SUPPLEMENTARY DATA

NAMPT (visfatin), a direct target of hypoxia-inducible factor-2α, is an essential catabolic regulator of osteoarthritis

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Supplementary Materials and Methods

Histology and immunohistochemistry

Human OA cartilage was frozen, sectioned at a thickness of 10 μm, and fixed in paraformaldehyde. Sulfate proteoglycan was detected with alcian blue staining.[1-4] Cartilage destruction in mice was examined using safranin-O staining. Briefly, knee joints were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA, and embedded in paraffin. The paraffin blocks were sectioned at a thickness of 5 μm. Sections were deparaffinized in xylene, hydrated with graded ethanol, and stained with safranin-O. Cartilage destruction was scored by two blinded observers using the OARSI scoring system[5]. Synovitis was determined by safranin-O and hematoxylin staining, and synovial inflammation (grade 0-3) was scored as described previously.[1–4] HIF-2α was immunostained using 2 μg/ml of mouse monoclonal antibody (clone 190b; Santa Cruz Biotechnology Inc.). NAMPT was detected using 10 μg/ml of mouse monoclonal antibody (clone OMN1379; AdipoGen). A rabbit polyclonal anti-mouse NAMPT antibody was used to neutralize mouse e NAMPT (BioVision, catalog #5908-100).

Primary culture of articular chondrocytes

Chondrocytes were isolated from femoral condyles and tibial plateaus of mice by digesting cartilage tissue with 0.2% collagenase (Sigma).[1–4 6] The cells were maintained as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (AusgeneX) and antibiotics (penicillin G and streptomycin). Cells at culture day 3 were infected with adenoviruses or treated with recombinant mouse NAMPT protein (AdipoGen).

Microarray analysis

Mouse articular chondrocytes were infected with Ad-*Epas1* or empty virus (Ad-C) at a multiplicity of infection (MOI) of 800 for 48 hours. Total RNA was extracted using the TRI reagent (Molecular Research Center Inc.). Microarray analyses were performed by Seoulin Bioscience using Agilent 44 K mouse 60-mer oligonucleotide microarrays (Agilent Technologies) and processed according to the manufacturer's instructions (Agilent 60-mer oligomicroarray processing protocol, http://www.Agilent.com).

Adenoviruses, infection of chondrocytes, and intra-articular (IA) injection of mice

The adenovirus expressing mouse *Epas1* (Ad-*Epas1*) was described previously.[1-2] Briefly, Ad-*Nampt* was constructed by inserting the mouse *Nampt* cDNA into the *Not*I site of the pShuttle-CMV vector. Ad-*Nampt* was produced by Seoulin Bioscience using the pAdEasy System (QBiogene). Mouse articular chondrocytes were cultured for 3 days, infected with Ad-*Nampt*,

Ad-*Epas1* or empty virus (Ad-C) for 3 hours at the indicated MOI, and cultured in the presence or absence of pharmacological agents. For IA injection of adenovirus, Ad-*Nampt*, Ad-*Epas1,* or Ad-C (1×10^9 PFU) was injected into the knee joints of mice once per week for 3 weeks. The mice were co-injected (IA) with the non-competitive NAMPT inhibitor FK866 (10 mg/kg body weight; Cayman) in a total volume of 10 μl.

Enzyme-linked immunosorbent assay (ELISA)

Mouse articular chondrocytes were infected with Ad-C (800 MOI) or Ad-*Nampt* (at the indicated MOI). After 36 hours of culture, the amount of cellular iNAMPT and secreted eNAMPT was determined in whole-cell lysates and culture supernatants, respectively, using a mouse NAMPT ELISA kit (Abnova, KA0095) according to the manufacturer's instructions. Briefly, 100 μl of lysates and purified culture supernatants were added to each well of an ELISA plate at an appropriate dilution. After rigorous washing, samples were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse NAMPT antibody and allowed to undergo a colorimetric reaction with tetra-methylbenzidine (TMB) substrates. Following a 15-minute incubation, the reaction was stopped by the addition of H_2SO_4, and absorbance at 450 nm was measured using a VERSAmax microplate reader (Molecular Devices). NAMPT concentrations were estimated by reference to a standard curve generated using serially diluted recombinant mouse NAMPT protein.

Reverse transcription-polymerase chain reaction (RT-PCR), quantitative RT-PCR (qRT-PCR), and small inhibitory RNA (siRNA)

Total RNA was extracted from primary cultured chondrocytes using the TRI reagent (Molecular Research Center Inc.). For cartilage tissues, knee joints of wild-type (WT), transgenic (TG), or knockout (KO) mice were scraped with a blade after DMM surgery or adenovirus injection to remove cartilage, and RNA was isolated using the TRI reagent. The RNA was reverse transcribed, and the resulting cDNA was amplified by PCR. PCR primers and experimental conditions are summarized in online supplementary table S1. Transcript levels were quantified by qRT-PCR. qRT-PCR reactions were performed using an iCycler thermal cycler (Biorad) and SYBR premix Ex Taq (TaKaRa Bio). For each target gene, transcript levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold changes relative to the indicated controls. The siRNA sequences that knocked down *Epas1* or *Nampt* most effectively were used in this study (online supplementary table S2). Non-targeting (scrambled) siRNA was used as a negative control. Chondrocytes were transfected by incubating for 6 hours with siRNA and Lipofectamine 2000 (Invitrogen), and infected with adenoviruses as described

above. Transfection of siRNA (100 nM) did not cause any cytotoxicity in primary cultures of chondrocytes.

Western blotting

Total cell lysates were prepared in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, 0.2% sodium dodecyl sulfate [SDS], 5 mM NaF) and used to detect iNAMPT. Secreted proteins (eNAMPT, MMP3, MMP12, and MMP13) were detected after trichloroacetic acid (TCA) precipitation from 900 μl of serum-free conditioned medium. All lysis buffers contained a cocktail of protease inhibitors and phosphatase inhibitors (Roche). The following antibodies were used for Western blotting: mouse monoclonal anti-NAMPT (clone OMN1379) from AdipoGen, and mouse monoclonal anti-MMP3 (clone EP1186Y), anti-MMP12 (clone EP1261Y), and anti-MMP13 (clone EP1263Y) from Epitomics.

