SUPPLEMENTARY MATERIALS AND METHODS

Experimental Design

The objective of this study was to determine the role of metformin in the development and progression of OA and the mechanism in AMPK signaling dependency using post-traumatic OA and genetic mouse models. The effects that metformin was able to limit OA development and delay OA progression were observed in DMM-induced OA WT mouse model but not in DMM-induced OA in AMPKα1 KO mice; this was analyzed using histological staining and the OARSI, synovial hyperplasia and osteophyte formation histological scoring system. Related protein expression was further confirmed in these two mouse models using IHC and IF analyses. Moreover, osteophyte formation was quantitatively analyzed by μCT in mice 6- and 12-weeks after DMM surgery. In addition, OA pain sensitivity reduced after administration of metformin in DMM-induced OA WT mouse model, but not in the AMPKα1 KO mouse model, was analyzed using mechanical allodynia testing using a calibrated set of von Frey filaments. Meanwhile, the results of spontaneous activity, including travel distance, average walking speed, rearing frequency and duration, measured using the Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS, Metris, Netherlands) system, were consistent with those of the von Frey test. Furthermore, we found that metformin could upregulate the expression of pAMPKα1 and total AMPKα1 in DRG cells in mice 6- and 12- weeks after DMM surgery using IF staining. For *in vitro* experiments, expression of TNF-α and IL-1β-induced chondrocyte marker genes in articular chondrocytes derived from WT mice and AMPKα1 KO mice were analyzed using RT-qPCR. In addition, TNF-α and IL-1β inhibited AMPK phosphorylation in human articular chondrocytes, which was rescued by treatment with metformin, was detected by western blot analysis. The number of samples meeting statistical

requirements for each experiment is indicated in the figure legend.

For the non-human primate study, we performed PMM surgery on the left knee joint of nine male rhesus macaques and sham operations on the right knee joint. One month after surgery, five rhesus macaques were administrated metformin 7 times a week and another four were provided placebo. Cartilage surface lesions were observed 7 months after PMM surgery and were semi-quantified by macroscopic scoring of cartilage. These samples were also scanned using μCT and the bone volume of subchondral sclerosis was quantified through CTAn software. In addition, knee joint cartilage thickness was measured using MRI scanning and the behavior activity of animals was recorded using a digital camera at different time points, including 1 month before surgery, and 1, 3, and 7 months after surgery.

Experimental Post-Traumatic OA in Mice

The animal protocol of this study has been approved by the Institutional Animal Care and Use Committee (IACUC) of Rush University Medical Center and all experimental methods and procedures were carried out in accordance with the approved guidelines to comply with all relevant ethical regulations for animal testing and research. AMPKα1 KO mice were generously provided by Dr. Benoit Viollet (INSERM, U1016, Paris, France). In this study, 32 AMPKα1 KO mice and 40 congenic wild-type (WT) mice with C57BL/6/129 background were housed in static, polysulfone, microisolation caging on corncob and cellulose bedding and maintained on a 12:12-h light: dark cycle. Caging, food, and water bottles were changed weekly. Post-traumatic OA was induced by DMM surgery as previously described.[1] Briefly, after anesthesia with 1.2% tribromoethanol (Sigma-Aldrich, #T48402) 240 mg/kg body weight i.p. injection, surgery was completed in 10-week-old male AMPKα1 KO mice (8 in each group) and WT mice (10 in each group) by transection of the anteromedial meniscotibial ligament and the medial collateral

ligament. Sham-operated mice were used as controls. The mice were administered metformin (MP Biomedicals LLC, OH, USA) either 2 weeks before (limit group) or 2 weeks after (delay group) DMM surgery.

