SUPPLEMENTAL METHODS

Animals

Ten-week-old, male and female, *PKCδ* null *(PKCδ$^{-/-}$)* and wild type (WT) mice with a C57BL/6 background were used for all animal experiments. Mice were housed and handled in accordance with federal animal welfare guidelines and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002) and the Guide for the Use and Care of Laboratory Animals (8th Edition). All animal protocols and practices were reviewed and approved in advance by the Rush University Institutional Animal Care and Use Committee.

Human Tissue Acquisition

Adult human knee cartilage and synovia were obtained within 24 h of death from donors (age range: 40–65, mean 52) via the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL), with approval by the local ethics committee and informed consent obtained from the families (ORA#: L03090306). Surgically removed cartilage from OA patients (age range: 40-65) were obtained from the Orthopedic Tissue and Implant Repository Study with consent from the patients. Prior to dissection, each specimen was graded for overall degenerative changes based on the modified 5-point scale of Collins [1]. Details of the human tissues are described in the Supplemental Table 1. Human tissues were handled strictly according to the guidelines of the Human Investigation Committee of Rush University Medical Center.

Induction of Osteoarthritis in Mice

OA was induced by a medial meniscal destabilization (DMM) as we previously described [2]. Briefly, mice were anesthetized with 5% isoflurane (Abbott Laboratories) in oxygen and the left knee was shaved and prepared for aseptic surgery. A medial para-patellar arthrotomy exposed the anterior medial meniscotibial ligament, which was elevated with a microprobe and severed using curved dissecting forceps. Complete disruption of the ligament was confirmed visually by manually displacing the medial meniscus with fine forceps. The patella was repositioned, and the skin incision closed with 5-0 polypropylene sutures. Sham surgery was performed on the left knee of a separate group of mice; it consisted of a skin incision and medial capsulotomy only, followed by skin closure as described above.

Animal Behavioral Tests

The method of testing for mechanical allodynia (von Frey sensitivity) followed that of Chaplan and colleagues [3]. To obtain consistent results, we allowed animals to adapt to the grid environment for 15 minutes. A calibrated set of von Frey filaments (Stoelting, Wood Dale, IL) was applied from below to the plantar hind paw to determine the 50% force withdrawal threshold using an iterative method. For exploratory locomotor activity, animals were tested in clear vivarium plastic cages (42×25×20 cm) surrounded by a cage rack Photobeam Activity System (San Diego Instruments, San Diego, CA) in which beam interruptions are automatically recorded. All behavioral tests were performed by an investigator blinded to the study groups and to the identification of animals.

Histology, Immunohistochemistry and Histomorphometry

At 4 and 8 weeks post-DMM or post-sham surgery, animals were terminally anesthetized and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). Entire knee joints were then dissected for histology, macroscopic analyses, and μCT imaging. Knee joints (n=10 per group) were serially sectioned in the sagittal plane, and 3-4 representative mid-sagittal 7-μm-thick sections were selected and stained with Safranin-O for histological evaluation. OA grade was determined using the OARSI scoring system [4]. The L3-5 DRGs were dissected and post-fixed for 24 h at 4°C, then processed for paraffin embedding.

Human synovial tissues obtained within 24 h of death from donors were harvested immediately after opening the knee joint capsule by an experienced technician. Synovial tissues obtained during arthroscopic knee procedures were selected by the orthopedic surgeon. Synovial tissues adjacent to the medial tibiofemoral compartment were selected for the immunohistological analyses because prior study showed higher prevalence and severity of OA synovium in these sites [5]. Importantly, efforts were made to ensure that all synovial tissues were selected from the same area. After harvesting, fat tissues from synovial tissues were removed and immediately fixed in 4% paraformaldehyde. After dehydration in ethanol, specimens were embedded in paraffin, taking care to orientate them so that whole thickness of the synovial membrane can be obtained in the section. Serial transverse 7-μm-thick sections were cut and analyzed for the immunohistological evaluation.

Primary antibodies used were ATF4, CD11b (Santa Cruz Biotechnology Inc, Santa Cruz, CA), CD31, CD68, CGRP, NGF, TrkA, VEGF (Abcam, Cambridge, MA), IL-1β, TNFα (Novus Biologicals, Littleton, CO), MAP2, NeuN, PKCδ, phospho-PKCδ (Ser645) (Millipore, Billerica, MA), PGP 9.5 (Ultracone, Cambrige, UK), pERK1/2 (Cell Signaling, Danvers, MA). Immunohistochemical staining was performed using the standard avidin-biotin-peroxidase complex technique. Sections were then visualized using a Vectastain Kit (Vector Laboratories, Burlingame, CA) followed by counterstaining with hematoxylin. For immunofluorescence staining, an appropriate secondary antibody conjugated to a fluorescence probe was added, incubated at room temperature for 1 hour, rinsed in PBS, and mounted in an anti-fading mounting media (Vector Laboratories, Burlingame, CA). Results were obtained using an Olympus BX43 upright microscope (Olympus Optical, Tokyo, Japan). Double immunofluorescence staining was performed by the sequential addition of primary and secondary antibodies followed by incubation with appropriate secondary antibodies coupled to Alexa-488 or Alexa-543 fluorochromes (Life Technologies, Carlsbad, CA).