Cloning, reporter gene assays, promoter binding assays, and chromosome immunoprecipitation (ChIP) assays

The expression vectors for *Epas1* and dominant-negative *Epas1* (Δ*Epas1*) have been previously described.[1] *Nampt* promoter regions were cloned using primers specific for the genomic DNA of C57BL/6 mice (online supplementary Table S1). Amplified products were inserted into the pGL3 vector (Promega), and reporter gene activity was assayed. HIF-2α binding sites (CGTG) in the *Nampt* promoter were mutated to AAAG using a site-directed mutagenesis kit (iNtRON Biotech), and the reporter gene activity of the mutant promoter was compared with that of the WT reporter gene. The HIF-2α reporter gene was constructed in a pGL3 vector by inserting four tandem repeats of 5′-GATCGCCCTACGTGCTGTCTCA-3′ into the upstream region of the SV40 promoter. Chondrocytes were transfected for 3 hours with HIF-2α reporter gene (1 μg) and a β-galactosidase expression vector (0.1 μg) using Lipofectamine 2000 (Invitrogen). Transfected cells were infected with Ad-*Epas1* for 3 hours and treated with the indicated concentrations of FK866. Cells were harvested 36 hours after treatment, and luciferase activity was measured and expressed relative to β-galactosidase activity to normalize for transfection efficiency.

ChIP assays of mouse articular chondrocytes treated with IL1β or infected with Ad-*Epas1* at a MOI of 800 were performed using a kit (Millipore). The primers for the ChIP assay were designed to allow amplification of the three different HRE-containing regions of the *Nampt* promoter (online supplementary table S1). Cross-linking of DNA-proteins was induced by the addition of formaldehyde (1% final concentration) directly to the culture medium for 10 minutes at 37°C. Cells were lysed, and DNA in the supernatant was sheared by sonication. Sonicated chromatin was incubated overnight with 2 μg of rabbit anti-mouse HIF-2α antibody (Novus

Biologicals, NB100-122) or control anti-IgG antibody (Santa Cruz Biotechnology, SC-2027) at 4°C. Antibody–protein–DNA complexes were isolated with protein A–agarose. Eluted DNA was purified using phenol extraction and used as a template for PCR.

Skeletal staining

Skeletons of whole-mouse embryos were stained with alcian blue and alizarin red, as described previously.[7] Briefly, whole embryos were skinned, eviscerated, and fixed with 95% ethanol for 4 days and immersed in acetone for 3 days. Samples were stained in a freshly prepared staining solution composed of 1 volume of 0.3% alcian blue 8GX in 70% ethanol, 1 volume of 0.1% alizarin red S in 95% ethanol, 1 volume of 100% acetic acid, and 17 volumes of 100% ethanol. The samples were sequentially destained with 1% KOH for up to 48 hours and with 20% glycerol containing 1% KOH for 14 days. Images were acquired using a Zeiss SteREO Discovery V12 microscope.

MMP activity assays

Chondrocytes infected with Ad-*Nampt* for 2 hours were incubated in serum-free DMEM for an additional 36 hours. Conditioned medium was harvested and centrifuged for 3 minutes at 3000 rpm. MMP activity in collected supernatants was assayed using an EDANS/DabcylPlus fluorescence resonance energy transfer (FRET) peptide (SensoLyte 520 MMP assay kit; ANASPEC) according to the manufacturer's instructions. Briefly, supernatant was incubated with 1 mM 4-aminophenyl-mercuric acetate for 40 minutes at 37°C and then mixed with MMP substrate solution for 30 minutes at 37°C on a 96-well plate. The FRET substrate can be cleaved by MMP2, MMP3, MMP8, MMP9, MMP12, and MMP13. Upon cleavage into two separate fragments by MMP, the fluorescence of EDANS is restored and fluorescence intensity can be monitored. Fluorescence intensity was determined at excitation/emission wavelengths of 340 nm/520 nm using a fluorescence microplate reader.

References

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2. **Ryu JH**, Shin Y, Huh H, *et al*. Hypoxia-inducible factor-2α regulates Fas-mediated chondrocyte apoptosis during osteoarthritic cartilage destruction. *Cell Death Differ*. 2012;**19**:440–50.
3. **Ryu JH**, Yang S, Shin Y, *et al*. Interleukin-6 plays an essential role in hypoxia-inducible factor 2α-induced experimental osteoarthritic cartilage destruction in mice. *Arthritis Rheum*. 2011;**63**:2732–43.

4. **Oh H**, Chun CH, Chun JS. Dkk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. *Arthritis Rheum.* 2012;**64**:2568–78.
5. **Glasson SS**, Chambers MG, van den Berg WB, *et al*. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartilage.* 2010;Suppl.9:S17–23.
6. **Gosset M**, Berenbaum F, Thirion S, et al. Primary culture and phenotyping of murine chondrocytes. *Nat Protoc*. 2008;3:1253–60.
7. **Oh H**, Chun CH, Chun JS.. Misexpression of Dickkopf-1 in endothelial cells, but not in chondrocytes or hypertrophic chondrocytes, causes defects in endochondral ossification. *J Bone Miner Res*. 2012;27:1335–44.