Metformin Administration

Metformin was dissolved in drinking water (205 mg/kg body weight) 7 times a week; the metformin dosage was converted from the human equivalent dose (1000 mg per day) to the mouse dose based on body surface area.[2] We measured water consumption and body weight of mice once a week and we made an adjustment on the concentration of metformin in drinking water every week based on changes in water consumption and body weight of mice. Mice were sacrificed 6 and 12 weeks after DMM surgery. Since approval by the U.S. Food and Drug Administration (FDA) in 1995, the dosage of metformin for type 2 diabetes therapy was established at from 1000-1700 mg per day (500-850 mg, twice a day), not exceeding the maximum recommended daily doses of 2000 mg per day because of its potential risk of moderate renal impairment.[3] In this study, we converted the human dosage of 1000 mg per day, which was the most common and safe oral dose, to animal equivalent doses based on body surface area. By multiplying a conversion factor, the dosage of metformin was 205 mg/kg per day for mouse and 51.7 mg/kg/day for rhesus macaque.

Behavioral Assessment

The mechanical allodynia test was performed using a calibrated set of von Frey filaments (North Coast Medical Inc., CA, USA). Prior to von Frey hind paw test, the mice were allowed to accommodate for 15 minutes on a wire mesh grid. The filaments (typical force range used in mouse is from 0.04 to 6.0 g, beginning with 0.4 g) were applied to the plantar surface of the hind paw to determine the 50% force withdrawal threshold using the classical up-down iterative

method as previously described.[4] A response is considered positive if the animal exhibits any nocifensive behavior, including brisk paw withdrawal, licking, or shaking of the paw, either during application of the stimulus or immediately after the filament is removed. The tests were performed in a blind manner in that the investigator was not aware of the identification of animals as well as the study groups. The assessment of spontaneous behavior was measured by the Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS, Metris, Netherlands). Briefly, after animals were weighed, we simultaneously used 4 platforms to test 4 mice, which were from the same group, for 15 hours at the same time frame from 18:00 pm to 9:00 am the next day. The following parameters were assessed: distance of locomotion, average speed of locomotion, rearing frequency, and rearing duration.

Micro-CT, Histology, Immunohistochemistry (IHC) and Histomorphometry

We used a Scanco μCT35 scanner (Scanco Medical, Brüttisellen, Switzerland) with 55 kVp source and 145 μAmp current for formalin-fixed mouse legs with a resolution of 10 μm. The scanned images from each group were evaluated at the same thresholds to allow 3-dimensional structural rendering of each sample.

For histological staining, slides of mouse knee joints coronal sections with 3 μm thick were stained with Alcian blue/Hematoxylin & Orange G (AB/H&OG) for morphologic analysis. The severity of OA-like phenotype was analyzed using the OARSI score, cartilage area, synovitis score, osteophyte size and osteophyte maturity. For each sample, we analyzed three-levels of each section (50 μM apart) through the medial compartment of the knee. The severity of OA-like phenotype was analyzed using the OARSI scoring system using three-level sections of the joints, including medial femoral condyle and medial tibial plateau by two blinded observers.[4] For each sample, nine sections were cut, 3 for morphometric analysis and 6 for IHC analysis.

The articular cartilage area of the medial tibia plateau was quantified by tracing the Alcian blue-positive staining areas using the OsteoMeasure system (OsteoMetrics, Inc., Atlanta, GA, USA). As previously described,[5,6] changes in synovial tissue (synovitis score) was semi-quantified by the number of synovial lining layers and osteophyte formation was evaluated semi-quantitatively using osteophyte formation scores, including osteophyte size and osteophyte maturity. In addition, slides of dorsal root ganglion (DRG) sections with 3 μm thick were stained with hematoxylin and eosin (H&E) for histological analysis.

