EXTENDED REPORT

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

Siyoung Yang,[1,2] Je-Hwang Ryu,[3] Hwanhee Oh,[1] Jimin Jeon,[1] Ji-Sun Kwak,[1] Jin-Hong Kim,[1] Hyun Ah Kim,[4] Churl-Hong Chun,[5] Jang-Soo Chun[1]

ABSTRACT
Objective Hypoxia-inducible factor 2α (HIF-2α), encoded by *Epas1*, causes osteoarthritic cartilage destruction by regulating the expression of matrix-degrading enzymes. We undertook this study to explore the role of nicotinamide phosphoribosyltransferase (NAMPT or visfatin) in HIF-2α-mediated osteoarthritic cartilage destruction.
Methods The expression of HIF-2α, NAMPT and matrix-degrading enzymes was determined at the mRNA and protein levels in human osteoarthritis (OA) cartilage, mouse experimental OA cartilage and primary cultured mouse chondrocytes. Experimental OA in mice was induced by destabilisation of the medial meniscus (DMM) surgery or intra-articular injection of Ad-*Epas1* or Ad-*Nampt* in wild-type, *Epas1*^{+/−}, *Epas1*^{fl/fl};*Col2a1-Cre* and *Col2a1-Nampt* transgenic (TG) mice. Primary cultured mouse chondrocytes were treated with recombinant NAMPT protein or were infected with adenoviruses.
Results We found that the *Nampt* gene is a direct target of HIF-2α in articular chondrocytes and OA cartilage. NAMPT protein, in turn, increased mRNA levels and activities of MMP3, MMP12 and MMP13 in chondrocytes, an action that was necessary for HIF-2α-induced expression of catabolic enzymes. Gain-of-function studies (intra-articular injection of Ad-*Nampt*; *Col2a1-Nampt* TG mice) and loss-of-function studies (intra-articular injection of the NAMPT inhibitor FK866) demonstrated that NAMPT is an essential catabolic regulator of osteoarthritic cartilage destruction caused by HIF-2α or DMM surgery.
Conclusions Our findings indicate that NAMPT, whose corresponding gene is a direct target of HIF-2α, plays an essential catabolic role in OA pathogenesis and acts as a crucial mediator of osteoarthritic cartilage destruction caused by HIF-2α or DMM surgery.

INTRODUCTION
Osteoarthritis (OA) is a degenerative joint disease that is primarily characterised by cartilage destruction. A variety of aetiological risk factors and pathophysiological processes contribute to the progressive nature of the disease. Important among potential OA-causing mechanisms are mechanical stresses, including joint instability and injury, and factors that predispose toward OA, such as ageing. These factors lead to the activation of biochemical pathways in chondrocytes that result in degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS).[1–3] We have

previously demonstrated that hypoxia-inducible factor (HIF)-2α, encoded by *EPAS1*, triggers osteoarthritic cartilage destruction by upregulating chondrocyte expression of matrix-degrading enzymes.[4–6]

In a preliminary study, we identified *Nampt* as a HIF-2α-upregulated gene in mouse chondrocytes. *Nampt* encodes nicotinamide phosphoribosyltransferase, which functions as both an intracellular form (iNAMPT) and an extracellular form (eNAMPT). eNAMPT, also known as visfatin, acts as an adipokine, whereas the nicotinamide phosphoribosyltransferase enzymatic activity of iNAMPT regulates salvage pathways of NAD^+ synthesis.[7 8] eNAMPT displays pro-inflammatory functions and appears to be associated with inflammatory diseases, such as rheumatoid arthritis (RA).[9 10] For instance, levels of eNAMPT in the synovial fluid of RA patients are positively related to disease severity.[11–13] eNAMPT regulates the expression of chemokines and cytokines in cell types involved in RA pathogenesis.[14 15] Additionally, inhibition of iNAMPT enzymatic activity with FK866[16] blocks experimental RA.[17 18] Similar to their association with RA, elevated eNAMPT levels are positively linked to OA pathogenesis.[19] eNAMPT in human OA chondrocytes inhibits proteoglycan synthesis and increases the expression of matrix-degrading enzymes.[20 21] Additionally, interleukin (IL)-1β acts through overexpression of NAMPT to inhibit collagen type II (COL2A1) expression in chondrocytes.[22] Although these observations suggest a possible role for NAMPT in OA pathogenesis, the contribution of NAMPT in OA pathogenesis in vivo remains to be established. In this study, we investigated the role and underlying molecular mechanisms of HIF-2α-upregulated NAMPT in OA pathogenesis.

MATERIALS AND METHODS
OA cartilage and experimental OA
Human OA cartilage was sourced from individuals undergoing arthroplasty.[4–6] Male C57BL/6, STR/ort, CBA/CaCrl, *Col2a1-Nampt* transgenic (TG), *Epas1*^{+/−}, *Epas1*^{fl/fl};*Col2a1-Cre* and *Il6*^{−/−} mice were used for experimental OA studies. *Col2a1-Nampt* TG mice were generated using the *Col2a1* promoter and enhancer.[4–6] *Epas1*^{+/−}, *Epas1*^{fl/fl} and *Col2a1-Cre* TG mice were obtained from the Jackson Laboratory. *Epas1*^{fl/fl};*Col2a1-Cre* and *Il6*^{−/−} mice were described previously.[5 6] Spontaneous OA in STR/ort[23] and *Nampt* TG mice was examined at 28 and 55 weeks of age,
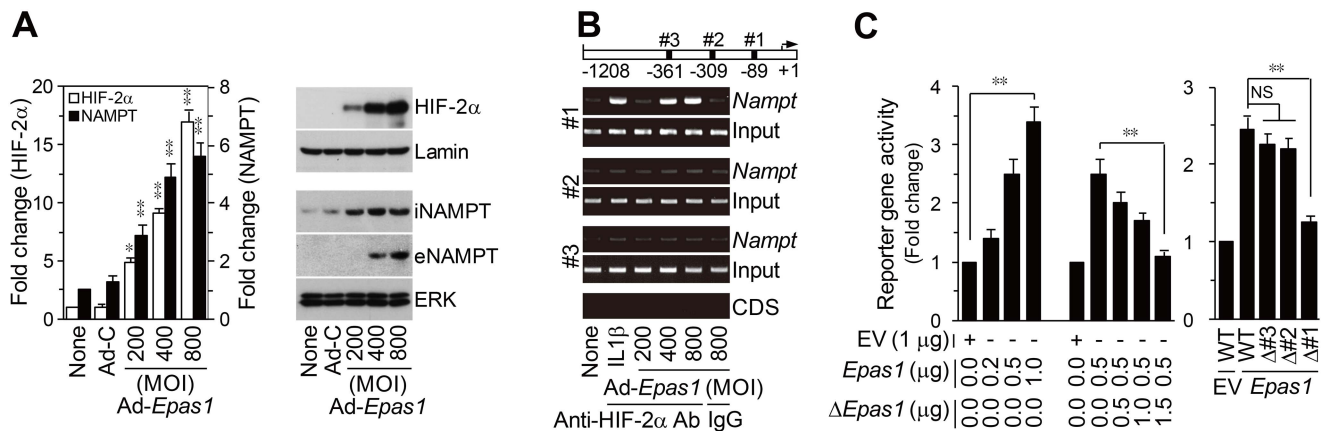
Figure 1 *Nampt* is a direct target gene of HIF-2α in mouse articular chondrocytes. (A) qRT-PCR (n=6) and western blotting of HIF-2α and NAMPT in chondrocytes infected with Ad-C (800 MOI) or Ad-*Epas1* for 24 h. (B) HIF-2α binding to the *Nampt* promoter was detected by ChIP assay in chondrocytes treated with IL-1β (1 ng/mL) or infected with Ad-*Epas1*. The *Nampt* coding sequence (CDS) was used as a negative control. IgG was used as an isotype control for the anti-HIF-2α antibody. (C) *Nampt* reporter gene activity in chondrocytes transfected with empty vector (EV), *Epas1* vector or Δ*Epas1* vector (n=6) (left). Reporter gene activity of wild-type (WT) and HIF-2α binding site-mutated reporter genes (n=6) (right). *p<0.01, **p<0.001.

respectively. Experimental OA was induced by destabilisation of the medial meniscus (DMM) surgery. Mice were also injected intraperitoneally with FK886 (10 mg/kg) once every 3 days and sacrificed 8 weeks after DMM surgery. Experimental OA was additionally induced by intra-articular injection (once weekly for 3 weeks) of Ad-*Nampt* or Ad-*Epas1* (1×10^9 plaque forming units) with or without FK886. Mice were sacrificed 21 days after the first intra-articular injection.[4–6 24]