Supplementary Tables

Supplementary Table S1. PCR primers and conditions

Gene	Strand	Primer sequences	Size (bp)	AT (°C)	Origin
Acan	S AS	5′-GAAGACGACATCACCATCCCAG-3′ 5′-CTGTCTTTGTCACCCACACATG-3′	581	55	Mouse
Adamts4	S AS	5′-CATCCGAAACCCTGTCAACTTG-3′ 5′-GCCCATCATCTTCCACAATAGC-3′	281	58	Mouse
Adamts5	S AS	5′-GCCATTGTAATAACCCTGCACC-3′ 5′-TCAGTCCCATCCGTAACCTTTG-3′	292	58	Mouse
Adipoq	S AS	5′-TCTTAATCCTGCCCAGTCATGC-3′ 5′-TGCTGCCGTCATAATGATTCTG-3′	420	58	Mouse
Ccl2	S AS	5'-GGCCTGCTGTTCACAGTTGGC-3' 5'-GCTGAAGACCTTAGGGCAGATGCA-3'	314	60	Mouse
Ccl5	S AS	5'-CTCACCATCATCCTCACTG-3' 5'-CTAGCTCATCTCCAAATAGTTG-3'	255	60	Mouse
Ccl7	S AS	5'-GCTTTCAGCATCCAAGTGTG-3' 5'-TTCAGCACAGACTTCCATGC-3'	180	60	Mouse
Col2a1	S AS	5′-CACACTGGTAAGTGGGGCAAGA-3′ 5′-GGATTGTGTTGTTTCAGGGTTCG-3′	173	55	Mouse
Cxcl1	S AS	5'-CGCCTATCGCCAATGAGCTG-3' 5'-CCAAGGGAGCTTCAGGGTCAAG-3'	174	60	Mouse
Cxcl2	S AS	5'-AGTGAACTGCGCTGTCAATG-3' 5'-GCCTTGCCTTTGTTCAGTATC-3'	203	60	Mouse
Cxcl10	S AS	5'-TTTCTGCCTCATCCTGCTG-3' 5'-GATGGTCTTAGATTCCGGATTC-3'	223	60	Mouse
Epas1	S AS	5′-CGAGAAGAACGACGTGGTGTTC-3′ 5′-GTGAAGGCGGGCAGGCTCC-3′	370	62	Mouse
Gapdh	S AS	5′-TCACTGCCACCCAGAAGAC-3′ 5′-TGTAGGCCATGAGGTCCAC-3′	450	62	Mouse
Il1b	S AS	5'-TTGACAGTGATGAGAATGACC-3' 5'-GCAGGTTATCATCATCATCC-3'	300	60	Mouse

Il6	S AS	5'-ACCACTCCCAACAGACCTGTCTATACC-3' 5'-CTCCTTCTGTGACTCCAGCTTATCTGTTAG-3'	435	60	Mouse
Il12	S AS	5'-GGAAGCACGGCAGCAGAATA-3' 5'-AACTTGAGGGAGAAGTAGGAATGG-3'	180	60	Mouse
Il17	S AS	5'-TTCATCTGTGTCTCTGATGCT-3' 5'- TTGACCTTCACATTCTGGAG-3'	131	59	Mouse
Il21	S AS	5'-ATGGAGAGGACCCTTGTCTGTCTG-3' 5'-TATGTGCTTCTGTTTCTTTCCTCC-3'	336	60	Mouse
Lep	S AS	5′-CCAAAACCCTCATCAAGACC-3′ 5′-ATCCAACTGTTGAAGAATGTCC-3′	395	58	Mouse
Nampt	S AS	5′-ACAGATACTGTGGCGGGATTG-3′ 5′-TGATATCCACGCCATCTCCTTG-3′	424	58	Mouse
NAMPT	S AS	5′-GAACAGGATCTTTCGTTCCATA-3′ 5′-TTCCTGAGGGCTTTGTCATTCC-3′	357	58	Human
Nampt #1 (ChIP)	S AS	5′-GGTGACGGTCGGCTTTAGGC-3′ 5′-CTGCGCGTGCGCAGCGCAGG-3′	120	58	Mouse
Nampt #2 (ChIP)	S AS	5′-GTGGGTGGCTCCTTGGCCTTAG-3′ 5′-CCGCCCCAACCCGGACCTTCCT-3′	100	58	Mouse
Nampt #3 (ChIP)	S AS	5′-GAGGATCGGAATCCACAAGACG-3′ 5′-ACCCACTGTCGTCTCCCTGTTC-3′	100	58	Mouse
Nampt promoter	S AS	5′-CGCATAAAACAGCCTAAAATGG-3′ 5′-CTCGGCCCGGACCGGAGACGCCG-3′	1,230	55	Mouse
Nampt TG	S AS	5′-ATAAGAATGCGGCCGCATGAATGCTGCT GCGGCAGAAGCCGA-3′ 5′-ATAAGAATGCGGCCGCCGCCTAATGAGG TGCCACGTCCTGC-3′	1,476	55	Mouse
Mmp2	S AS	5′-CCAACTACGATGATGAC-3′ 5′-ACCAGTGTCAGTATCAG-3′	233	60	Mouse
Mmp3	S AS	5′-TCCTGATGTTGGTGGCTTCAG-3′ 5′-TGTCTTGGCAAATCCGGTGTA-3′	102	55	Mouse
Mmp9	S AS	5′-ACCACATCGAACTTCGA-3′ 5′-CGACCATACAGATACTG-3′	212	58	Mouse
Mmp12	S AS	5′-CCCAGAGGTCAAGATGGATG-3′ 5′-GGCTCCATAGAGGGACTGAA-3′	482	60	Mouse

Mmp13	S AS	5′-TGATGGACCTTCTGGTCTTCTGG-3′ 5′-CATCCACATGGTTGGGAAGTTCT-3′	473	55	Mouse
Mmp14	S AS	5′-GTGCCCTAGGCCTACATCCG-3′ 5′-TTGGGTATCCATCCATCACT-3′	580	55	Mouse
Mmp15	S AS	5′-GAGAGATGTTTGTGTTCAAGGG-3′ 5′-TGTGTCAATGCGGTCATAGGG-3′	260	62	Mouse
Pf4	S AS	5'-GAGCGTCGCTGCGGTGTTTC-3' 5'-TAGGGGTGCTTGCCGGTCCAG-3'	280	66	Mouse
Retn	S AS	5′-TTCTTCCTTGTCCCTGAACTG-3′ 5′-TGTCCAGTCTATCCTTGCACAC-3′	276	58	Mouse
Tnfa	S AS	5'-CTTGTCTACTCCCAGGTTCTCTTC-3' 5'-ACAGAGCAATGACTCCAAAGTAGACC-3'	301	58	Mouse