For IHC staining, knee joint sections were heated at 95°C in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) for 15 min, and then sequentially treated with 3% H_2O_2, 0.5% Triton X-100, Avidin/Biotin Blocking Kit (Invitrogen, Carlsbad, CA, USA). After blocking with 10% normal goat serum (Vector Laboratories) for 1 h, sections were treated with primary antibodies, including phospho-AMPKα (Thr172) (ab23857), total AMPKα (ab131512), MMP13 (ab39012), Adamts5 (ab41037), and Collagen X (ab58632) antibody (Abcam, Cambridge, Massachusetts, USA) overnight at 4°C and incubated with secondary biotinylated goat anti-rabbit or anti-mouse antibody (Vector Laboratories) for 30 min, followed by treatment with the VECTASTAIN Elite ABC Kit (Vector Laboratories). IHC signals were revealed by ImmPACT DAB Peroxidase Substrate (Vector Laboratories). Information about antibody concentrations and dilutions is provided in Table S1.

For fluorescence immunostaining, bilateral L3-5 DRGs were harvested after mice were deeply anaesthetized with 1.2% tribromoethanol (240 mg/kg) and perfused through the ascending aorta with 50 ml of saline followed by 200 ml of 4% paraformaldehyde (PFA) in 0.01 M PBS, PH 7.4. We used the ipsilateral (DMM surgery leg) DRGs for IHC studies. The DRGs were postfixed in the 4% PFA for 3 hours and then placed in 30% sucrose (in 0.1 M PBS)

overnight. Samples were sectioned at a 10 μm thickness on a freezing microtome (Leica, CM3050S, Germany). DRG sections were incubated with 1/250 phospho-AMPKα (Thr172) (ab23857) and 1/200 total AMPKα (ab131512) antibody overnight at 4°C and then incubated with secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) for 30 min. Information about antibody concentrations and dilutions is provided in Table S1. Images of histology and IHC were captured using CellSens Imaging Software (Olympus) on an Olympus BX43 microscope, or a Zeiss LSM700 confocal microscope.

Cell Culture and RT-PCR Analysis

Primary mouse knee chondrocytes were isolated from articular cartilage of 4-day-old neonatal mice, as described previously.[7] After being incubated with metformin (5 mM) for 12 hours, the isolated cells were treated with TNF-α (100 ng/ml) and IL-1β (10 ng/ml) for 6 hours. Total mRNA was extract with Trizol (Invitrogen Life Technologies, CA, USA). 1 μg total RNA was used to synthesize complementary DNA (cDNA) using an iScripts cDNA Synthesis kit (Quanta Biosciences, MD, USA). Real-time PCR amplification was performed using specific primers of genes encoding for matrix degradation enzymes and a SYBR Green real-time PCR kit (Quanta Biosciences). Data were collected from cells isolated from 3 independent mice (n = 3). The primer sequences of *Mmp3*, *Mmp13*, *Adamts4*, *Adamts5* are listed in a supplementary table (Table S2).

Nitric Oxide Release

Primary mouse knee chondrocytes isolated from WT and AMPKα1 KO mice were pre-treated with metformin at 1-2 mM concentrations for 1 hours before stimulation with IL-1β (10 ng/ml) for 18 hours. Nitric oxide (NO) production was measured by the concentration of nitrite in conditioned media, using $NaNO_2$ as a standard, as described before.[8]

Experimental Post-Traumatic OA in Non-Human Primates

Care and experimental procedures of non-human primates were approved by the Institutional Animal Care and Use Committee in the Tianjin International Joint Academy of Biomedicine (Tianjin, China). This study was conducted in compliance with relevant Chinese law and regulations on the management of laboratory animals promulgated by the State Science and Technology Commission. Nine male rhesus macaques (*Macaca mulatta*, Hengshu Biotechnology Co., Sichuan, China), 8.5-11.4 years and 9-15 kg, were kept in an indoor facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were housed in individual stainless-steel cages in a specific room where an environmental temperature of 21-25°C and relative humidity of 40-60% were maintained. Although individually housed, animals were provided continuous auditory, visual and olfactory contact with neighboring conspecifics. In addition to the standard non-human primate diet (Beijing Keao Xieli Feed Co., Ltd., Beijing, China), water and fresh fruits were available *ad libitum*. Small amounts of primate treat and various cage-enrichment devices were supplied.

