Supporting Information

Li et al. 10.1073/pnas.0911377107

SI Materials and Methods

Materials and Reagents. Fresh human osteoarthritic cartilage was obtained from the National Disease Research Interchange (Philadelphia, PA) under institutionally approved protocols. These samples were procured from donors who had undergone knee replacement surgery within the previous 36 h. Recombinant human oncostatin M (OSM) and IL-1β were purchased from R&D Systems. DMEM (DMEM)/F-12 was purchased from Invitrogen. FBS was purchased from Sigma. Dimethylmethylene blue (DMMB) was purchased from Polysciences. Shark chondroitin sulfate was obtained from Fluka Biochemika. Proteinase K and Nutridoma-SP were from Roche Applied Science. Chondroitinase ABC, keratanase, and keratanase II were from Seikagaku. A monoclonal neoepitope antibody that binds the N-terminal sequence NH2-AGEG was generated by immunizing mice with a peptide containing eight amino acids derived from the aggrecan protein sequence starting at one of the aggrecanase cleavage sites, ^{1820}AGEG, by Abgent for Wyeth. This antibody specifically recognizes the ^{1820}AGEG neoepitope on aggrecan fragments generated by cleavage at the Glu^{1819}–Ala^{1820} bond. Anti-mouse IgG conjugated with alkaline phosphatase and Western Blue substrate for alkaline phosphatase was purchased from Promega. LXR-specific agonist GW3965 was synthesized by Wyeth according to the published structure (1). Reagents and primer/probes for real-time PCR (TaqMan low-density array, or TLDA) were obtained from Applied Biosystems. pY-STAT1 and STAT1 antibodies were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated–anti-β-actin antibody was from Santa Cruz Technology.

RNA Extraction from Mouse Cartilage and Real-Time Quantitative RT–PCR. Femoral-head cartilage samples from hip joints were harvested from 3-week-old wild-type and Lxrβ$^{-/-}$ C57BL/6 mice. Twenty hips from each group were randomly pooled into three tubes. RNA was extracted from cartilage as described in *Materials and Methods*. Taqman primer/probes for the mouse Lxrα gene (Mm00443451_m1) and the control β-glucuronidase (GUSB) gene (Mm00446953_m1) were purchased from Applied Biosystems. Reverse transcription and real-time PCR were performed using the One-Step Quantitative RT–PCR System (Applied Biosystems on an ABI Prism 7900 Sequence Detection System (Applied Biosystems).

Real-Time Quantitative TaqMan Analysis Using a Custom-Designed TaqMan Low-Density Array. To assess the relative mRNA expression levels of several human cartilage genes, a custom-designed TLDA (Applied Biosystems) was constructed to include genes such as microsomal PGE synthase 1 (mPGES-1) (ABI assay ID: Hs00610420_m1), ADAMTS4 (aggrecanase-1; Hs00192708_m1), PTGS2 (COX-2; Hs00153133_m1), ABCG1 (Hs00245154_m1), TIMP3 (Hs00165949_m1), MMP-1 (Hs00899658_m1), and MMP-13 (Hs00233992_m1). Thermal cycling was performed using the ABI 7900 Real-Time PCR System. Cartilage RNA was first converted to cDNA using a high-capacity cDNA RT kit (Applied Biosystems). Each port on the TLDA card received 100 μL TaqMan reaction consisting of cDNA corresponding to 100 ng of total RNA combined with 1× TaqMan Universal Master Mix (Applied Biosystems). The level of expression was calculated on the basis of the PCR cycle number (C_T). For each donor, C_T numbers for both GUSB (Hs99999908_m1) and GAPDH (Hs99999905_m1) genes in each sample of the four treatments were used as endogenous normalizers. For each sample, the relative gene expression level was determined according to the $2^{-\Delta\Delta C}$T method. In this case, $\Delta\Delta C_T = \Delta C_T$ treated sample – ΔC_T (GUSB–DMSO). The GUSB level in the DMSO-treated sample (vehicle control) for each donor was chosen to serve as the calibrator for these calculations because we found levels of the GUSB gene in different donors to be relatively consistent, and no marked effect of treatment on GUSB expression was observed. Therefore, the relative quantity numbers for each gene under different treatments are normalized to GUSB levels in the DMSO-treated sample.