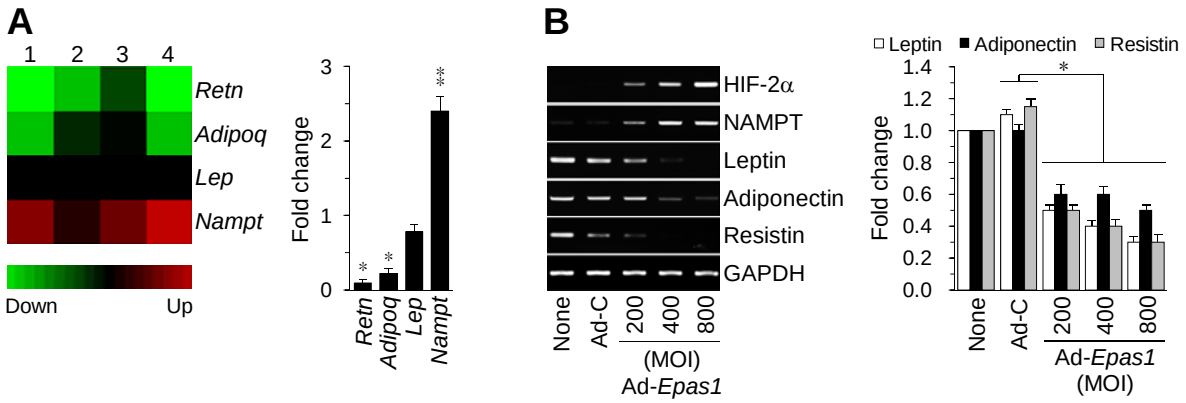
AT, annealing temperature; S, sense; AS, antisense.

Supplementary Table 2. siRNA sequences

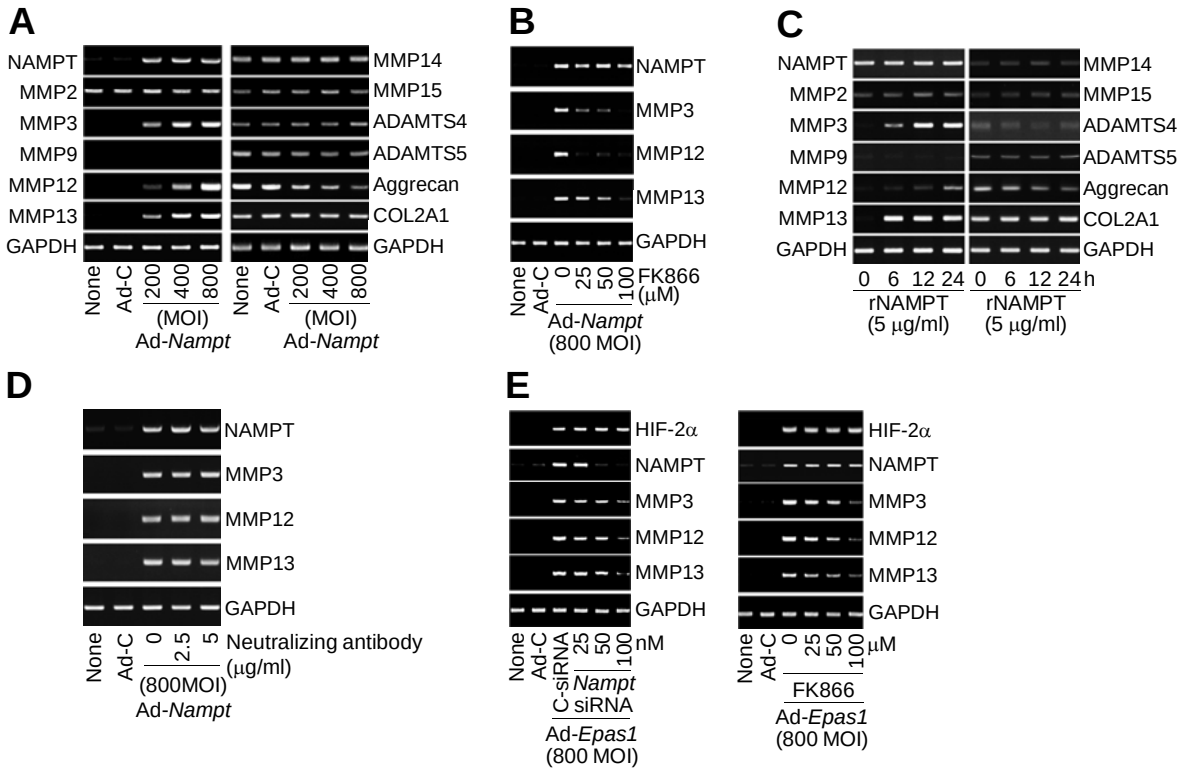
Target	Strand	Sequences
Control siRNA	S AS	5′-CCUACGCCACCAAUUUCGU-3′ 5′-ACGAAAUUGGUGGCGUAGG-3′
Epas1	S AS	5′-CUCAGUUACAGCCACAUCGUCACUG-3′ 5′-CAGUGACGAUGUGGCUGUAACUGAG-3′
Nampt	S AS	5′-UUCUCAAGGAUGUAGUUCCAUCCUC-3′ 5′-GAGGAUGGAACUACAUCCUUGAGAA-3′

S, sense; AS, antisense.