Prior to surgery, animals were anaesthetized with Zoletil (Virbac, France), 4-6 mg/kg; the surgical knees were shaved with razors and prepared with povidone-iodine. The surgery was performed using aseptic technique. The skin above the medial collateral ligament was incised about 5 cm. The articular capsule was longitudinally cut to expose the medial meniscus. Partial medial meniscectomy (PMM) was performed by removing the anterior horn and the posterior horn of the medial meniscus was loosened. The incision was closed in layers and the animals were returned to their home cages after recovery from anesthesia. To prevent post-operative infection, animals were treated with cefazolin (25 mg/kg, i.m., twice a day for 3 days). To alleviate acute post-operative pain, animals were treated with flurbiprofen axetil (10 mg/kg, i.v.,

once a day for 3 days). Metformin was mixed with a powdered diet and cold pressed into a shape resembling that of the standard NHP diet. One month after surgery, rhesus macaques were administered metformin (51.7 mg/kg) as the treatment group (n=5) or provided placebo as the control group (n = 4) 7 times a week.

Macroscopic Scoring of Cartilage

Knee joint samples were harvested and the central cartilage of the medial tibial condyle (MTC) and the medial femoral condyle (MFC) was assessed. The scoring system for macroscopic grading of cartilage damage was segmented into normal (0), surface roughening (1), fibrillation and fissures (2), small erosions down to subchondral bone (3), and large erosions down to subchondral bone (4).[5]

MRI and MicroCT

MRIs of knee joints were performed longitudinally at one month before surgery, and 1, 3, and 7 months after surgery on a 3T MRI scanner (GE, Discovery 750, USA) with a dedicated peripheral knee coil using high resolution T1- and T2-weighted protocols. Tibial and femoral cartilage plates were manually divided into sub-regions and the entire cartilage thickness was assessed quantitatively.[9] Knee joints were collected seven months after surgery. μCT was performed using a Skyscan 1276 scanner (Skyscan, Bruker, Belgium) and images were analyzed by CTAn V1.15.4 software (Skyscan). Regions of interest (ROIs) of subchondral sclerosis were reconstructed and their bone volumes were quantitatively assessed. The growth rate of subchondral sclerosis was calculated by volume ratio of PMM alone or PMM/Met group to sham group.

Behavioral Assessment in Non-Human Primates

Animal behavior was recorded for 6 hours from 9 am to 3 pm at one month before surgery, and 1, 3 and 7 months after surgery using a digital camera (Sony IMX307, Japan). The total time of duration of standing and walking were counted by two researchers blinded to the study groups.

Western Blot Analysis in Human Chondrocytes

To determine if metformin reverses the effects of inflammatory cytokines in human chondrocytes, western blot assays were performed. Briefly, isolated primary human knee chondrocytes, kindly provided by Dr. Martin Lotz (The Scripps Research Institute), were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, and 100 IU/ml of penicillin at 37°C. No later than first passage chondrocytes were used for all experiments in order to retain their proper phenotype. The chondrocytes were treated with metformin (2 mM) either for the times indicated or for 2 hours and then followed by stimulation with TNF-α (100 ng/ml) and IL-1β (10 ng/ml) for 18 hours. Cells were lysed in RIPA buffer with 2 mM sodium vanadate and protease inhibitor cocktails (Roche, Mannheim, Germany). Cell lysates (10-15 µg) were separated by gradient 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, California, USA) probed with antibodies according to manufacturer's instruction, exposed to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, Massachusetts, USA) and visualized by radiography.

Power Analysis and Statistical Analysis

For the power analysis, we used the formula $n = 2(z_{alpha} + z_{beta})^2/d^2$ to calculate sample numbers, where d = (u1-u2)/sigma (sigma = SD). If we assume a probability of type I error, or alpha of 0.05, and a probability of type II error, or beta of 0.20, we will obtain values of

z_{alpha}=1.645, and z_{beta}= 0.842, so n = $2(1.645+0.842)^2/d^2$. The details of animal number calculations for histomorphometric analysis are presented in Table S3.