Histology and immunohistochemistry

Human OA cartilage sections were stained with alcian blue. Cartilage destruction in mice was examined using safranin-O
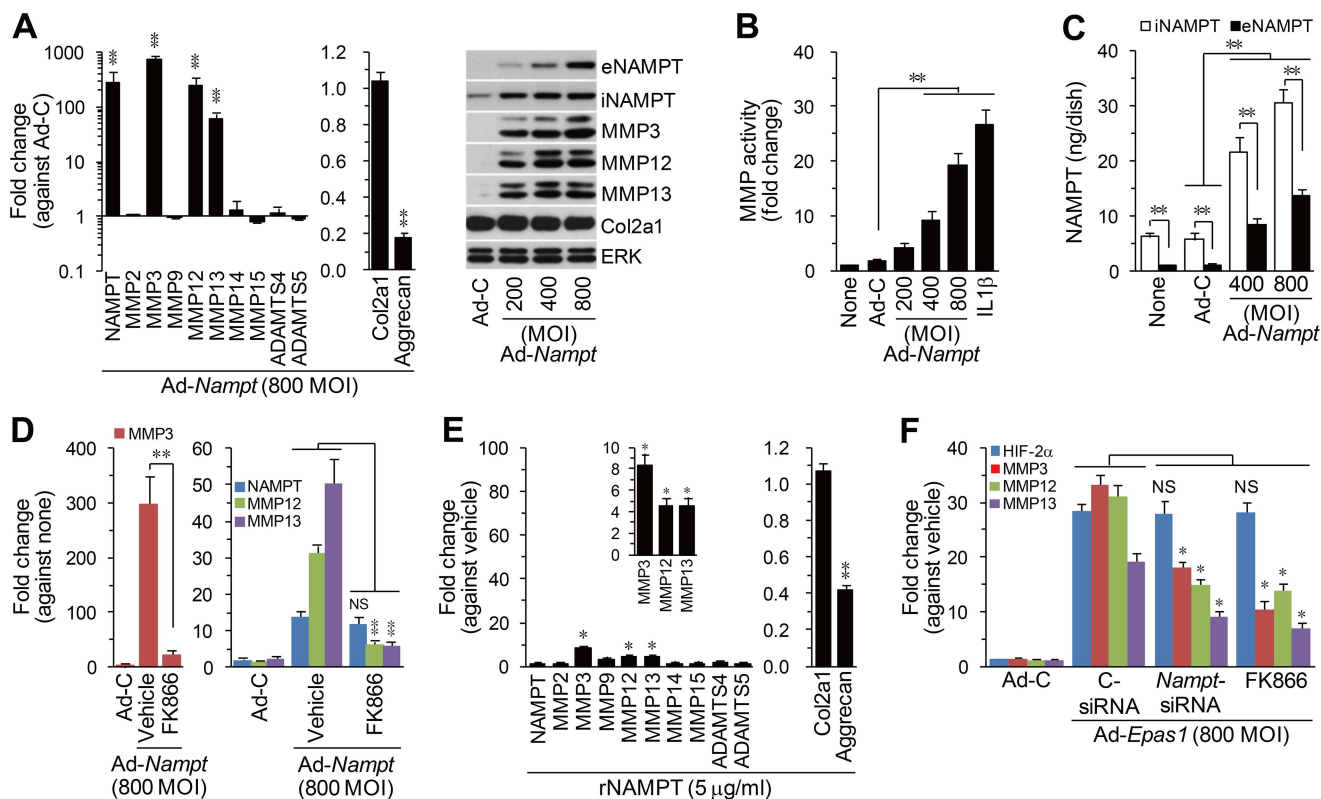

Figure 2 NAMPT is required for HIF-2α-induced matrix metalloproteinase (MMP) expression in chondrocytes. (A) Detection of the indicated mRNAs/proteins by qRT-PCR/western blotting in chondrocytes infected with Ad-C or Ad-*Nampt* (n=6). (B) MMP activity assays in chondrocytes infected with Ad-C (800 MOI) or Ad-*Nampt* (n=5). (C) NAMPT levels quantified by ELISA (n=5) in chondrocytes infected with Ad-C (800 MOI) or Ad-*Nampt*. (D) Expression levels of catabolic factors determined by qRT-PCR (n=6) in chondrocytes infected at an MOI of 800 with Ad-C or Ad-*Nampt* in the absence or presence of FK866 (100 μM). (E) qRT-PCR (n=6) in chondrocytes treated with recombinant NAMPT (rNAMPT). (F) Chondrocytes were transfected with 100 nM control siRNA (C-siRNA) or *Nampt* siRNA, or were treated with or without FK866 (100 μM) and exposed to Ad-*Epas1* for 36 h. mRNA levels were determined by qRT-PCR (n=6). *p<0.01, **p<0.001.
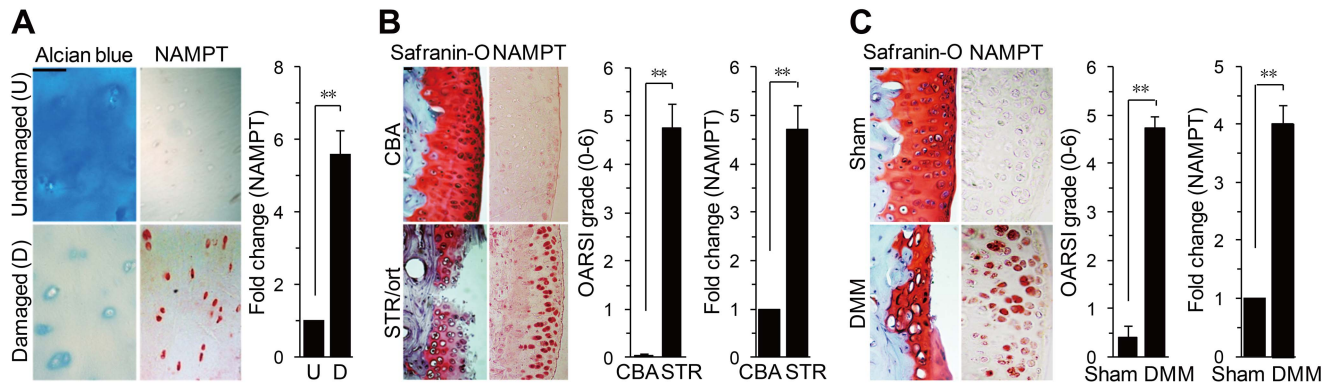
Yang S, *et al. Ann Rheum Dis* 2015;**74**:595–602. doi:10.1136/annrheumdis-2013-204355

Figure 3 NAMPT is overexpressed in human and mouse osteoarthritis (OA) chondrocytes. (A) Alcian blue staining, NAMPT immunostaining and NAMPT mRNA levels quantified by qRT-PCR of human OA cartilage. (B and C) Spontaneous OA cartilage from STR/ort mice and CBA control mice (B), and sham- and destabilisation of the medial meniscus (DMM)-operated C57BL/6 mice (C). mRNA levels in cartilage tissue were quantified by qRT-PCR (n=10). **p<0.001. Scale bar: 50 μm.

staining and scored using the OARSI grading system.[25] Synovitis was determined by haematoxylin staining. Detailed procedures are presented in online supplementary materials and methods.

Other materials and methods

Additional methods are described in online supplementary materials and methods. These include histology and immunohistochemistry; primary culture of articular chondrocytes; microarray analysis; adenoviruses, infection of chondrocytes and intra-articular injection of mice; ELISA; reverse transcription-PCR (RT-PCR), quantitative RT-PCR (qRT-PCR) and small inhibitory RNA; western blotting; cloning, reporter gene assays and ChIP assays; skeletal staining; and MMP activity assays. PCR primer and siRNA sequences are summarised in online supplementary Table S1 and Table S2, respectively.

Statistical analysis

Values are presented as means±SEM. The n numbers indicated for each figure correspond to the number of independent experiments or the number of mice used. Data quantified based on an ordinal grading system, such as OARSI grade, were analysed using non-parametric statistical methods. For qRT-PCR, data expressed as relative fold changes, Student t test and analysis of variance with post hoc tests were used for pair-wise comparisons and multi-comparisons, respectively, after first confirming a normal distribution using the Shapiro–Wilk test. Significance was accepted at the 0.05 level of probability (p<0.05).

RESULTS

HIF-2α directly targets the *Nampt* gene in articular chondrocytes

Microarray analyses of chondrocytes infected with Ad-*Epas1* indicated upregulation of *Nampt* (visfatin) and downregulation of *Adipoq* (adiponectin), *Retn* (resistin) and *Lep* (leptin) (online supplementary figure S1). Differential adipokine expression was additionally confirmed in primary cultured chondrocytes (figure 1A). We focused on the role of NAMPT, whose corresponding gene is positively regulated by HIF-2α, in OA pathogenesis. ChIP assays revealed that HIF-2α binds to the proximal site among the three HIF-2α binding sites (CGTG) in the mouse *Nampt* promoter (figure 1B). HIF-2α stimulated *Nampt* promoter activity, an effect that was inhibited by co-transfection of dominant-negative (Δ) HIF-2α (figure 1C). Mutation of the proximal site, but not other HIF-2α binding sites, inhibited reporter gene activity (figure 1C). These results

collectively show that the *Nampt* gene is a direct target of HIF-2α in chondrocytes.

NAMPT is required for HIF-2α-induced MMP expression

We next investigated the possible role of NAMPT in the expression of matrix-degrading enzymes and ECM proteins in chondrocytes. Overexpression of NAMPT by Ad-*Nampt* infection induced upregulation and activation of the MMP3, MMP12 and MMP13, and downregulation of aggrecan without significant effects on COL2A1 expression (figure 2A,B and online supplementary figure S2A). ELISAs revealed that both iNAMPT and eNAMPT were produced by Ad-*Nampt* infection (figure 2C). The significance of NAMPT enzymatic activity was determined using the specific inhibitor FK866.[16] FK866 significantly blocked Ad-*Nampt*-induced upregulation of MMP, MMP12 and MMP13 (figure 2D and online supplementary figure S2B). eNAMPT action was assessed by treating chondrocytes with recombinant mouse NAMPT protein (rNAMPT). rNAMPT stimulated the expression of MMPs (MMP3, MMP12 and MMP13) and downregulated aggrecan (figure 2E and online supplementary figure S2C). However, addition of a neutralising antibody against eNAMPT did not affect Ad-*Nampt*-induced upregulation of MMP3, MMP12 and MMP13 (online supplementary figure S2D). Additionally, HIF-2α-induced MMP expression was significantly blocked by knockdown or inhibition of NAMPT (figure 2F and online supplementary figure S2E). NAMPT overexpression in chondrocytes also upregulated various cytokines and chemokines (online supplementary figure S3). Among these, IL-6 induced upregulation of MMP3 and MMP13, consistent with a previous report.[6] Experiments using chondrocytes from Il6^{−/−} mice demonstrated that NAMPT-induced MMP3 expression was at least partly mediated by IL-6 production (online supplementary figure S3). However, treatment of Ad-*Epas1*-infected chondrocytes with other adipokines (ie, resistin, adiponectin and leptin) did not affect HIF-2α-induced upregulation of MMPs (online supplementary figure S4). Collectively, these results indicate that NAMPT promotes upregulation of MMP3, MMP12 and MMP13 in chondrocytes, an action that is necessary for HIF-2α-induced expression of catabolic enzymes.