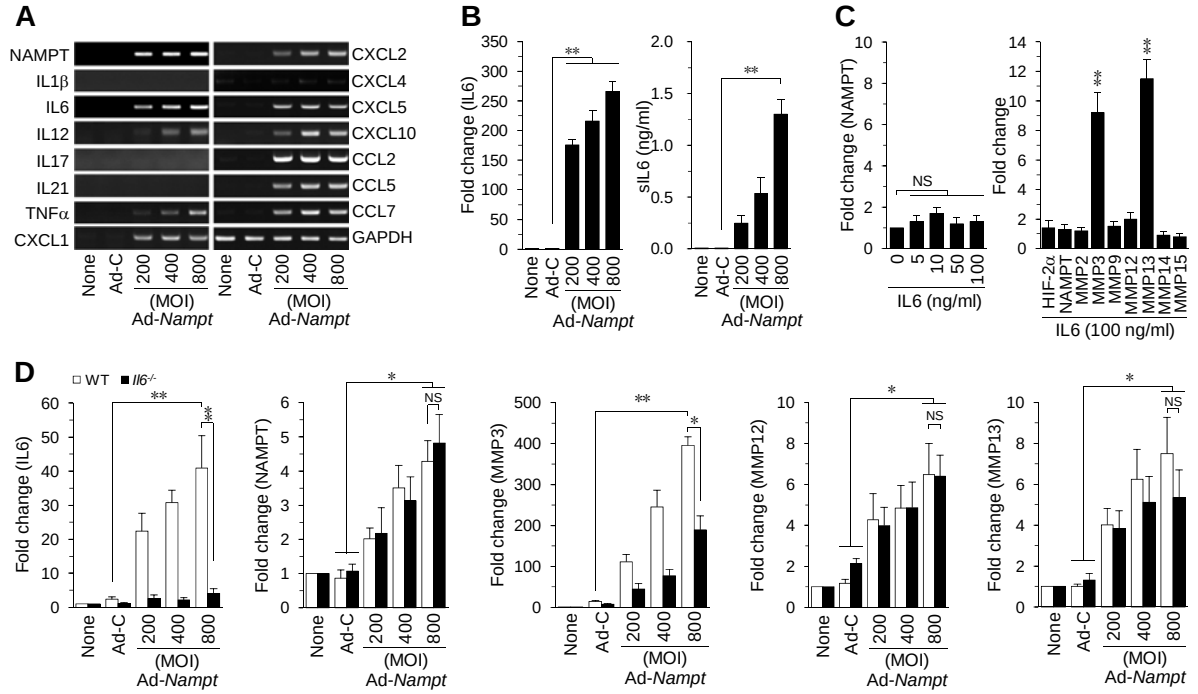
Supplementary Figures



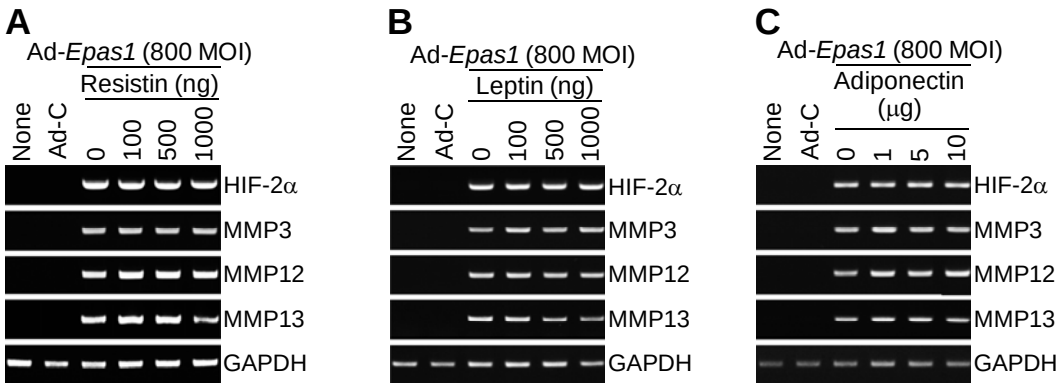
Supplementary Figure S1. HIF-2α regulation of adipokine expression in mouse articular chondrocytes. (A) Microarray analyses of primary cultures of mouse articular chondrocytes infected with Ad-*Epas1* at a MOI of 800 for 24 hours (n = 4). (B) Primary cultures of mouse articular chondrocytes were left untreated (None) or were infected with Ad-C (MOI 800) or Ad-*Epas1* (at the indicated MOI) for 24 hours. The indicated adipokines were detected by RT-PCR and their expression levels were quantified by qRT-PCR (n = 6). Values are presented as means ± SEM (*P < 0.01, **P < 0.001).



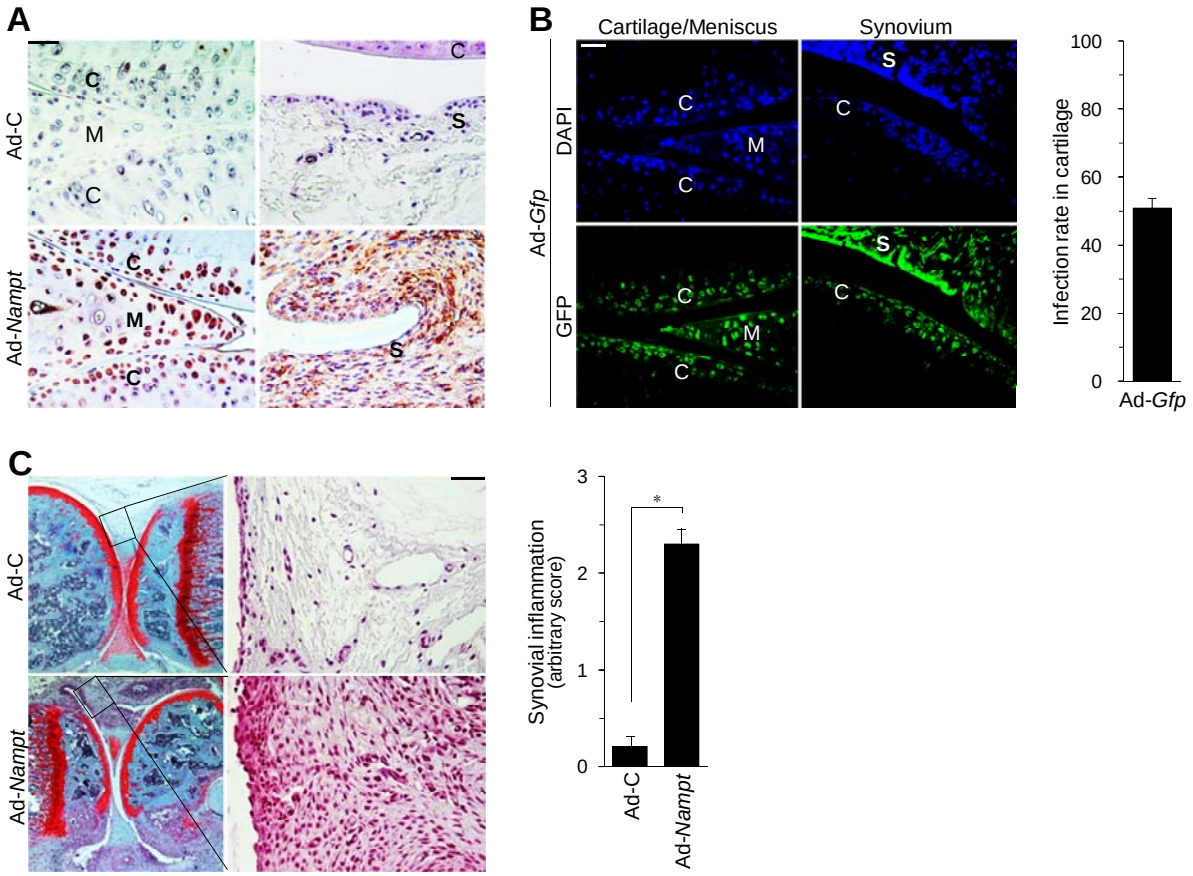
Supplementary Figure S2. RT-PCR analysis of catabolic factor expression by NAMPT in primary cultures of articular chondrocytes. (A) Representative RT-PCR results of the indicated catabolic and anabolic factors in chondrocytes infected with Ad-C (800 MOI) or Ad-*Nampt* (at the indicated MOI) ($n \geq 6$). (B) Representative RT-PCR results ($n = 6$) of the indicated catabolic and anabolic factors in chondrocytes infected at an MOI of 800 with Ad-C or Ad-*Nampt* in the absence or presence of FK866 (100 μM). (C) Representative RT-PCR results ($n = 6$) in chondrocytes treated with recombinant NAMPT (rNAMPT). (D) Chondrocytes were infected with Ad-*Nampt* (800 MOI) and treated with the indicated concentrations of neutralizing antibody against mouse NAMPT. Representative RT-PCR results are shown ($n = 4$). (E) Chondrocytes were transfected with 100 nM control siRNA (C-siRNA) or *Nampt* siRNA, or were treated with or without the indicated concentrations of FK866, and exposed to Ad-*Epas1* for 36 hours. mRNAs were detected by RT-PCR ($n \geq 6$). Values are presented as means ± SEM (*$P < 0.01$, **$P < 0.001$; NS, not significant).