All data are expressed as mean ± 95% CI or mean ± s.d., as indicated in the figure legends. Statistical analyses were completed with Prism GraphPad. Unpaired Student's *t* test (for two groups), one-way or two-way ANOVA (for multiple groups) were used followed by the Tukey-Kramer test. $p<0.05$ was considered statistically significant.

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Table S1. Concentrations and dilutions of antibodies used in this study

Antibodies	Method	Concentrations	Dilutions
Anti-AMPKα1 (phospho Thr183) + AMPKα2 (phospho Thr172) antibody (ab23875)	IHC	4 μg/ml	1:100
	IF	1.6 μg/ml	1:250
Anti-AMPKα1 + AMPKα2 antibody (ab131512)	IHC	10 μg/ml	1:100
	IF	5 μg/ml	1:200
Anti-MMP13 antibody (ab39012)	IHC	5 μg/ml	1:200
Anti-Adamts5 antibody (ab41037)	IHC	2 μg/ml	1:500
Anti-Collagen X antibody (ab58632)	IHC	1 μg/ml	1:1000
Anti-TRPA1 antibody (NB110-40763SS, ab2721)	IF	4 μg/ml	1:250

Table S2. The names of sequences of primers used in this study

Genes	Primer sequence (forward primers)	Primer sequence (reverse primers)
Mmp3	5'-GGCCTGGAACAGTCTTGGC-3'	5'-TGTCCATCGTTCATCATCGTCA-3'
Mmp13	5'-CTTCTTCTTGTTGAGCTGGACTC-3'	5'-CTGTGGAGGTCACTGTAGACT-3'
Adamts4	5'-ATGGCCTCAATCCATCCCAG -3'	5'-GCAAGCAGGGTTGGAATCTTTG-3'
Adamts5	5'-GGAGCGAGGCCATTTACAAC-3'	5'-CGTAGACAAGGTAGCCCACTTT-3'
Col2a1	5′-CCTGGACCCCGTGGCAGAGA-3'	5′-CAGCCATCTGGGCTGCAAAG-3'
Aggrecan	5′-AGGATGGCTTCCACCAGTGC-3'	5′-TGCGTAAAAGACCTCACCCTCC-3'
Sox9	5′-TAAATTCCCAGTGTGCATCC-3'	5′-GCACCAGGGTCCAGTCATA-3'
IGF-1	5′-AAAGCAGCCCCGCTCTATCC-3′	5′-CTTCTGAGTCTTGGGCATGTCA-3′
Bmp7	5′-GGCAGAGCATCAACCCCAA-3′	5′-GACTTCCGTGGCCTTGAAGA-3′
MCP-1	5′-GCATCCACGTGTTGGCTCA-3′	5′-CTCCAGCCTACTCATTGGGATCA-3′
CCR-2	5′-ACAGCTCAGGATTAACAGGGACTTG-3′	5′-ACCACTTGCATGCACACATGAC-3′
NGF	5'-ACTGGACTAAACTTCAGCATTCC-3'	5'-GGGCAGCTATTGGTGCAGTA-3'
TNF-α	5′-CCGTCTCCTACCCGGAAAAG-3'	5′-CGGAATCGGCAAAGTCAAGG-3'
IL-1β	5'- GCAACTGTTCCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
β-Actin	5'-GGCTGTATTCCCCTCCATCG -3'	5'-CCAGTTGGTAACAATGCCATGT -3'

Table S3. Power Analysis (histomorphometric measurements)