Lactate Assay. Lactate levels were measured as an indicator of cellular viability and metabolic activity using a lactate assay kit from Trinity Biotech. Lactate level was measured in the human cartilage culture medium samples collected at 2-day intervals during the 10 days of culture as described in Fig. 2. Briefly, 5 μL of each sample was incubated with 250 μL of lactate reagent at room temperature for 5 min, and the absorbance was read at 540 nm. The lactate concentration was calculated using an equation derived from a standard curve.

Culture of SW982 Synovial Sarcoma Cells and RNA Inteference. SW982 cells were maintained in DMEM/F-12 medium containing 10% FBS. Cells were transfected with control siRNA and siRNA targeting nuclear receptor corepressor (NCoR) or silencing mediator of retinoid acid and thyroid hormone receptor (SMRT) (50 nM; Applied Biosystems) using DharmaFECT Reagent 4 (Dharmacon RNAi Technologies). Forty-eight hours after transfection, cells were cultured in serum-free DMEM/F-12 medium contain 1% Nutridoma (Roche Applied Science) and treated with GW3965 (1 μM) for 1 h before stimulation with IL-1β (10 ng/mL) for 16 h. Expression levels of mPGES-1 were evaluated by real-time PCR, using GAPDH as endogenous control.

Western Blot Analysis. SW982 cells were maintained in DMEM/F-12 medium containing 10% FBS. Before treatments, cells were grown to ~80% confluence and were starved in serum-free DMEM/F-12 medium contain 1% Nutridoma overnight. For time-course analysis of signal transducer and activator of transcription-1 (STAT1) phosphorylation, cells were treated with 10 ng/mL IL-1β for various periods of time. For another study, cells were first treated with GW3965 at various doses for 1 h, followed by IL-1β (10 ng/mL) treatment for 4 h. To collect total cell lysate, cells were rinsed with cold PBS and lysed with 2× SDS loading buffer. Proteins were resolved on a 4–12% SDS–PAGE gel and transferred to a nitrocellulose membrane using the iBlot dry blotting system (Invitrogen). The membrane was blocked for 60 min in tris-buffered saline (TBS) containing 0.05% Tween-20 and 5% nonfat milk and then incubated with primary antibodies. After washing, the membrane was incubated with the corresponding second antibody conjugated with horseradish peroxidase. Signals were detected using enhanced chemiluminescence (PerkinElmer).

Electrophoretic Mobility Shift Assay. SW982 cells were cultured and treated as above. Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce Biotechnology) following the manufacturer's instructions. Protein concentrations were determined by the Bradford assay (Pierce Biotechnology). Electrophoretic mobility shift assay (EMSA) was carried out using a LightShift chemiluminescent EMSA kit (Pierce Biotechnology) following the manufacturer's protocol. A double-stranded oligonucleotide containing the GAS-binding site in the human cyclooxygenase-2 (COX-2) promoter reagion (5′-CGC CTC TCT **TTC** CAA **GAA** ACA AGG AGG-3′) was used as

the probe. The probe was synthesized by Integrated DNA Technologies and chemically labeled with biotin at the 3′ end. Four micrograms of nuclear extract and 250 fmol of biotin-labeled probe were used in a 20-μL reaction using the binding buffer provided in the kit. Specific binding was controlled by competition with unlabeled probe (50 pmol). In one reaction, nuclear extract was incubated with 0.12 μg of STAT1 antibody (Cell Signaling Technology) on ice for 1 h before the addition of the labeled probe. The DNA protein complex formed was resolved on a 6% DNA retardation gel (Invitrogen) and transferred onto a nylon membrane. DNA was cross-linked to the membrane under UV light. The biotin end-labeled DNA was detected using the streptavidin–horseradish peroxide conjugate and LightShift chemiluminescent substrate.