NAMPT is upregulated in OA cartilage

To ascertain whether NAMPT plays a role in OA pathogenesis, we examined NAMPT expression in OA cartilage of humans
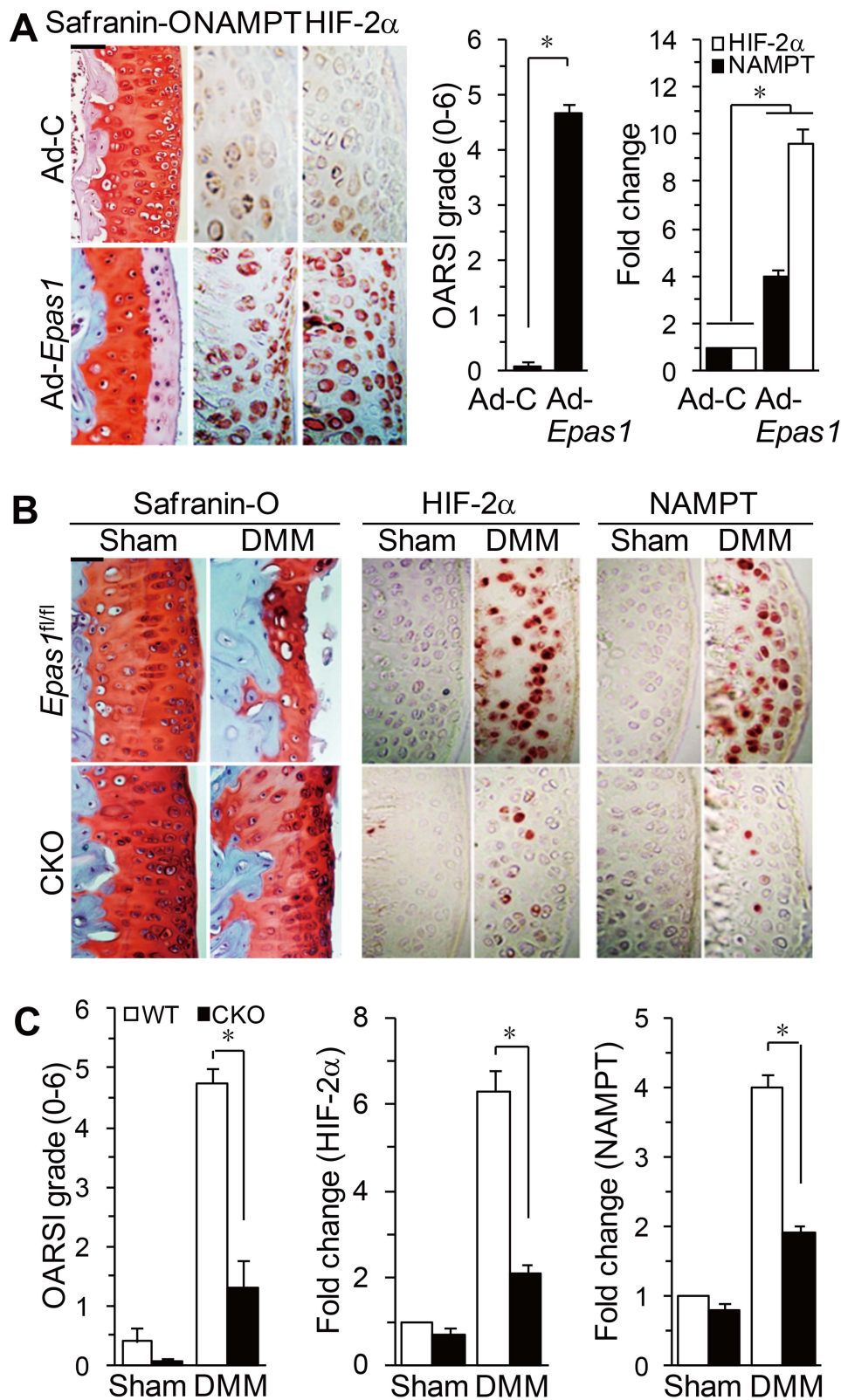
Figure 4 NAMPT expression in osteoarthritis cartilage is regulated by HIF-2α. (A) Cartilage tissue of mice, intra-articularly injected with Ad-C or Ad-*Epas1*, was used for safranin-O staining, OARSI grade, and immunodetection/qRT-PCR of HIF-2α and NAMPT proteins/mRNAs (n=6). (B and C) Safranin-O staining, immunostaining for HIF-2α and NAMPT, OARSI grade, and qRT-PCR of NAMPT and HIF-2α mRNAs (n=10) in wild-type (WT) and *Epas1* conditional knockout (CKO) mice following destabilisation of the medial meniscus (DMM) surgery. *p<0.001. Scale bar: 100 μm.

and mice. NAMPT mRNA and protein were markedly elevated in OA-affected human cartilage compared with undamaged regions of arthritic cartilage (figure 3A). NAMPT levels were also increased in mouse OA models, including STR/ort mouse OA cartilage (figure 3B) and OA cartilage induced by DMM surgery (figure 3C). OA cartilage in both humans and mouse
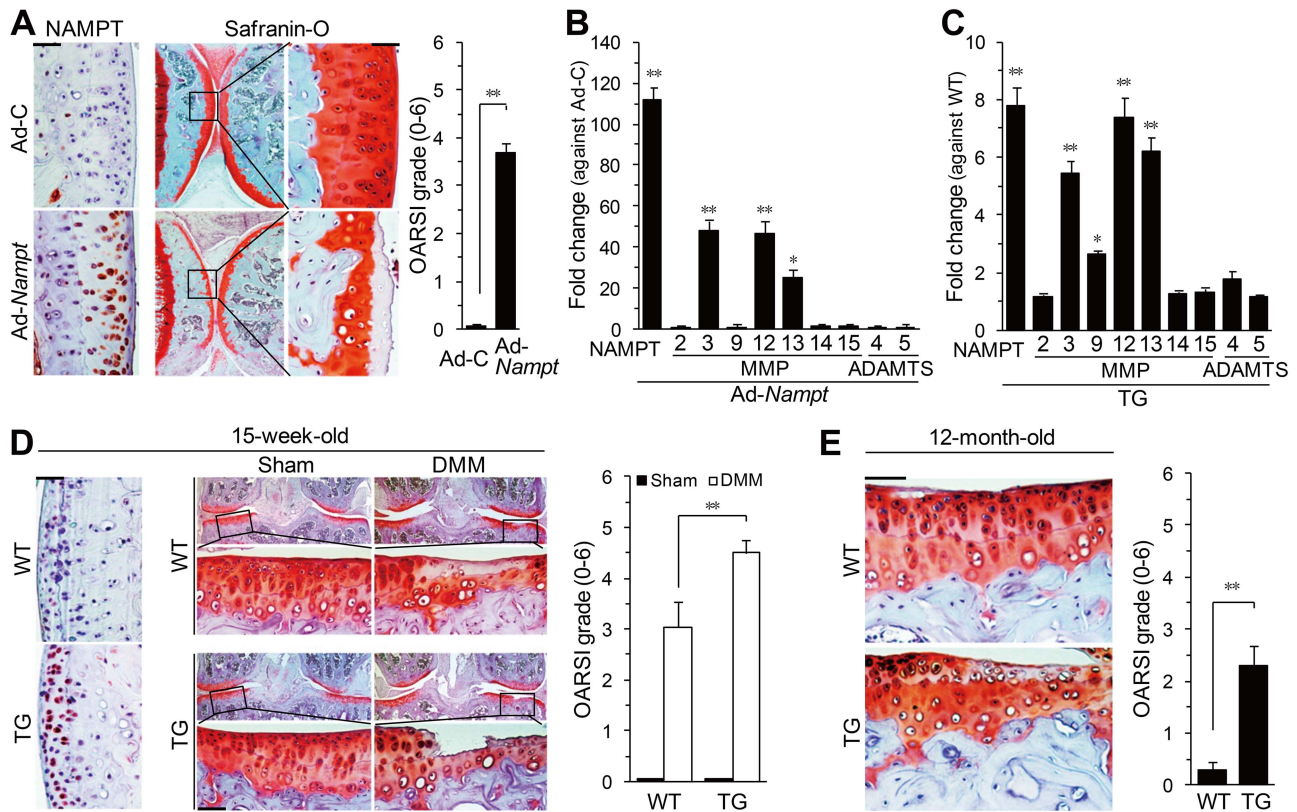
Figure 5 Overexpression of NAMPT in chondrocytes causes osteoarthritis cartilage destruction. (A and B) Mice were Intra-articularly injected with Ad-C or Ad-*Nampt* and evaluated by (A) NAMPT immunostaining, safranin-O staining and OARSI grading (n=6), and (B) qRT-PCR (n=6) of catabolic factors in cartilage tissues. (C) qRT-PCR of mRNAs in chondrocytes isolated from wild-type (WT) or *Col2a1-Nampt* transgenic (TG) mice (n=7). (D) NAMPT immunostaining, safranin-O staining and OARSI grade (n=13) in destabilisation of the medial meniscus (DMM)- or sham-operated WT and *Col2a1-Nampt* TG mice. (E) Spontaneous cartilage destruction in 12-month-old WT and TG mice determined by safranin-O staining and OARSI grade (n=10). *p<0.01, **p<0.001. Scale bar: 100 μm.

models shows increased expression of HIF-2α.[4–6] Consistent with this, overexpression of HIF-2α in articular cartilage by intra-articular injection of Ad-*Epas1* increased NAMPT levels, with concomitant osteoarthritic cartilage destruction (figure 4A). Additionally, DMM-induced NAMPT upregulation and OA cartilage destruction were significantly blocked in chondrocyte-specific *Epas1* conditional knockout (CKO) mice (figure 4B,C), indicating that NAMPT expression in chondrocytes of OA cartilage is regulated by HIF-2α.