Parameters	Mean ± SD	20% of mean	d	d^2	Mouse number
OARSI score	0.625 ± 0.09	0.125	1.39	1.93	6.4 (6)
Cartilage area	0.22 ± 0.034	0.044	1.29	1.66	7.4 (7)
Synovitis score	1.0 ± 0.15	0.2	1.33	1.77	7.0 (7)
Osteophyte size	0.175 ± 0.026	0.035	1.35	1.82	6.8 (7)
Osteophyte maturity	0.25 ± 0.038	0.05	1.32	1.74	7.1 (7)

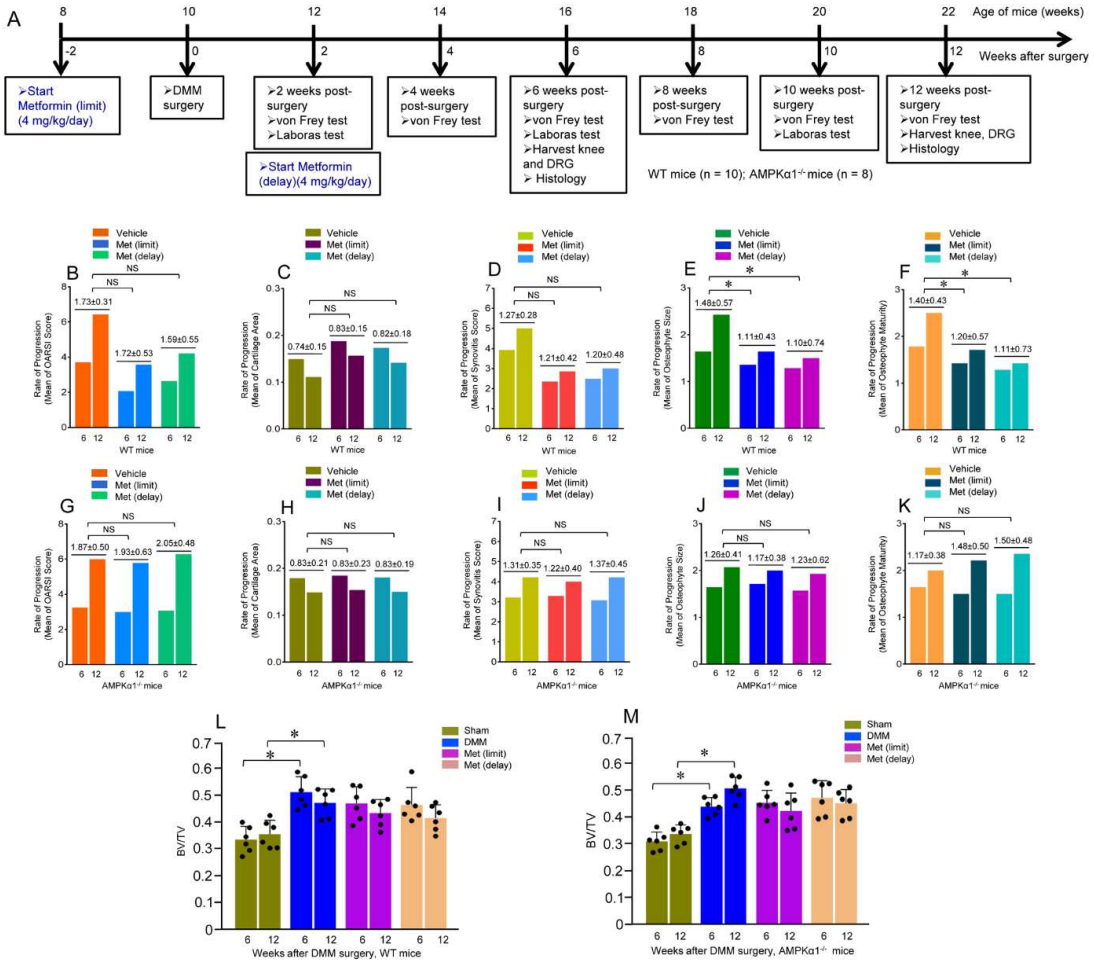

Figure S1. Metformin limited osteoarthritis (OA) development and delayed OA progression in the mouse destabilization of medial meniscus (DMM) model. (**A**) Experimental flow chart. DMM surgery was performed in 10-week-old wild type (WT) mice. Metformin was administered 2 weeks before (limit group) or after (delay group) DMM surgery. (**B-F**) OA progression rates, including the progression rates of Osteoarthritis Research Society International (OARSI) score, cartilage area, synovitis score, osteophyte size and osteophyte maturity, during 6-12-weeks after DMM surgery, were analyzed in WT mice. (**G-K**)

OA progression rates, including the progression rates of OARSI score, cartilage area, synovitis score, osteophyte size and osteophyte maturity, during 6-12-weeks after DMM surgery, were