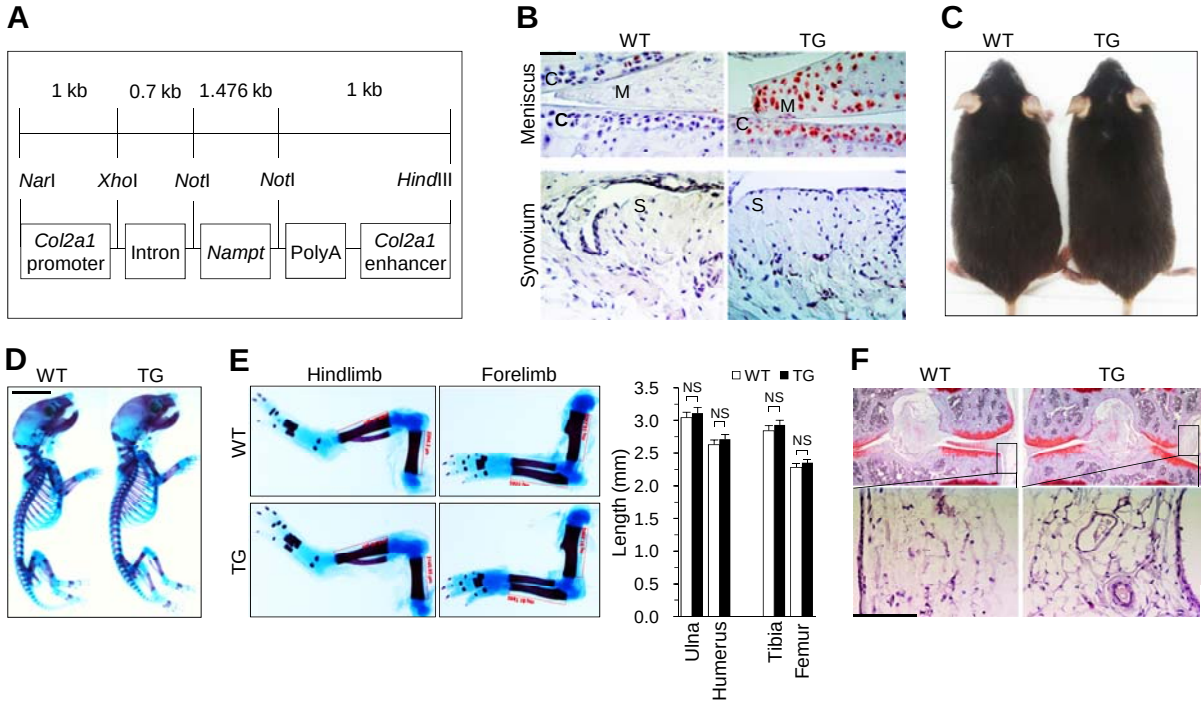
Supplementary Figure S3. NAMPT induces upregulation of cytokines and chemokines in articular chondrocytes. (A) Representative RT-PCR results for the indicated catabolic factors in chondrocytes infected with Ad-C (800 MOI) or Ad-*Nampt* (at the indicated MOI) ($n \geq 6$). (B) Chondrocytes were infected with Ad-C (800 MOI) or Ad-*Nampt* (at the indicated MOI) for 36 hours. IL6 mRNA levels and secreted IL6 protein (sIL6) were determined by RT-PCR and ELISA, respectively ($n = 5$). (C) Chondrocytes were treated with the indicated concentrations of recombinant IL6 protein for 24 hours, and NAMPT mRNA was quantified by qRT-PCR ($n = 6$) (left). Chondrocytes were treated with 100 ng/ml of IL6 for 24 hours, and expression levels (fold changes relative to vehicle controls) of the indicated genes were quantified by qRT-PCR ($n = 10$) (right). (D) Primary cultures of chondrocytes isolated from WT and *Il6*$^{-/-}$ mice were infected with Ad-C (800 MOI) or Ad-*Nampt* (at the indicated MOI) for 36 hours. Expression of the indicated mRNAs was quantified by qRT-PCR ($n = 5$). Values are presented as means ± SEM (*$P < 0.001$, **$P < 0.0001$; NS, not significant).