NAMPT causes experimental OA cartilage destruction

The role of NAMPT in OA pathogenesis in mice was examined by intra-articular injection of Ad-*Nampt*, which caused NAMPT overexpression in cartilage, meniscus and synovium (figure 5A and online supplementary figure S5A). Gene delivery by intra-articular injection of adenovirus was further confirmed by monitoring injected Ad-*Gfp* (online supplementary figure S5B). Ad-*Nampt* injection triggered cartilage destruction with significantly increased expression of the NAMPT targets MMP3, MMP12 and MMP13 in cartilage tissue (figures 5A,B). Ad-*Nampt* injection additionally resulted in synovitis (online supplementary figure S5C). The role of NAMPT was further validated by generating *Col2a1-Nampt* TG mice. Chondrocytes from these mice, which showed no developmental defects (online supplementary figure S6), exhibited elevated mRNA levels of NAMPT and its target genes compared with wild-type (WT) chondrocytes (figure 5C). Consistent with this, *Col2a1-Nampt* TG mice showed significantly enhanced

osteoarthritic cartilage destruction following DMM surgery (figure 5D). Moreover, aged (12-month-old) TG mice showed spontaneous cartilage destruction compared with WT mice of the same age (figure 5E). Thus, gain-of-function studies of NAMPT collectively indicate that NAMPT is an important catabolic regulator of OA pathogenesis.

NAMPT is required for experimental OA cartilage destruction

Because iNAMPT enzymatic activity is necessary for MMP expression, we examined whether iNAMPT regulates OA pathogenesis. Injection of FK866 (intraperitoneally or intra-articularly) significantly inhibited cartilage destruction induced by Ad-*Nampt* injection (figure 6A), with concomitant reduction of the mRNA levels NAMPT targets MMP3, MMP12 and MMP13 in cartilage tissue (online supplementary figure S7A). Intraperitoneal injection of FK866 also inhibited DMM-induced cartilage destruction (figure 6B) and upregulation of NAMPT target MMPs (online supplementary figure S7B). Finally, injection of FK866 (intraperitoneally or intra-articularly) blocked Ad-*Epas1*-induced cartilage destruction and expression of NAMPT target MMPs (figure 6C and online supplementary figure S7C). Additionally, synovitis caused by Ad-*Epas1* or Ad-*Nampt* injection was blocked by co-injection of FK866 (online supplementary figure S8). However, knockdown of *Epas1* in *Epas1*^{+/−} or *Epas1*^{fl/fl};*Col2a1-Cre* mice did not affect the cartilage destruction induced by Ad-*Nampt* injection (online supplementary figure S9), suggesting that NAMPT is downstream
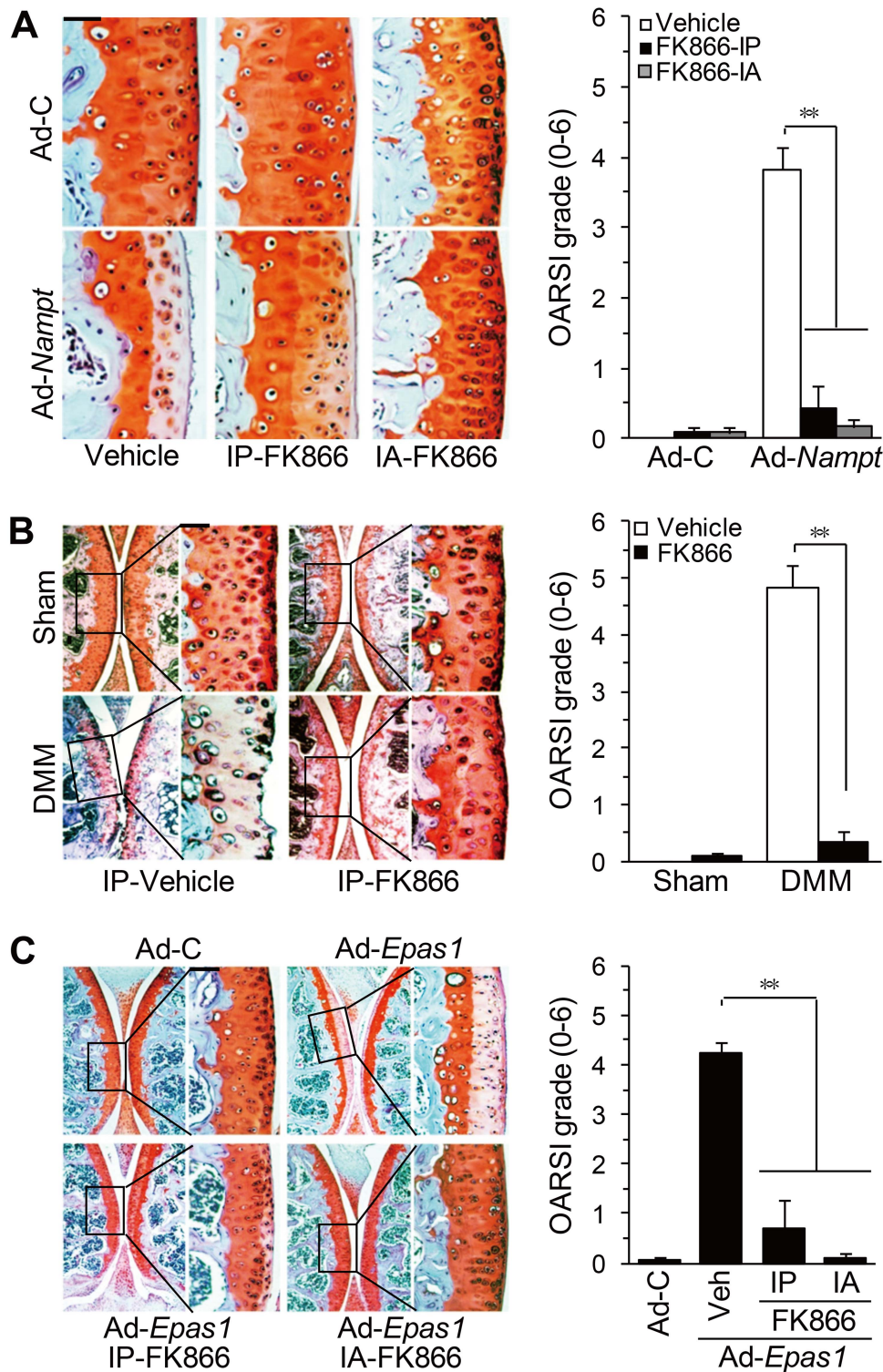
Figure 6 Inhibition of NAMPT enzymatic activity blocks osteoarthritis cartilage destruction. (A) Mice were intra-articularly injected with Ad-C or Ad-*Nampt* and co-injected (intra-articularly or intraperitoneally) with FK866. Cartilage destruction was detected by safranin-O staining and quantified by OARSI grade (n=10). (B) Mice were intraperitoneally injected with FK866 after destabilisation of the medial meniscus (DMM) surgery. Cartilage destruction was detected and quantified (n=10). (C) Mice were intra-articularly injected with Ad-C or Ad-*Epas1* and co-injected (intra-articularly or intraperitoneally) with FK866. Cartilage destruction was detected and quantified (n=10). **p<0.001. Scale bar: 100 μm.

of HIF-2α. These results collectively indicate that iNAMPT enzymatic activity is necessary for OA pathogenesis induced by DMM surgery or intra-articular injection of Ad-*Nampt* or Ad-*Epas1*.

DISCUSSION
Catabolic signals in OA pathogenesis are categorised into two main pathways: upregulation of tissue-destructive enzymes such as MMP3, MMP13 and ADAMTS5, and downregulation of

cartilage-specific ECM proteins such as type II collagen and aggrecan. HIF-2α serves as an essential catabolic regulator of OA pathogenesis by upregulating tissue-destructive genes.[4–6] We demonstrated here that NAMPT, whose corresponding gene is a direct target of HIF-2α, plays an essential role in OA pathogenesis by upregulating matrix-degrading enzymes and downregulating aggrecan expression. Our gain-of-function studies (intra-articular injection of Ad-*Nampt*; *Col2a1-Nampt* TG mice) and loss-of-function studies (intra-articular injection of FK866) demonstrated that NAMPT is an essential catabolic regulator of OA pathogenesis. NAMPT appears to exert its catabolic functions by downregulating aggrecan and upregulating MMP3 and MMP13, which are critical effectors of OA pathogenesis.[26 27] The mechanism by which NAMPT regulates MMP expression was not elucidated in this study. However, because NAMPT acts as an enzyme and adipokine, we speculate that NAMPT regulates the expression of MMP genes indirectly, rather than by directly modulating the activity of their promoters. Possible mechanisms include modulation of chromatin functions by NAMPT-regulated members of the Sirtuin family[7] [28] and regulation by secondary mediators such as cytokines and chemokines that are upregulated by NAMPT. Indeed, we found that NAMPT-induced MMP3 expression is mediated, at least in part, by IL-6. In addition to the NAMPT target MMPs, MMP9 was also upregulated in OA cartilage induced in response to DMM surgery or Ad-*Epas1* injection, and intra-articular injection of FK866 inhibited both cartilage destruction and MMP9 expression. Because NAMPT does not directly modulate MMP9 expression, we speculate that the inhibitory effect of FK866 on MMP9 expression is attributable to inhibition of osteoarthritic cartilage destruction by FK866.