To quantitatively assess changes in the density of nerve fibers sprouting and in neovascularization in the mouse knee joint, images from five different sections per group were analyzed using Image J software (NIH, Bethesda, MD). First, sections were observed at low power (X10) to identify areas with the highest nerve fiber or capillary density in the synovium. Nerve sprouting and neovascularization were consistently present in the synovium adjacent to the medial meniscus. Three images per section were acquired within the medial synovium and analyzed for quantitative histomorphometric analyses. To ensure consistency, for all different animal groups, the same regions were selected for the immunohistological analyses. In order to reduce non-referred fluorescence signals, all specimen staining and image acquisition were done in parallel for the entire set, with identical image acquisition settings and exposure times. PGP 9.5-positive nerve fibers and CD 31-positive blood vessels were calculated as the density of nerve fibers or blood vessels area divided by the total area examined ($\mu m^2/\mu m^2$). For nerve fiber sprouting and neovascularization quantitation, all sections were analyzed in three different selected areas of 75 μm × 50 μm. For fluorescence intensity (FI) measurement, values from mice or humans (5 different tissue slides per group) were measured. Background intensity was subtracted, and the results are presented in arbitrary units (a.u.). For histomorphometric analyses, the percent of positively stained cells for an average of 3 fields in each slide were counted using Image J (NIH). Immunostained pictures obtained at 200X magnification

(n=5 per group), representing the medial femoral condyle and the tibial plateau of mice tissues, and medial femoral condyle of human tissues, were used for histomorphometric analyses. For each immunostaining experiment, a negative control without primary antibody was included. Two different blinded investigators performed all histomorphometric analyses.

Macroscopic Imaging and µCT

Gross knee-joint pathology was evaluated by India ink application followed by surface photography using a Nikon dissecting microscope (SMZ1000, X6) (Nikon Instruments Inc, Melville, NY). Abnormalities were evaluated by two blinded observers. Structural alterations of articular cartilage surface and subchondral bone architecture were evaluated by µCT scans using standard procedures we described previously [6]. Scanning was conducted within a 10-mm region of the intact mouse knees at 10-µm resolution (20-mm tube, high resolution at 55 kVP, 145 µA, 300 msec integration time) using a Scanco Model 40 Desktop µCT (Scanco Medical, Basserdorf, Switzerland).

Human Primary Cell Isolation and Culture

Human articular cartilage cells and synovia were obtained through the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL). As we previously described [7], human chondrocytes were released by enzymatic digestion, and the cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (1:1) containing 10% fetal bovine serum and antibiotics (complete media). For isolation of human synovial fibroblasts, synovial tissues were finely minced and tissue suspensions were seeded in DMEM media supplemented with 10% FBS. Human primary pulmonary artery endothelial cells were cultured as we previously described [8].

Transient Transfection of siRNA

Human primary cells were transiently transfected with validated *PKCδ* siRNA (LifeTechnologies, Carlsbad, CA) or mock (transfection reagents only) using either the Nucleofector™ kit (Lonza, Walkersville, MD) or Lipofectamine 2000 system (Life Technologies, Carlsbad, CA) as described previously [9]. After 48 h, cells were harvested, and total RNA isolation was performed for further experimentation.

Whole Knee Joint RNA isolation

Total RNA was isolated from whole mouse knee joints. Soft tissues were cleared from knee epiphyses and the knee joints were cut with a scalpel at standardized sites of the tibial and femoral heads. The tissues (including synovium, bone and adjacent tissues) were then snap frozen in liquid nitrogen, pulverized and total RNA was extracted from individual homogenized joints (a non-pooling strategy was used) using Trizol reagent (Life Technologies, Carlsbad, CA), following the manufacturer's instructions.

Reverse Transcription and Real-Time Polymerase Chain Reaction

Reverse transcription (RT) was carried out with 1 µg total RNA using the ThermoScript ^{TM} RT-PCR system (Life Technologies, Carlsbad, CA) for first strand cDNA synthesis. For real-time PCR, cDNA was amplified using the MyiQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative mRNA expression was determined using the $^{\Delta\Delta}C_T$ method detailed by the manufacturer (Bio-Rad, Hercules, CA). GAPDH was used for the internal control. The values represent the mean of 3-5 different donor samples in three separate experiments. The primer sequences will be provided upon request.

Intra-articular Anti-NGF-2.5S injection and Retrograde NGF-Biotin transport

Ten-week old *PKC*δ null and WT mice underwent either DMM surgery or sham surgery in the left knee joint. From the second week after DMM surgery, the animals received intra-articular (IA) injections of 30µg Anti-NGF-2.5S antibody (Sigma, St. Louis, MO) in 5 µL saline (n=10) or saline control (n=10), twice weekly until 8 weeks post-DMM surgery. The sham group (n=10) also received twice a week IA injections of saline for 8 weeks. Due to the shorter duration of action of the anti-NGF antibody in prior mouse studies [10], bi-weekly IA anti-NGF injection was performed in our experimental protocol. For retrograde NGF transport studies, mNGF2.5S-Biotin (1µg/knee joint) was administered IA in the left knee joint of DMM surgery or sham surgery mice (n=4/per group) 3 days before the animals were euthanized. mNGF 2.5S-Biotin was kindly provided by Alomone Labs (Jerusalem, Israel). Mice were terminally anesthetized and perfused transcardially with 0.9 % saline followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS, pH 7.4). Lumbar dorsal root ganglions were obtained at 8 weeks post-DMM for NGF-

Biotin retrograde transport analysis. L3-5 DRGs were post-fixed for 24 h at 4°C and then processed for paraffin embedding. Five-µm sections were incubated with 20µg/mL proteinase K for 10 min at 37°C followed by several washes in PBS. Sections were then incubated in a 1:10 dilution of Streptavidin-Alexa Fluor 488 (Life Technologies, Carlsbad, CA) for 5 h at room temperature followed by NeuN double immunofluorescence staining. Slides were mounted in antifading mounting media (Vector Laboratories, Burlingame, CA) and examined using an Olympus BX43 upright microscope (Olympus Optical, Tokyo, Japan).

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