Bone & Joint Research

Supplementary Material

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Supplementary Methods

Animal experiments in this study were conducted in compliance with nationally or internationally recognized guidelines, and were approved by the institutional Animal Care and Use Committees of Xiangya Hospital of Central South University. All the mice were housed in animal facility of Central South University with controlled temperature and light cycles (24°C and 12 hr/12 hr light cycle).

Mechanical loading

In the present study, mice were assigned to run on a motor-powered treadmill to generate a mechanical stimulation to the rotator cuff (RC) tendon-bone interface (TBI). Briefly, all mice received a one-week acclimatization training course of treadmill running before surgery. The speed (5 m/min to 10 m/min) and duration (5 mins to 20 mins) of the treadmill were increased gradually until all mice were able to tolerate running at 10 m/min for 20 minutes on the treadmill. On postoperative day 7, the mice that were assigned for treadmill running began to run at a speed of 10 m/min on a 0° declined lane for 20 minutes per day, five days per week.

Mesenchymal stem cell fate tracing

To trace mesenchymal stem cell (MSC) chondrogenesis at the TBI healing site, the Prx1-CreER-GFP mice (Stock No: 029211; Jackson lab, USA) were crossed with the R26R-EYFP mice (Stock No: 006148; Shanghai Model Organisms Center, China) to generate Prx1-CreER-GFP; R26R-EYFP mice and they were injected intraperitoneally with tamoxifen (75 mg/kg; MilliporeSigma, USA) for five days before surgery. At four weeks after surgery, the mice were sacrificed to harvest the SST and humeral head composite for immunofluorescence.

Immunofluorescence

The specimens were fixed, decalcified, and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, USA). The 5 µm frozen sections were blocked with 5% bovine serum albumin (BSA) (Gibco, USA) and incubated with the primary antibodies at 4°C overnight, After washing, the sections were then incubated with the respective secondary antibodies (1:500; Abcam, UK) for one hour at room temperature while avoiding light and sealed with 4’,6-diamidino-2-phenylindole (DAPI). To trace MSC chondrogenesis, the primary antibodies were anti-GFP (1:200) and anti-SOX9 (1:200); and

to test macrophage polarization, the primary antibodies were anti-F4/80 (1:200), anti-CD86 (1:200), and anti-CD206 (1:200; all Abcam). The images were captured using a fluorescence microscope. Positively stained cells in the TBI were quantified in three random visual fields of high-power field (HPF) (200 μm × 200 μm) using ImageJ (National Institutes of Health, USA).

Application of cyclic mechanical stretch in vitro

BMDMs were seeded on fibronectin (MilliporeSigma) coated plate, which could expand and contract under external forces. After 24 hours of culture, cells were washed twice with phosphate-buffered saline (PBS) and the media was changed to Dulbecco's Modified Eagle Medium (DMEM) without serum. Cells were then subjected to the cyclic stretch of low (5%), moderate (10%), and high (15%) magnitude at a frequency of 0.5 Hz with CELLOAD-300 (Hao Mian Technology, China). After 12 hours, cells were trypsinized for flow cytometry and quantitative real-time polymerase chain reaction (qRT-PCR). The conditioned media of statically cultured BMDMs (SC-CM) and optimal load-polarized BMDMs were collected, centrifuged, and then filtered via a 0.22 μm syringe filter. DMEM media without cellular exposure was used as a control.

Enzyme-linked immunosorbent assay

The concentrations of pro-chondrogenic cytokines in BMDMs conditioned media and DMEM media were quantified by enzyme-linked immunosorbent assay (ELISA) kits (CLOUD-CLONE CORP, Wuhan, China) according to the manufacturer's protocol. The measured cytokines included transforming growth factor (TGF)-β1 and TGF-β3, bone morphogenetic protein 2 (BMP2), fibroblast growth factor 2 (FGF2), and platelet-derived growth factor (PDGF)-BB.

Chondrogenic differentiation assay

The micromass culture system was conducted to assess the chondrogenic ability by using Mesenchymal Stem Cell Chondrogenic Differentiation kit (Cyagen Biosciences). In brief, 4×10^5 MSCs were suspended in a complete chondrogenic induction media and then pelleted in the centrifuge tube. The complete chondrogenic induction media was prepared by the addition of dexamethasone, ascorbate, Insulin-Transferrin-Selenium (ITS) supplement, sodium pyruvate, proline, and TGF-β3 into differentiation basal media, which was supplemented with conditional media from BMDMs or DMEM at a ratio of 2:1. After three weeks of induction, the micromasses were fixed, embedded with paraffin, and cut into 5 μm thick sections for Alcian blue staining and immunohistochemistry (IHC). Besides, chondrogenic pellets were collected for glycosaminoglycan (GAG) quantification and qRT-PCR.

Immunohistochemistry

Paraffin sections were rehydrated and retrieved for antigen using sodium citrate antigen retrieval solution (Solarbio, China), and immersed in 3% H_2O_2 to inactivate endogenous peroxidase. The sections were blocked with 5% bovine serum albumin, treated with anti-collagen type II alpha 1 primary antibody (Abcam) at 4°C overnight, and were then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for one hour. Subsequently, the sections were stained with DAB (ZSGB-BIO, China) for five minutes, counterstained with haematoxylin for three minutes, dehydrated, and imaged under light microscopy.

Glycosaminoglycan quantification

GAG content and DNA content in the chondrogenic pellet were determined by using an Acidic Mucopolysaccharide Kit (KAMIYA, USA) and PicoGreen dsDNA quantitation assay (YEASEN, China) based on their manual instruction. Next, the GAG content was normalized to the DNA content.

Table i. List of primer sequences used in this study.

Gene	Forward primer	Reverse primer
TNF-α	GCTCCTCCACTTGGTGGTTT	AGGCGGTGCCTATGTCTCAG
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
CCR7	TGTACGAGTCGGTGTGCTTC	GGTAGGTATCCGTCATGGTCTTG
Arg-1	GAGCCACCGTTTTACATTGTGA	CTCGCCCACTAGGCAGTTC
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
CD206	AGCTTCATCTTCGGGCCTTTG	GGTGACCACTCCTGCTGCTTTAG
TGF-β1	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
TGF-β3	CAGGCCAGGGTAGTCAGAG	ATTTCCAGCCTAGATCCTGCC
BMP2	GGGACCCGCTGTCTTCTAGT	TCAACTCAAATTCGCTGAGGAC
FGF2	TGGTGACCACAAGCTGAATG	TCCCTTGATAGACACAACTCCTC
PDGF-BB	CATCCGCTCCTTTGATGATCTT	GTGCTCGGGTCATGTTCAAGT
SOX9	GTGGATGTCGAAGCAGCAG	CTCAGCTGCTCCGTCTTGAT
COL2A1	GGTGTCAAGGGTCACAGAGG	CACTCTCACCCTTCACACCC
Aggrecan	ACCTGTGTGAGATCGACCAG	GGAGTGACAATGCTGCTCAG
β-actin	GGAGATCACAGCTCTGGCT	GTCGATTGTCGTCCTGAGG

Arg-1, Arginine-1; BMP2, bone morphogenetic protein 2; CCR7, chemokine receptor type 7; COL2A1, collagen type II alpha 1; FGF2, fibroblast growth factor 2; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; PDGF-BB, platelet-derived growth factor-BB; SOX9, SRY-related HMG box 9; TGF-β1, transforming growth factor-beta 1; TGF-β3, transforming growth factor-beta 3; TNF-α, tumour necrosis factor-a.