Our results suggest that iNAMPT enzymatic activity plays an important role in the regulation of catabolic factor expression and is sufficient to promote OA pathogenesis. Consistent with this, administration of FK866 abolished experimental OA induced by DMM surgery or injection of Ad-*Nampt* or Ad-*Epas1*. However, eNAMPT also appears to plays a role in OA pathogenesis. This is clearly demonstrated by the observations that Ad-*Nampt* or Ad-*Epas1* infection increased both iNAMPT and eNAMPT and that rNAMPT treatment caused upregulation of MMP3, MMP12 and MMP13. To date, the contribution of NAMPT to OA pathogenesis has not been established *in vivo*, although *in vitro* studies have suggested possible functions in OA pathogenesis.[19–22] Therefore, our results are the first evidence for in vivo catabolic functions of NAMPT in the pathogenesis of OA. Intra-articular injection of Ad-*Nampt* also caused synovitis, which was blocked by inhibition of NAMPT enzymatic activity. Because OA is a disease that involves all joint tissues, including cartilage, synovium and subchondral bone, the synovial inflammation may affect cartilage destruction; hence, the inhibitory effects of the NAMPT inhibitor FK866 on synovitis may contribute to the observed effects on cartilage destruction.

NAMPT enzymatic activity regulates salvage pathways of NAD^+ synthesis,[7 8] and NAD^+, in turn, is an essential cofactor of Sirtuin (SIRT) family protein deacetylases.[7 29] Indeed, we found that NAMPT and HIF-2α increased NAD^+ levels and SIRT1 activity in chondrocytes (data not shown). Among Sirtuin family members, SIRT1 is known to deacetylate HIF-2α,[30 31] whereas SIRT7 negatively regulates HIF-2α protein stability.[32] Evidence suggests a possible function of SIRT1 in OA pathogenesis through the regulation of cartilage ECM expression and chondrocyte apoptosis.[33 34] Additionally, *Sirt1*-CKO mice or *Sirt1* mutant mice exhibit enhanced osteoarthritic cartilage destruction,[34 35] suggesting that SIRT1 plays a cartilage-

protective role. This is a somewhat contradictory finding given the pro-inflammatory and catabolic functions of its upstream activator NAMPT. However, we found that SIRT activity is required for protein stability and transcriptional activity of HIF-2α, and thereby regulates the induction of matrix-degrading enzymes and OA pathogenesis in various mouse models (data not shown). This suggests that other member(s) of the Sirtuin family, rather than SIRT1, is associated with HIF-2α/NAMPT regulation of OA pathogenesis.

In summary, we found that *Nampt* is a direct target gene of HIF-2α in articular chondrocytes and is upregulated in OA cartilage. We also demonstrated by gain-of-function/loss-of-function studies that NAMPT plays an essential catabolic role in OA pathogenesis and acts as a crucial mediator of osteoarthritic cartilage destruction caused by HIF-2α or DMM surgery. Therefore, NAMPT could be an effective therapeutic target in the treatment of OA disease. Indeed, inhibition of NAMPT enzymatic activity by injection of FK866 (intra-articularly or intraperitoneally) inhibits osteoarthritic cartilage destruction caused by DMM surgery or intra-articular injection of Ad-*Epas1* or Ad-*Nampt*.

Author affiliations
[1]Cell Dynamics Research Center and School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju, Korea
[2]Division of Immunology, Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA
[3]Research Center for Biomineralization Disorders and Dental Science Research Institute, School of Dentistry, Chonnam National University, Gwangju, Korea
[4]Division of Rheumatology, Hallym University Sacred Heart Hospital, Kyunggi, Korea
[5]Department of Orthopedic Surgery, Wonkwang University School of Medicine, Iksan, Korea

REFERENCES
1. Abramson SB, Attur M. Developments in the scientific understanding of osteoarthritis. *Arthritis Res Ther* 2009;11:227–35.
2. Bian Q, Wang YJ, Liu SF, et al. Osteoarthritis: genetic factors, animal models, mechanisms, and therapies. *Front Biosci* 2012;E4:74–100.
3. Little CB, Hunter DJ. Post-traumatic osteoarthritis: from mouse models to clinical trials. *Nat Rev Rheumatol* 2013;9:485–97.
4. Yang S, Kim J, Ryu H, et al. Hypoxia-inducible factor-2α is a catabolic regulator of osteoarthritic cartilage destruction. *Nat Med* 2010;16:687–93.
5. 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.

6 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.

7 Zhang LQ, Heruth DP, Ye SQ. Nicotinamide phosphoribosyltransferase in human diseases. J Bioanal Biomed 2011;3:13–25.

8 Imai SI. Nicotinamide phosphoribosyl transferase (NAMPT): a link between NAD biology, metabolism, and diseases. Curr Pharm Design 2009;15:20–8.

9 Gomez R, Conde J, Scotece M, et al. What's new in our understanding of the role of adipokines in rheumatic diseases? Nat Rev Rhematol 2011;7:528–36.

10 Gomez R, Largo F, Gomez-Reino JJ, et al. Adipokines in the skeleton: influence on cartilage function and joint degenerative diseases. J Mol Endocrinol 2009;43:11–18.

11 Otero M, Lago R, Gomez R, et al. Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis. Ann Rheum Dis 2006;65:1198–201.

12 Klein-Wieringa IR, van der Linden MP, Knevel R, et al. Baseline serum adipokine levels predict radiographic progression in early rheumatoid arthritis. Arthritis Rheum 2011;63:2567–74.

13 Rho YH, Solus J, Sokka T, et al. Adipocytokines are associated with radiographic joint damage in rheumatoid arthritis. Arthritis Rheum 2009;60:1906–14.

14 Brentano F, Schorr O, Ospelt C, et al. Pre–B cell colony-enhancing factor/visfatin, a new marker of inflammation in rheumatoid arthritis with proinflammatory and matrix-degrading activities. Arthritis Rheum 2007;56:2829–39.

15 Adya R, Tan BK, Punn A, et al. Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. Cardiovasc Res 2008;78:356–65.

16 Khan JA, Tao X, Tong L. Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents. Nat Struct Mol Biol 2006;13:582–8.

17 Evans L, Williams AS, Hayes AJ, et al. Suppression of leukocyte infiltration and cartilage degradation by selective inhibition of pre–B cell colony-enhancing factor/visfatin/nicotinamide phosphoribosyltransferase: Apo866-mediated therapy in human fibroblasts and murine collagen-induced arthritis. Arthritis Rheum 2011;63:1866–77.

18 Busso N, Karababa M, Nobile M, et al. Pharmacological inhibition of nicotineamide phosphoribosyltransferase/visfatin enzymatic activity identifies a new inflammatory pathway linked to NAD. PLoS One 2008;3:e2267.

19 Duan Y, Hao D, Li M, et al. Increased synovial fluid visfatin is positively linked to cartilage degradation biomarkers in osteoarthritis. Rheumatol Int 2012;32:985–90.

20 Yammani RR, Loeser RF. Extracellular nicotinamide phosphoribosyltransferase (NAMPT/visfatin) inhibits insulin-like growth factor-1 signaling and proteoglycan synthesis in human articular chondrocytes. Arthritis Res Ther 2012;14:R23.

21 Gosset M, Berenbaum F, Salvat C, et al. Crucial role of visfatin/pre-B cell colony-enhancing factor in matrix degradation and prostaglandin E2 synthesis in chondrocytes: possible influence on osteoarthritis. Arthritis Rheum 2008;58:1399–409.

22 Hong EH, Yun HS, Kim J, et al. Nicotinamide phosphoribosyltransferase is essential for interleukin-1beta-mediated dedifferentiation of articular chondrocytes via SIRT1 and extracellular signal-regulated kinase (ERK) complex signaling. J Biol Chem 2011;286:28619–31.

23 Mason RM, Chambers MG, Flannelly J, et al. The STR/ort mouse and its use as a model of osteoarthritis. Osteoarthritis Cartilage 2001;9:85–91.

24 Oh H, Chun CH, Chun JS. Dkk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. Arthritis Rheum 2012;64:2568–78.

25 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;18(Suppl 3):S17–23.

26 Blom AB, van Lent PL, Libregts S, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum 2007;56:147–57.

27 Little CB, Barai A, Burkhardt D, et al. Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. Arthritis Rheum 2009;60:3723–33.

28 Zhang T, Kraus WL. SIRT1-dependent regulation of chromatin and transcription: linking NAD(+) metabolism and signaling to the control of cellular functions. Biochim Biophys Acta 2010;1804:1666–75.

29 Imai S. Dissecting systemic control of metabolism and aging in the NAD World: the importance of SIRT1 and NAMPT-mediated NAD biosynthesis. FEBS Lett 2011;585:1657–62.

30 Dioum EM, Chen R, Alexander MS, et al. Regulation of hypoxia-inducible factor 2α signaling by the stress-responsive deacetylase sirtuin 1. Science 2009;324:1289–93.

31 Chen R, Dioum EM, Hogg RT, et al. Hypoxia increases sirtuin 1 expression in a hypoxia-inducible factor-dependent manner. J Biol Chem 2011;286:13869–78.

32 Hubbi ME, Hu H, Kshitiz, et al. Sirtuin-7 inhibits the activity of hypoxia-inducible factors. J Biol Chem 2013;288:20768–75.

33 Asahara H. Switching gears to an arthritis gene expression network by SirT1 cleavage. Arthritis Rheum 2011;63:2161–3.

34 Gabay O, Sanchez C. Epigenetics, sirtuins and osteoarthritis. Joint Bone Spine 2012;79:570–3.

35 Matsuzaki T, Matsushita T, Takayama K, et al. Disruption of Sirt1 in chondrocytes causes accelerated progression of osteoarthritis under mechanical stress and during ageing in mice. Ann Rheum Dis 2014;73:1397–1404.

