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Synergistic promoting effects of bone morphogenetic protein 12/connective tissue growth factor on functional differentiation of tendon derived stem cells and patellar tendon window defect regeneration

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Current study investigated bone morphogenetic protein 12 (BMP12) and connective tissue growth factor (CTGF) activate tendon derived stem cells (TDSCs) tenogenic differentiation, and promotion of injured tendon regeneration. TDSCs were transfected with BMP12 and CTGF via recombinant adenovirus (Ad) infection. Gene transfection efficiency, cell viability and cytotoxicity, tenogenic gene expression, collagen I/III synthesis were evaluated *in vitro*. For the *in vivo* study, the transfected cells were transplanted into the rat patellar tendon window defect. At weeks 2 and 8 of post-surgery, the repaired tendon tissues were harvested for histological and biomechanical examinations. The transfected TDSCs revealed relatively stable transfection efficiency (80–90%) with active cell viability means while rare cytotoxicity in each group. During days 1 and 5, BMP12 and CTGF transfection caused tenogenic differentiation genes activation in TDSCs: type I/III collagen, tenascin-C, and scleraxis were all up-regulated, whereas osteogenic, adipogenic, and chondrogenic markers were all down-regulated respectively. In addition, BMP12 and CTGF overexpression significantly promote type I/III collagen synthesis. After *in vivo* transplantation, at 2 and 8 weeks post-surgery, BMP12, CTGF and co-transfection groups showed more integrated tendon tissue structure versus control, meanwhile, the ultimate failure loads and Young's were all higher than control. Remarkably, at 8 weeks post-surgery, the biomechanical properties of co-transfection group was approaching to normal rat patellar tendon, moreover, the ratio of type III/I collagen maintained about 20% in each transfection group, meanwhile, the type I collagen were significantly increased with co-transfection treatment. In conclusion, BMP12 and CTGF transfection stimulate tenogenic differentiation of TDSCs. The synergistic effects of simultaneous transfection of both may significantly promoted rat patellar tendon window defect regeneration.

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1. Introduction

Tendon injuries are a common clinical problem as damaged tendon tissue heals slowly and rarely regains the integrity or strength as of a normal tendon. In an effort to improve the care of patients with tendon injuries, cell therapies and tissue engineering strategies offer potential approaches toward tendon repair (Yin et al., 2010).

Recently, many cell types have been used with or without scaffold materials for tendon repair (Al-Ani et al., 2015; He et al., 2015; Hao et al., 2016). With the advancement in tissue engineering, clinical medicine is approaching a new phase of "regenerative medicine". The most common seed cell for regenerative medicine is mesenchymal stem cells (MSC), which have good capability of self-renewal and tenogenic differentiation. In addition, bone marrow mesenchymal stem cells (BMSCs) (He et al., 2015), adipose derived stem cells (ADSCs) (Uysal and Mizuno, 2010), embryonic stem cells (ESCs) (Chen et al., 2014) and induced pluripotent stem cells (iPSCs) (Xu et al., 2013) were reported to be used for tendon remodeling. These stem cells have certain advantages and

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disadvantages, such as uneven extracellular matrix arrangement, repair parts of ossification, and a certain incidence of teratoma. Previously we demonstrated a new type of stem/progenitor cells isolated from the tendon tissues called tendon derived stem cells (TDSCs) (Xu et al., 2015).

TDSCs were first reported by Bi, et al and then further characterized by Zhang and Wang as well as several other groups from varied species and tendon tissues (Bi et al., 2007; Zhang and Wang, 2010a,b; Goncalves et al., 2017; Pillai et al., 2017). Following transplantation of these stem cells to injured tendon, a fast proliferation and high affinity to tendon niches and microenvironment, TDSCs differentiation to functional tenocytes to promote damaged tendon regeneration (Al-Ani et al., 2015). Thus, how to rapidly and safely stimulate tenogenic differentiation of TDSCs play a important role to repair damaged tendons is still a question.

In the growth and development of tendon damage and repair process, there are many growth factors and cytokines involved in the activities particularly transforming growth factor beta (TGF-beta) superfamily (Pryce et al., 2009; Barsby and Guest, 2013), fibroblast growth factor (FGF) (Tokunaga et al., 2015), insulin-like growth factor (IGF-1) (Dahlgren et al., 2002), mechano-growth factor (MGF) (Zhang et al., 2016) and others. Currently, bone morphogenetic protein 12, also known as growth differentiation factor GDF-7 have been reported to induce tenogenic differentiation of stem cells (Shen et al., 2013), and connective tissue growth factor (CTGF) have the effects on cellular fibrosis (Kinashi et al., 2017). Reports said that CTGF-stimulated MSCs lost their surface mesenchymal epitopes, expressed broad fibroblastic hallmarks, and increased synthesis of collagen type I and tenacin-C led the cells failed to show osteogenic or chondrogenic differentiation. However, CTGF-treated MSCs were α-SMA negative, differentiated into α-SMA positive myofibroblasts only when stimulated subsequently with TGF-β1 (Lee et al., 2015).