1. Collins JL, et al. (2002) Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. *J Med Chem* 45:1963–1966.

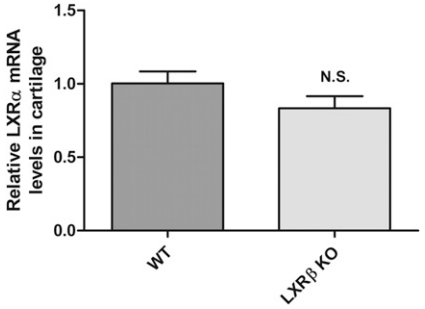

Fig. S1. No compensatory increase in LXRα mRNA expression was found in the cartilage from LXRβ KO mice. Shown are the relative mRNA levels of the LXRα gene in wt or Lxrβ$^{-/-}$ cartilage as determined by Taqman analysis using GUSB as an internal control. RNA samples extracted from 20 hips/group (pooled into 3 tubes) were analyzed.

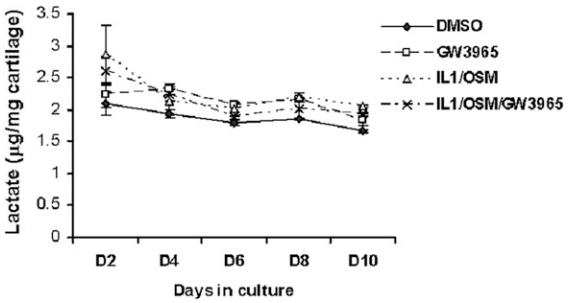

Fig. S2. GW3965 did not affect chondrocyte viability in the cartilage as measured by lactate assay. Cumulative lactate levels (mean ± SEM, triplicate wells) at each time point are shown.

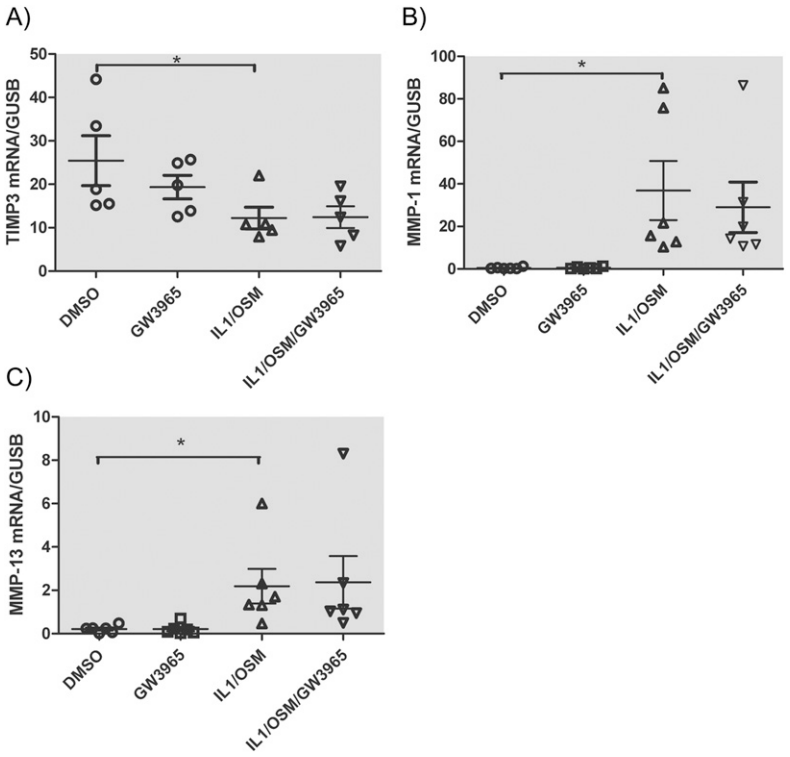

Fig. S3. Effects of LXR activation on the expression of (*A*) TIMP3, (*B*) MMP-1 and (*C*) MMP-13 in human cartilage. Real-time quantitative RT–PCR expression analysis of RNA samples from five donors. Human cartilage explants were pretreated with 2 μM GW3965 for 6 h and then stimulated with IL-1β/OSM (1 and 5 ng/mL, respectively) for an additional 18 h. The expression levels for each gene under various treatments are expressed as abundance relative to β-glucuronidase (GUSB) levels in the DMSO control samples. Relative expression levels of each gene with treatments in all five donors are shown as a scatter plot. *$P <$ 0.05 by paired Student's *t* test.