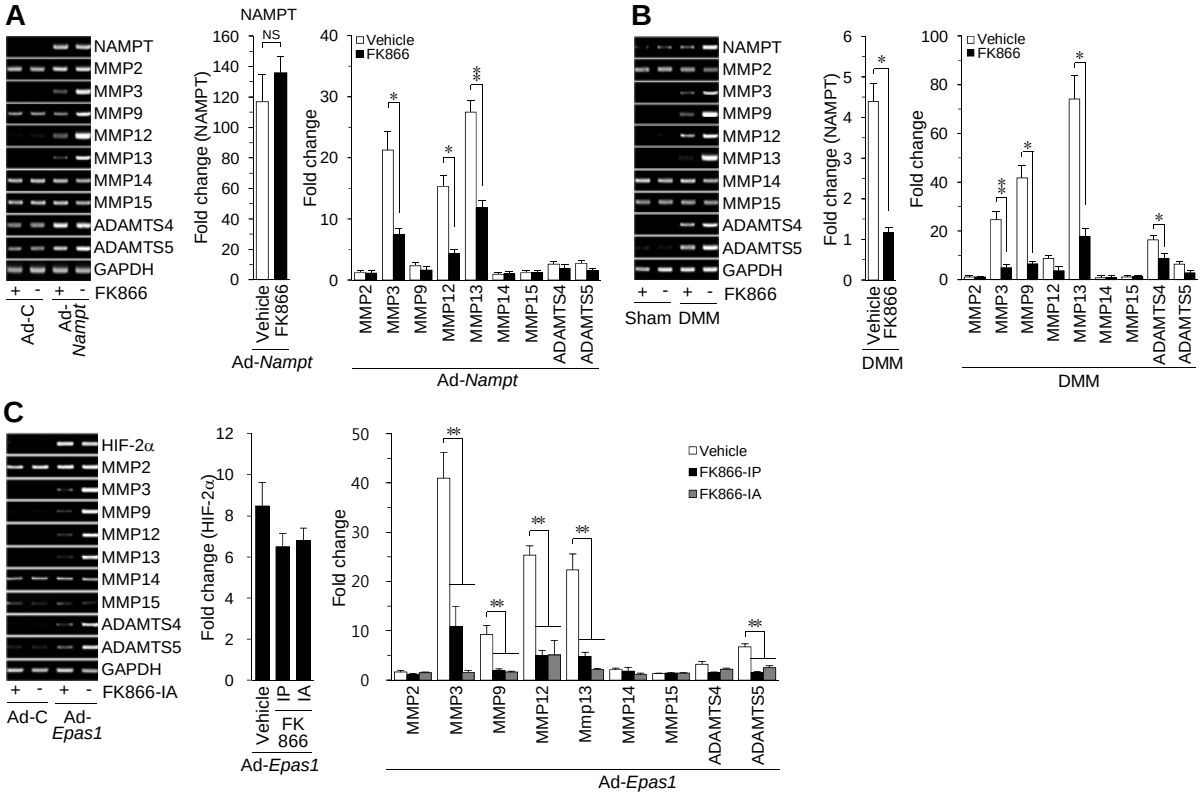
Supplementary Figure S4. Resistin, leptin, and adiponectin are not required for HIF-2α-induced upregulation of matrix-degrading enzymes. (A–C) Chondrocytes were untreated (None) or infected at an MOI of 800 with Ad-C or Ad-*Epas1* for 24 hours in the absence or presence of the indicated amounts of adipokines. mRNA levels of HIF-2α, MMP3, MMP12, and MMP13 were determined by qRT-PCR ($n \geq 6$).



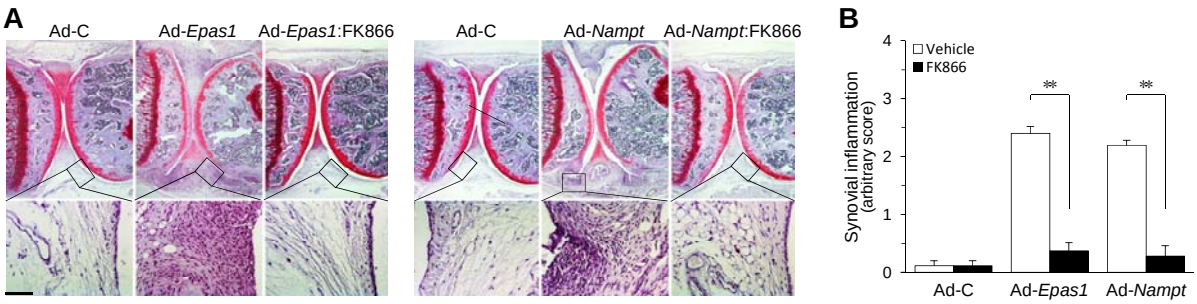
Supplementary Figure S5. Gene delivery into joint tissues by IA injection of adenovirus. (**A**) Mice were IA-injected with Ad-C or Ad-*Nampt* (1×10^9 PFU, once per week for 3 weeks), and sacrificed 21 days after the first injection. NAMPT expression in the meniscus, cartilage, and synovium was determined by immunostaining. (B) Mice were IA-injected with Ad-*Gfp* (1×10^9 PFU, once per week for 3 weeks), and sacrificed 21 days after the first injection. GFP expression was determined by fluorescence microscopy. Nuclei were detected by DAPI (4',6-diamidino-2-phenylindole) staining (left). The percentage of articular chondrocytes positive for GFP was determined from counts of more than 200 cells from four independent experiments (right). (C) Synovitis in mice injected with Ad-C or Ad-*Nampt* was detected by safranin-O and hematoxylin staining and quantified ($n = 10$). Values are presented as means ± SEM (*$P = 0.001$). Scale bar: 100 μm. C, cartilage; M, meniscus; S, synovium.