Therefore, in this study, we used recombinant adenovirus that over-expressing BMP12 and CTGF into TDSCs via infection. The results showed that BMP12 and CTGF transfection stimulate tenogenic differentiation of TDSCs by upregulating tenogenic whereas downregulating osteogenic, adipogenic, and chondrogenicgene expression levels, and stimulating collagen I/III synthesis. Also, the synergistic effects of simultaneous transfection with BMP12 and CTGF significantly promote rat patellar tendon window defect regeneration.

2. Materials and methods

2.1. Isolation and identification of rat TDSCs

Healthy Sprague-Dawley (SD) rats (200–250 g) were obtained from Daping Hospital of the Third Military Medical University (Chongqing, China). TDSCs were isolated according to our previous protocol (Xu et al., 2015). After 12 days the colonies formed, then were sub-cultured until third passage (P3) which were used for experiments. To demonstrate mesenchymal lineages differentiation potential of TDSCs, we carried out the protocol of TDSCs differentiation into osteoblasts, adipocytes, and chondrocytes as our previous study (Al-Ani et al., 2015). In addition, immunofluorescence was used for confirming TDSCs marker: nucleostemin (abcam ab32682, 1:200).

2.2. Recombinant adenovirus infection and experimental groups

The BMP12- and CTGF- recombinant adenoviruses were donated from Prof. Tong-Chuan He in Molecular Oncology Laboratory, The University of Chicago, and created using the AdEasy system according to the published protocol (Luo et al., 2007). All the recombinant adenoviruses system in this study conjuncted with green fluorescent protein (GFP) in order to trace the transfection. Based on the pilot experiments, we confirmed the best multiplicity of infection (MOI) for our recombinant adenoviruses to TDSCs was 100 MOI. Transfection efficiency for TDSCs were observed via fluorescence microscopy at week 2 post-infection. For *in vitro* and *in vivo* experiments, the transfected cells were cultured until specific assay, the medium was changed every 2–3 days. The experimental groups were: recombinant adenoviruses-GFP group (Ad-GFP indicates Control), recombinant adenoviruses -BMP12-GFP group (Ad-BMP12-GFP: "BMP12″ for short), recombinant adenoviruses-CTGF-GFP group (Ad-CTGF-GFP: "CTGF" for short), and simultaneous transfection with Ad-BMP12-GFP and Ad-CTGF-GFP (the co-transfection: "Co-trans").

2.3. Cell viability and cytotoxicity assay

Cell viability and cytotoxicity was performed by MTS assay (Promega), which detected whether BMP12 and CTGF transfection showed effects on cell proliferation. 5×10^3 TDSCs were seeded in each well of 24-well plate, after 24 h, non-adherent cells were discarded. Then, four groups were divided and set, followed by infection of different recombinant adenovirus with 100 MOI. After a period of 7 days culture, in each day, transfected cells were incubated with 10% of MTS reagent containing serum free medium for 3 hours at 37 °C with 5% CO_2 incubator, then aliquots were pipetted into a 96-well plate for measurement at 490 nm as a spectrophotometric reader.

2.4. Total RNA extraction and real-time RT-PCR

TDSCs were seeded in 6-well plates, and divided into four groups (Ad-GFP, Ad-BMP12-GFP, Ad-CTGF-GFP, and co-transfection). In 1 and 5 days after transfection, the transfected cells were directly lysed in the 6-well plates with RNA pure High-purity Total RNA Rapid Extraction Kit(Spin-column) (Bio Teke corporation, Beijing, China) according to the manufacturer's instruction. The total RNA was quantified spectrophotometrically and reverse-transcribed using the RT-PCR protocol (Thermo Scientific Rt-First Strand cDNA Synthesis kit, K1622) according to the manufacturer's instructions in two steps. The SsoAdvanced SYBR Green PCR supermix (Bio-Rad No.1725264) and CFX96 Real-Time PCR Detection System (Bio-Rad) was used to perform qRT-PCR. All the primers were designed via NCBI primer blast and synthesized by Invitrogen (Table 1). The data was analyzed by the $2^{-\Delta\Delta ct}$ method.

Table 1
List of Primer Sequences for Real-Time PCR.

Genes	Primer sequences (forward/reverse)	Product size (bp)
Collagen I	5′-AAGGTGACAGAGGCATAAAG-3′ 5′-GGAAGCTGAAGTCATAACCA-3′	297
Collagen III	5′-CATGATGAGCTTTGTGCAAT-3′ 5′-CTGCTGTGCCAAAATAAGAG-3′	76
Ten-C	5′-CCAACATCACAGACTCAGAA-3′ 5′-CAATTCTCTTGGGGAATCCA-3′	257
SCX	5′-CTGTGAACAGAGAGATGGAC-3′ 5′-CAGCTACTTAGAGTCAAGCC-3′	51
Runx2	5′-GTTAATCTCTGCAGGTCACT-3′ 5′-AAAGAAGTTTTGCTGACACG-3′	363
PPARγ	5′-ATCAGTGGGAATTAAGGCAA-3′ 5′-TCTTGTGAAGTGCTCATAGG-3′	122
SOX-9	5′-AAGTGAAGGTAACGATTGCT-3′ 5′-CTCACTAACTCTGAAGGAGC-3′	153
GAPDH	5′-CAAGGTCATCCATGACAACT-3′ 5′-CAGATCCACAACGGATACAT-3′	183

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