 M Na_2CO_3 at 100 °C for 60 min, and then dissolved in 9.3 M LiBr for 4 h at 60 °C. HAp was mixed with NaCl particles and then the mixture was added into the silk solution (16% w/v). Once the scaffold solidified, the salt was subsequently extracted by immersion in water. Col1 was isolated and purified from porcine Achilles' tendon using neutral salt and dilute acid extractions [17,38]. Collagen solution (10 mg/ml) was added onto the HAp-silk scaffold and then lyophilized in a freeze dryer (Heto Power Dry LL1500) for 7 days to allow the formation of collagen microsponges. Scaffolds were cut into cylinders of 4 mm × 3 mm, sterilized in 75% ethanol and washed with PBS before use.