SUPPLEMENTARY DATA

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

Siyoung Yang,[1][*] Je-Hwang Ryu,[2][*] Hwanhee Oh,[1] Jimin Jeon,[1] Ji-Sun Kwak,[1] Jin-Hong Kim,[1] Hyun Ah Kim,[3] Churl-Hong Chun,[4] and Jang-Soo Chun[1]

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′-GATCGCCCTA<u>CGTG</u>CTGTCTCA-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

1. **Yang S**, Kim J, Ryu H, *et al*. Hypoxia-inducible factor-2α is a catabolic regulator of osteoarthritic cartilage destruction. *Nat Med*. 2010;**16**:687–93.
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	5'-ACCACTCCCAACAGACCTGTCTATACC-3'	435	60	Mouse
	AS	5'-CTCCTTCTGTGACTCCAGCTTATCTGTTAG-3'			
Il12	S	5'-GGAAGCACGGCAGCAGAATA-3'	180	60	Mouse
	AS	5'-AACTTGAGGGAGAAGTAGGAATGG-3'			
Il17	S	5'-TTCATCTGTGTCTCTGATGCT-3'	131	59	Mouse
	AS	5'- TTGACCTTCACATTCTGGAG-3'			
Il21	S	5'-ATGGAGAGGACCCTTGTCTGTCTG-3'	336	60	Mouse
	AS	5'-TATGTGCTTCTGTTTCTTTCCTCC-3'			
Lep	S	5´-CCAAAACCCTCATCAAGACC-3´	395	58	Mouse
	AS	5´-ATCCAACTGTTGAAGAATGTCC-3´			
Nampt	S	5´-ACAGATACTGTGGCGGGATTG-3´	424	58	Mouse
	AS	5´-TGATATCCACGCCATCTCCTTG-3´			
NAMPT	S	5´-GAACAGGATCTTTCGTTCCATA-3´	357	58	Human
	AS	5´-TTCCTGAGGGCTTTGTCATTCC-3´			
Nampt #1 (ChIP)	S	5´-GGTGACGGTCGGCTTTAGGC-3´	120	58	Mouse
	AS	5´-CTGCGCGTGCGCAGCGCAGG-3´			
Nampt #2 (ChIP)	S	5´-GTGGGTGGCTCCTTGGCCTTAG-3´	100	58	Mouse
	AS	5´-CCGCCCCAACCCGGACCTTCCT-3´			
Nampt #3 (ChIP)	S	5´-GAGGATCGGAATCCACAAGACG-3´	100	58	Mouse
	AS	5´-ACCCACTGTCGTCTCCCTGTTC-3´			
Nampt promoter	S	5´-CGCATAAAACAGCCTAAAATGG-3´	1,230	55	Mouse
	AS	5´-CTCGGCCCGGACCGGAGACGCCG-3´			
Nampt TG	S	5´-ATAAGAATGCGGCCGCATGAATGCTGCT GCGGCAGAAGCCGA-3´	1,476	55	Mouse
	AS	5´-ATAAGAATGCGGCCGCCGCCTAATGAGG TGCCACGTCCTGC-3´			
Mmp2	S	5´-CCAACTACGATGATGAC-3´	233	60	Mouse
	AS	5´-ACCAGTGTCAGTATCAG-3´			
Mmp3	S	5´-TCCTGATGTTGGTGGCTTCAG-3´	102	55	Mouse
	AS	5´-TGTCTTGGCAAATCCGGTGTA-3´			
Mmp9	S	5´-ACCACATCGAACTTCGA-3´	212	58	Mouse
	AS	5´-CGACCATACAGATACTG-3´			
Mmp12	S	5´-CCCAGAGGTCAAGATGGATG-3´	482	60	Mouse
	AS	5´-GGCTCCATAGAGGGACTGAA-3´			

	Strand	Sequence			
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.

_____
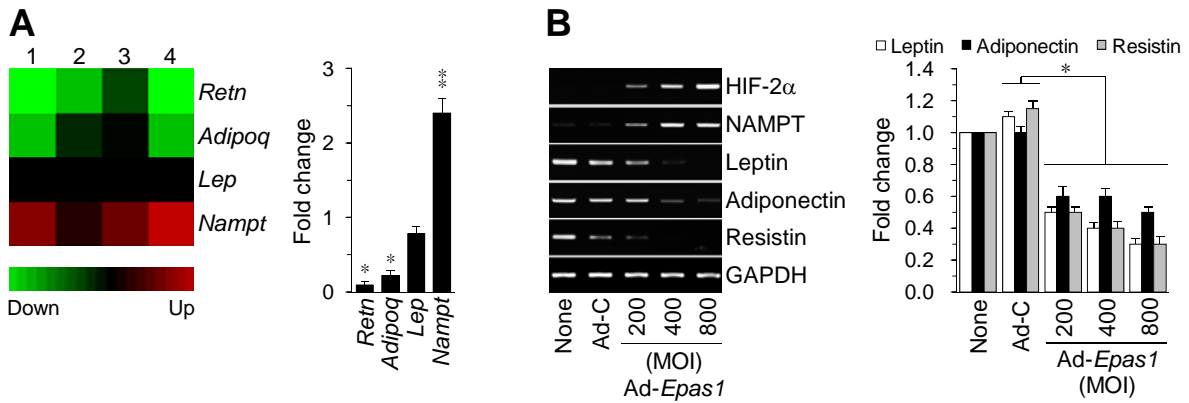
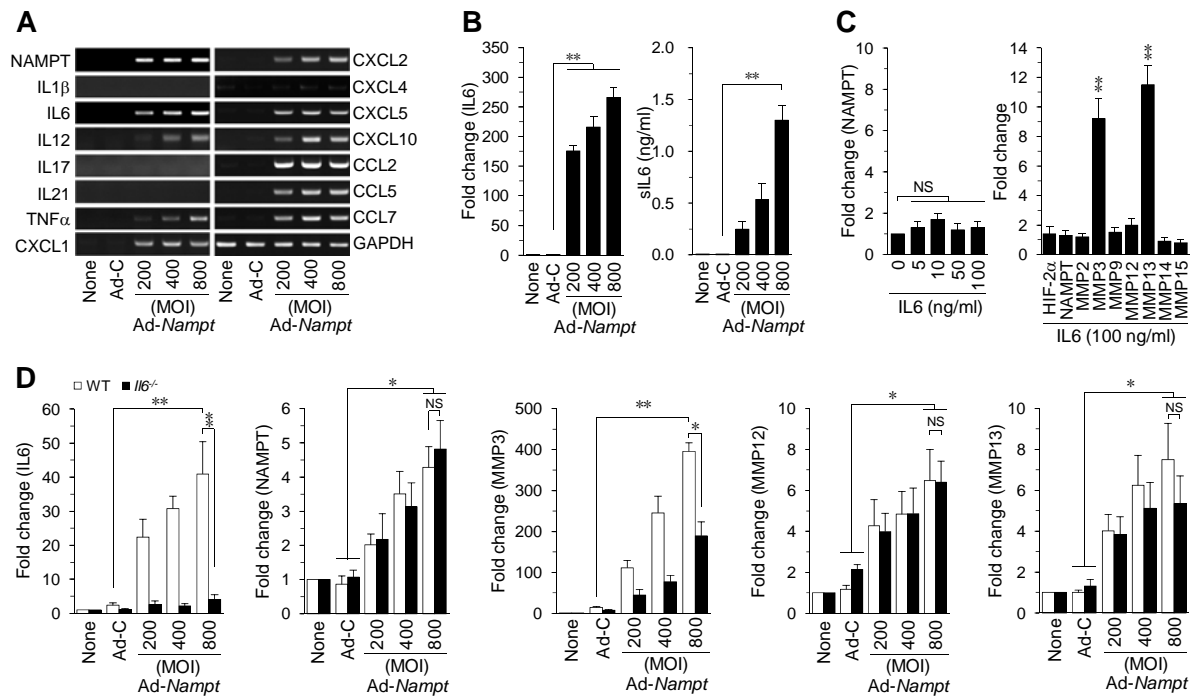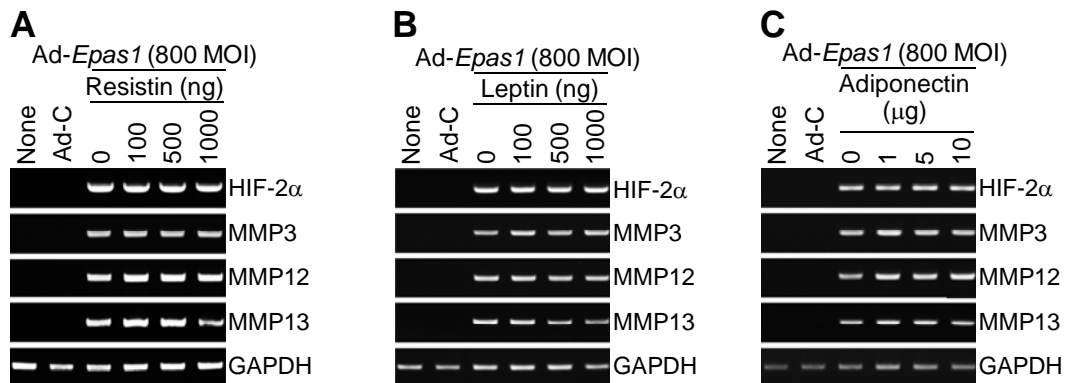**Supplementary Figure S1.** HIF-2α regulation of adipokine expression in mouse articular chondrocytes. (A) Microarray analyses of primary cultures of mouse articular chondrocytes infected with Ad-*Epas1* at a MOI of 800 for 24 hours ($n = 4$). (B) Primary cultures of mouse articular chondrocytes were 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 (*$P < 0.01$, **$P < 0.001$).
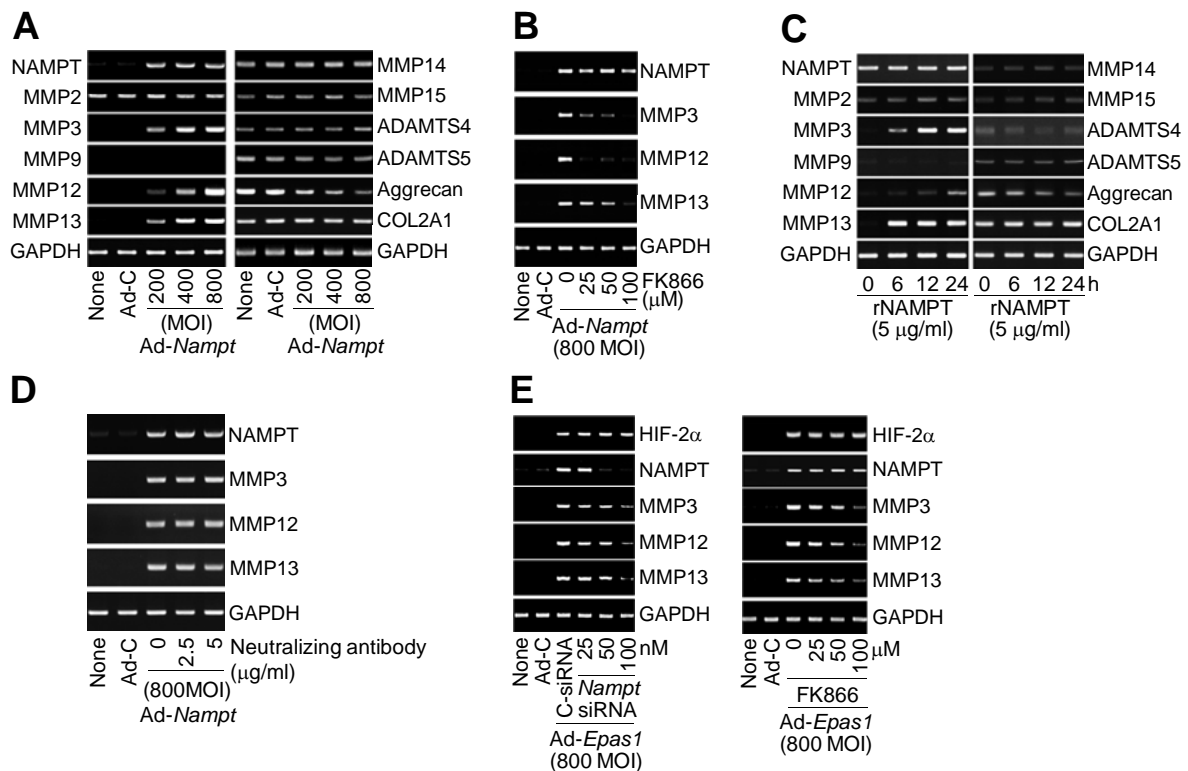
Supplementary Figure S2. RT-PCR analysis of catabolic factor expression by NAMPT in primary cultures of articular chondrocytes. (A) Representative RT-PCR results of the indicated catabolic and anabolic factors in chondrocytes infected with Ad-C (800 MOI) or Ad-*Nampt* (at the indicated MOI) ($n \geq 6$). (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).