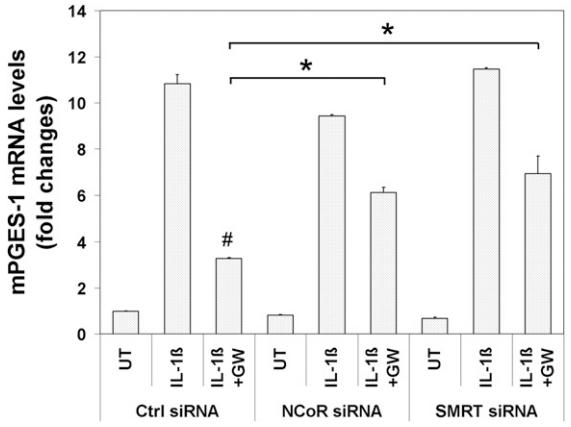

Fig. S4. LXR ligand GW3965 inhibits IL-1β–induced mPGES-1 gene expression in SW982 cells; NCoR- or SMRT-specific siRNA reverses GW3965-mediated repression of IL-1β–induced mPGES-1 gene expression. siRNA-transfected SW982 cells were pretreated with GW3965 (1 μM) for 1 h and then stimulated with IL-1β (10 ng/mL) for 16 h. mPGES-1 expression was evaluated by quantitative PCR. $^{\#}P < 0.05$ versus IL-1β + control siRNA; *$P < 0.05$ versus GW3965 + IL-1β control siRNA.

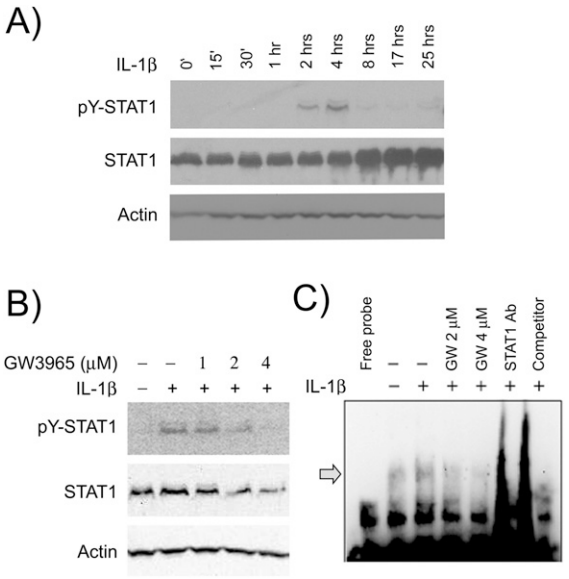

Fig. S5. LXR ligand GW3965 suppresses IL-1β–induced STAT1 phosphorylation and expression, as well as binding to a DNA probe containing the GAS element found in COX-2 promoter. (*A*) Phosphorylation of STAT1 protein (p-Tyr701) was detected at 2 h after IL-1β treatment. Maximal phosphorylation was observed at 4 h. (*B*) SW982 cells were treated with GW3965 at indicated doses for 1 h, followed by IL-1β treatment for 4 h. Total cell lysates were analyzed for pY-STAT1 and total STAT1 levels. (*C*) SW982 cells were treated as in *B*. Nuclear extracts were assayed by EMSA as described in *SI Materials and Methods*. Arrow indicates the specific protein binding to the biotin-labeled double-stranded COX-2 GAS probe, which was blocked by a STAT1 antibody and was competed out by an excess of unlabeled probe.