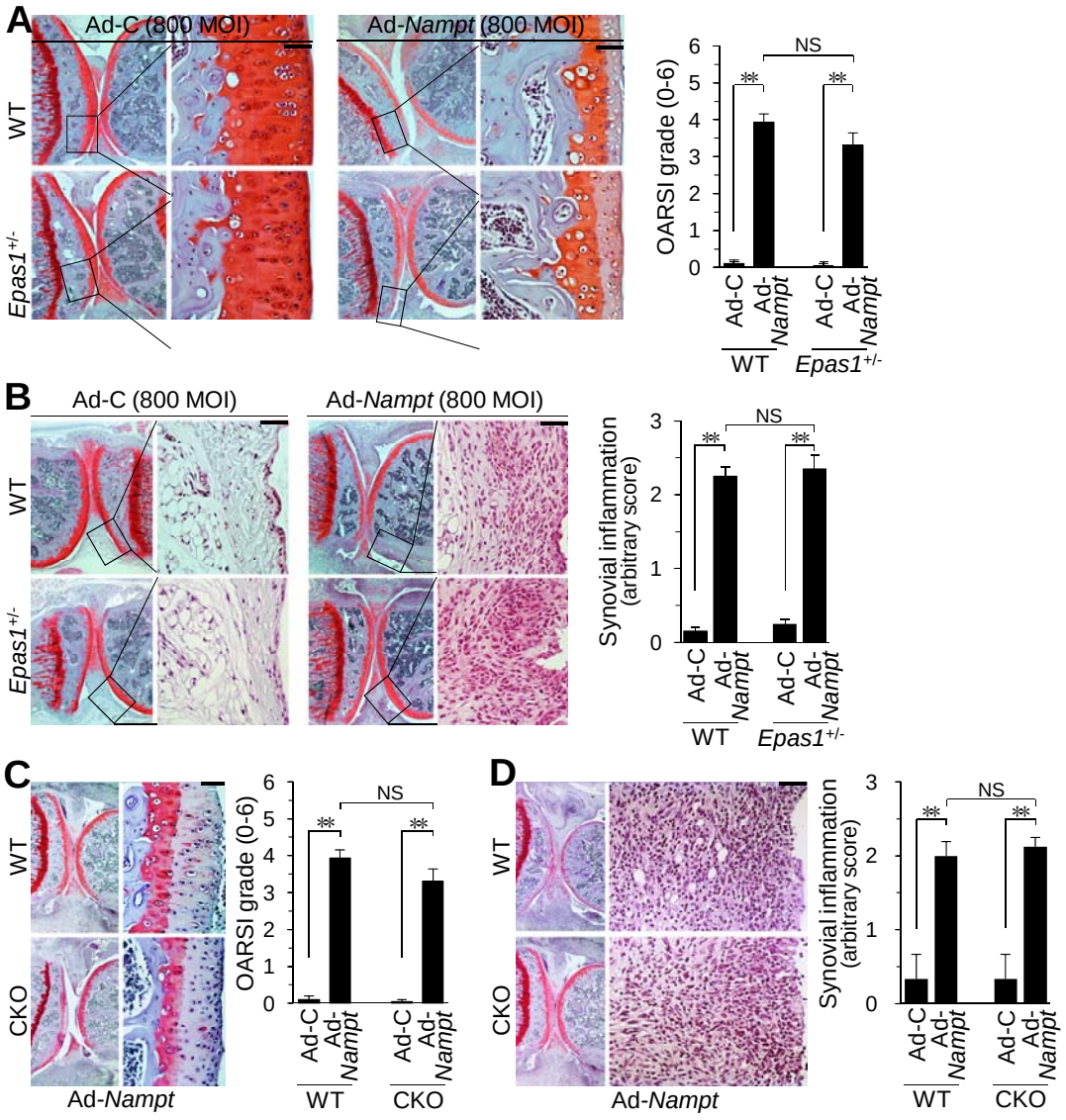
Supplementary Figure S6. Characterization of *Col2a1-Nampt* TG mice. (A) Vector constructs used for the generation of TG mice. (B) Immunostaining of NAMPT in joint tissues (cartilage, meniscus, and synovium) of *Col2a1-Nampt* TG mice and their WT littermates. (C) Images of 6-month-old *Col2a1-Nampt* TG mice and their WT littermates. (D) Alcian blue and alizarin red staining of E18.5 embryos of *Col2a1-Nampt* and their WT littermates. (E) Forelimb and hindlimb of *Col2a1-Nampt* TG mice and their WT littermates (post-natal day 1) were stained with alizarin red and alcian blue, and the lengths of their long bones were measured (n = 10). (F) Synovitis in 12-month-old *Col2a1-Nampt* TG mice and their WT littermates was detected by safranin-O and hematoxylin staining. Values are presented as means ± SEM (NS, not significant). Scale bars: 200 μm (B), 5 mm (D), and 200 μm (F). C, cartilage; M, meniscus; S, synovium.



Supplementary Figure S7. FK866 inhibits the *in vivo* expression of catabolic factors. (A) Mice were IA-injected with Ad-C or Ad-*Nampt* (1×10^9 PFU) with or without FK866 (10 mg/kg). mRNA levels in cartilage tissue were determined by RT-PCR and quantified by qRT-PCR ($n = 10$). (B) Following DMM or sham surgery, FK866 (10 mg/kg) was IP-injected once every 3 days for 8 weeks. Levels of the indicated mRNAs in cartilage tissue were determined by RT-PCR and quantified by qRT-PCR ($n = 10$). (C) Mice were IA-injected with Ad-C or Ad-*Epas1* (1×10^9 PFU) and co-injected (IA or IP) with FK866 (10 mg/kg). Levels of the indicated mRNAs in cartilage tissue were determined by RT-PCR and quantified by qRT-PCR ($n = 6$). Values are presented as means ± SEM (*$P < 0.01$, **$P < 0.001$; NS, not significant). Scale bar: 100 μm.



Supplementary Figure S8. FK866 inhibits synovitis in mice. (A and B) Mice were IA-injected with Ad-C, Ad-*Nampt*, or Ad-*Nampt* (1×10^9 PFU) with or without FK866 (10 mg/kg). Synovitis was detected by safranin-O and hematoxylin staining (n = 10). Values are presented as means ± SEM (**$P < 0.001$). Scale bar: 100 μm.



Supplementary Figure S9. *Epas1* knockout in mice does not affect OA cartilage destruction induced by Ad-*Nampt* injection. (A and B) Ad-C or Ad-*Nampt* (1×10^9 PFU) was injected into WT or *Epas1*$^{+/-}$ mice. Cartilage destruction was determined by safranin-O staining and quantified by Mankin's method (A, $n = 10$). Synovitis in mice injected with Ad-C or Ad-*Nampt* was detected by safranin-O/hematoxylin staining and scored (B, $n = 10$). (C and D) Ad-C or Ad-*Nampt* (1×10^9 PFU) was injected into WT or *Epas1*-CKO mice (*Epas1*$^{fl/fl}$;*Col2a1-Cre*). Cartilage destruction was determined by safranin-O staining and quantified by OARSI grade (C, $n = 10$). Synovitis in mice injected with Ad-C or Ad-*Nampt* was detected by safranin-O/hematoxylin staining and scored (D, $n = 10$). Values are presented as means ± SEM (**$P < 0.001$; NS, not significant). Scale bar: 100 μm.