11

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 ($n \geq 6$).
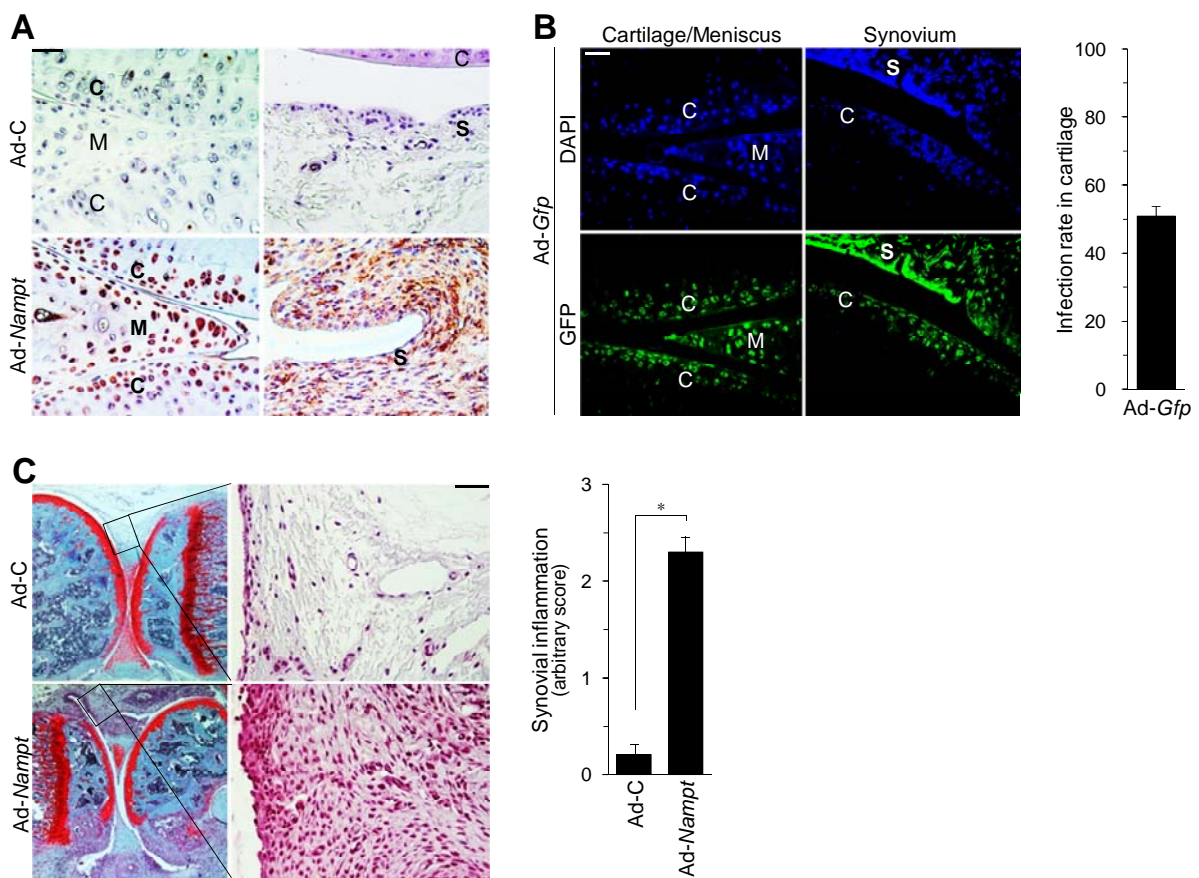
Supplementary Figure S5. Gene delivery into joint tissues by IA injection of adenovirus. (**A**) Mice were IA-injected with Ad-C or Ad-*Nampt* (1×10^9 PFU, once per week for 3 weeks), and sacrificed 21 days after the first injection. NAMPT expression in the meniscus, cartilage, and synovium was determined 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.
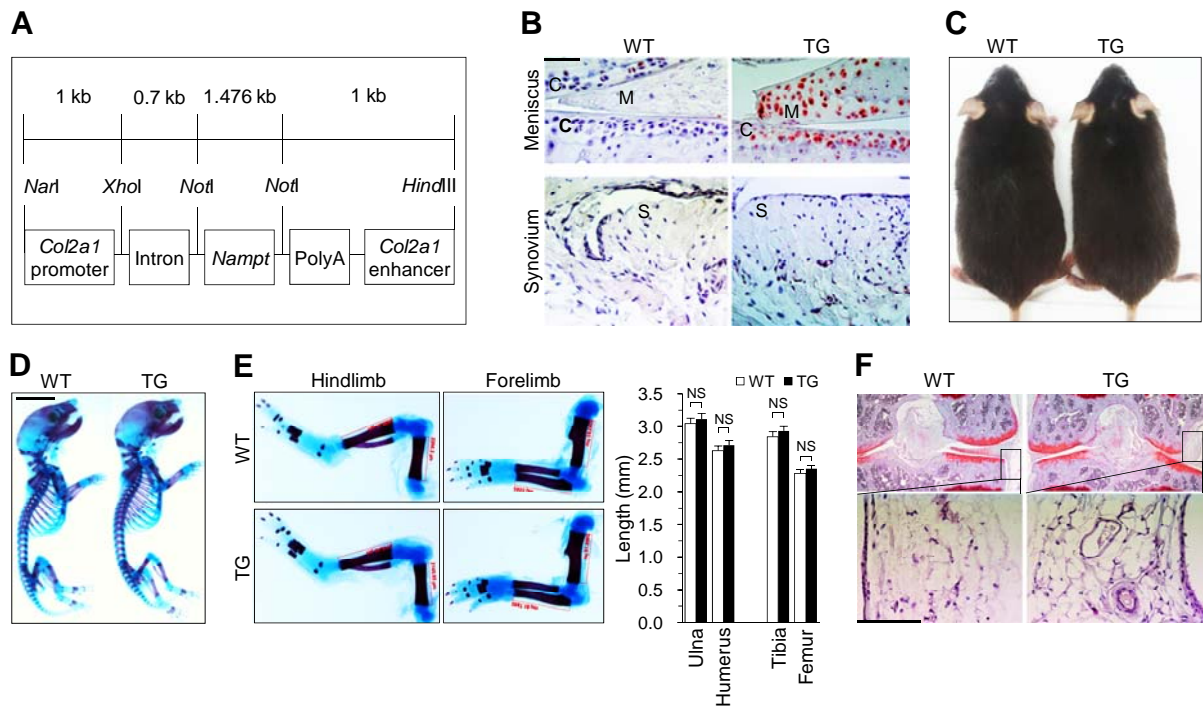
Supplementary Figure S6. Characterization of *Col2a1-Nampt* TG mice. (A) Vector constructs used for the generation of TG mice. (B) Immunostaining of NAMPT in joint tissues (cartilage, meniscus, and synovium) of *Col2a1-Nampt* TG mice and their WT littermates. (C) Images of 6-month-old *Col2a1-Nampt* 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.
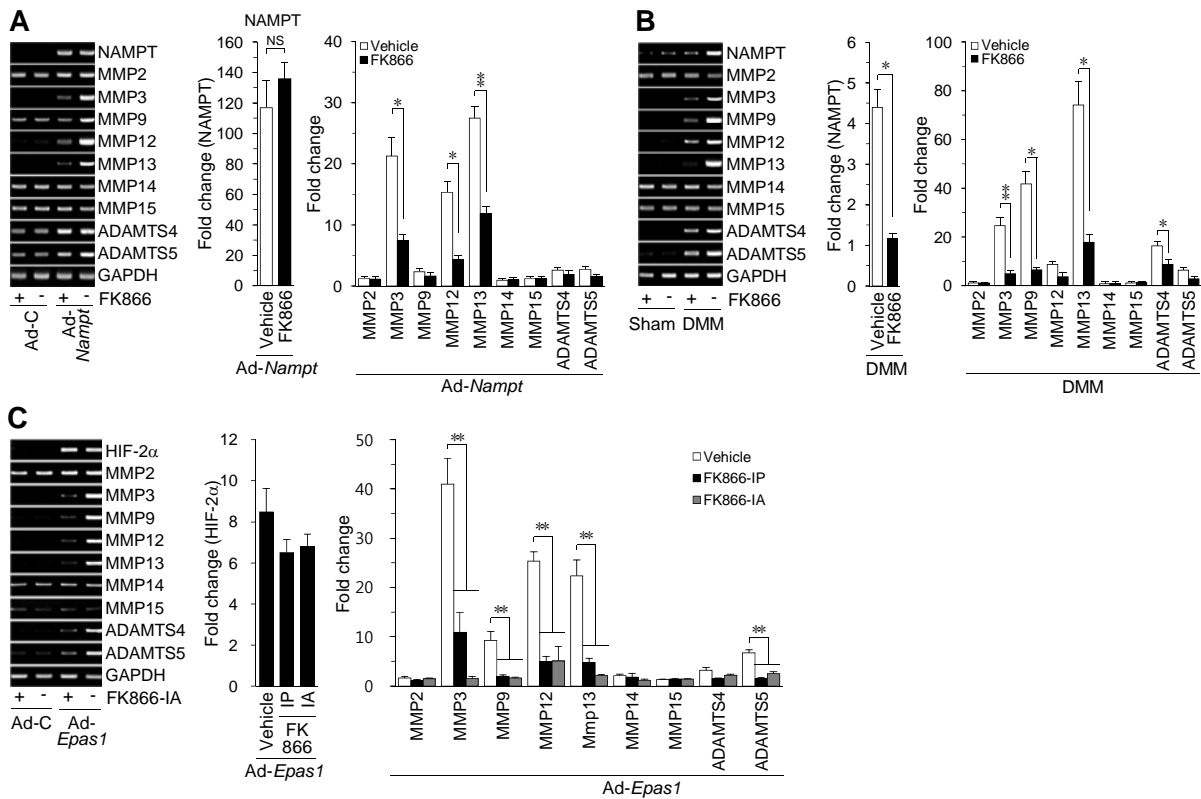