analyzed in AMPKα1 KO mice. (**L, M**) Changes in subchondral bone volume (BV/TV) were analyzed by using micro-computed tomography (μCT) 6- and 12-weeks after DMM surgery in WT and AMPKα1 KO mice.

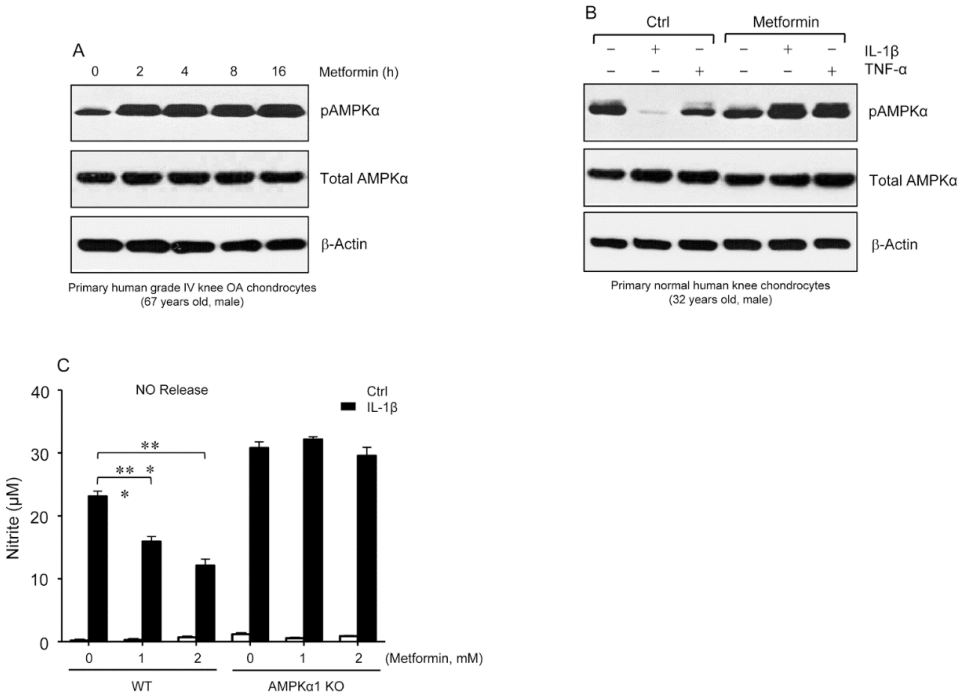

Figure S2. Metformin enhances AMPKα1 phosphorylation in human articular chondrocytes. (**A**) Primary human chondrocytes from a male 67-year-old advanced knee OA donor (underwent knee replacement surgery). Cells were treated with metformin (2 mM) for 2-16 hours. Metformin enhanced AMPKα1 phosphorylation starting at 2 hours until 16 hours. (**B**) Primary human knee chondrocytes were isolated from tibial cartilage from a normal 32-year-old male donor. Cells were treated with metformin (2 mM) for 2 hours and then incubated with TNF-α (100 ng/ml) or IL-1β (10 ng/ml) for 4 hours. Expression of pAMPKα protein was determined by western blot analysis. Treatment with IL-1β or TNF-α significantly inhibited AMPKα1 phosphorylation. Addition of metformin completely reversed the inhibitory effects on AMPKα1 phosphorylation by IL-1β a and TNF-α. (**C**) Metformin inhibits IL-1β-induced nitric oxide (NO)

release in an AMPK-dependent mechanism. Primary articular chondrocytes isolated from WT or AMPKα1 KO mice were treated with IL-1β (10 ng/ml) in the presence or absence of metformin (1 or 2 mM) for 18 hours. IL-1β-induced NO release was inhibited by metformin in articular chondrocytes derived from WT mice, but not in those derived from AMPKα1 KO mice.

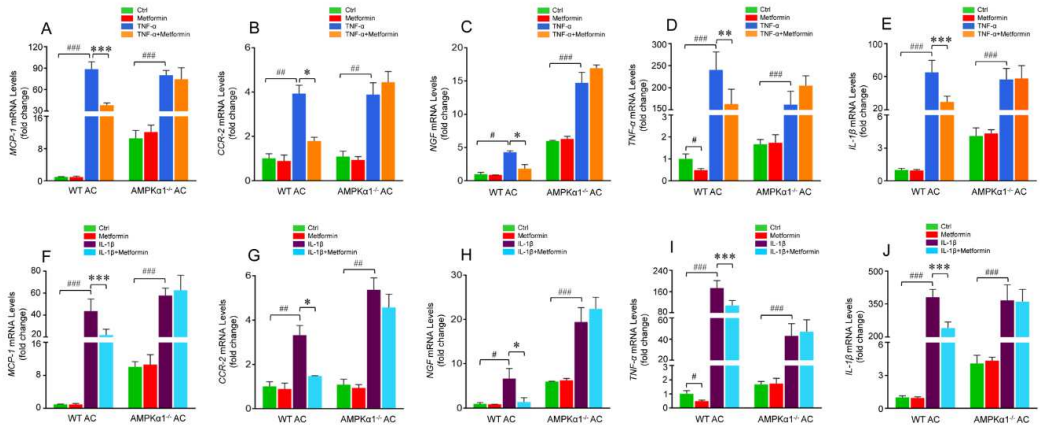

Figure S3. Metformin inhibits pain-related marker genes in articular chondrocytes. Primary articular chondrocytes were isolated fom 4-day-old WT mice and AMPKα1 KO mice. The cells were treated with TNF-α (A-E) or IL-1β (F-J) without or with metformin. Effects of TNF-α, IL-1β and metformin on expressions of pain-related marker genes in articular chondrocytes were examined by real-time PCR analysis.

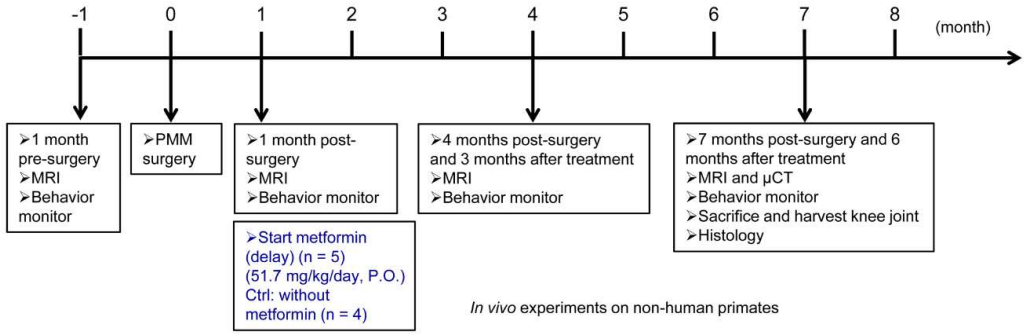

Figure S4. Flow chart of experiments with non-human primates. Rhesus macaques were administered metformin 1 month after PMM surgery and continued for 6 months.