Supplementary Figure S7. FK866 inhibits the *in vivo* expression of catabolic factors. (A) Mice were IA-injected with Ad-C or Ad-*Nampt* (1×10^9 PFU) with or without FK866 (10 mg/kg). mRNA levels in cartilage tissue were determined by RT-PCR and quantified by qRT-PCR ($n = 10$). (B) Following DMM or 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.
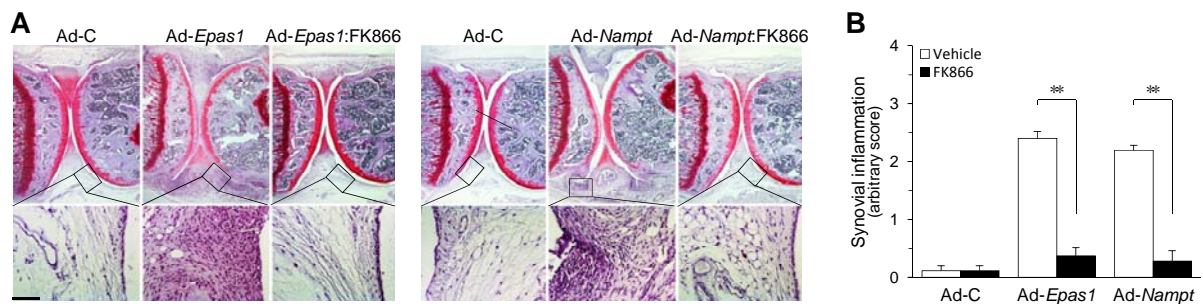
Supplementary Figure S8. FK866 inhibits synovitis in mice. (A and B) Mice were IA-injected with Ad-C, Ad-*Nampt*, or Ad-*Nampt* (1×10^9 PFU) with or without FK866 (10 mg/kg). Synovitis was detected by safranin-O and hematoxylin staining ($n = 10$). Values are presented as means $\pm$ SEM (*P < 0.001). Scale bar: 100 μm.
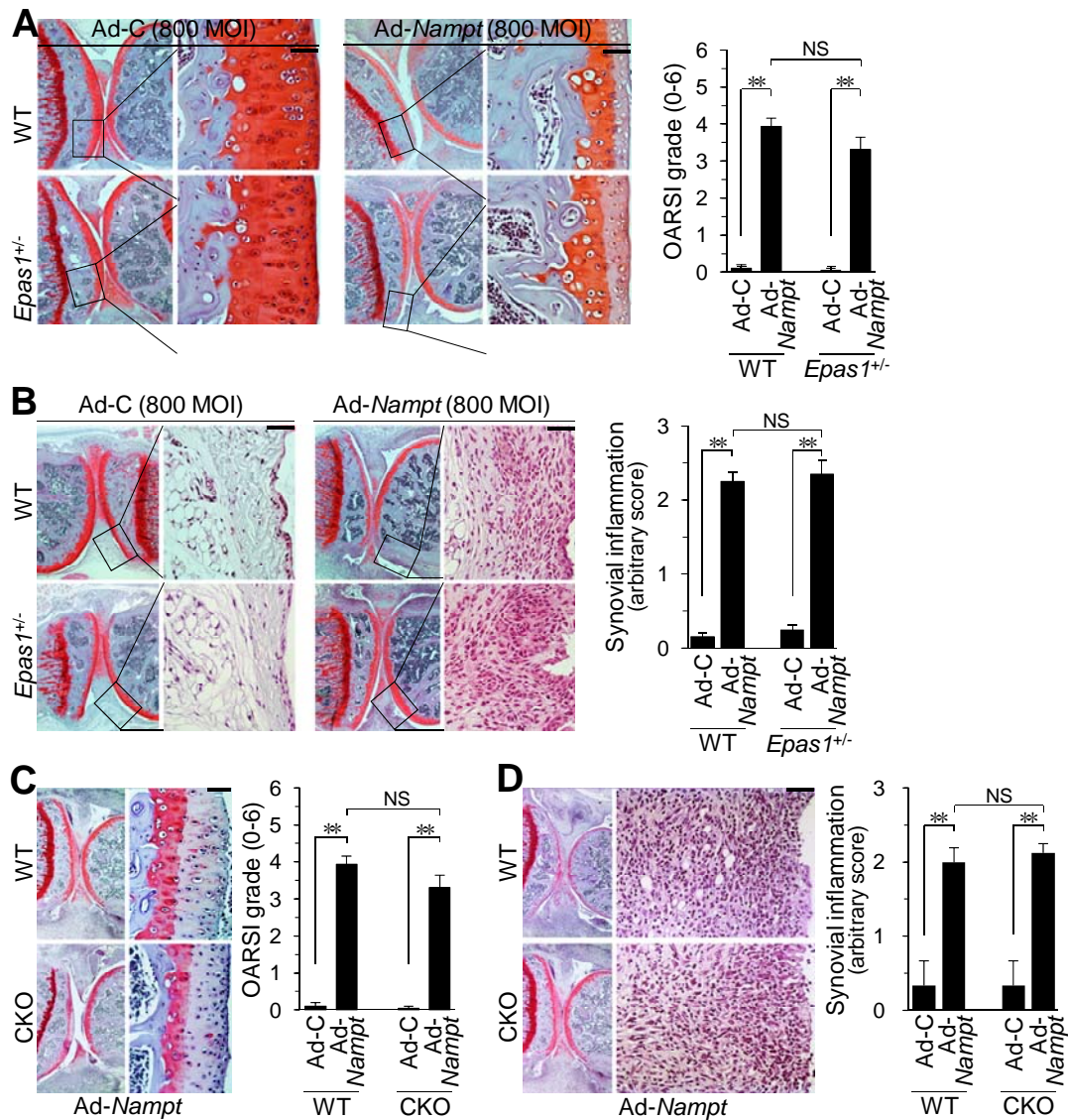
Supplementary Figure S9. *Epas1* knockout in mice does not affect OA cartilage destruction induced by Ad-*Nampt* injection. (A and B) Ad-C or Ad-*Nampt* (1×10^9 PFU) was injected into WT or *Epas1*^{+/-} mice. Cartilage destruction was determined by safranin-O staining and quantified by Mankin's method (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.