Transcriptional Repression of Matrix Metalloproteinase Gene Expression by the Orphan Nuclear Receptor NURR1 in Cartilage*§

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The NR4A orphan receptors (Nur77, NURR1, and NOR-1) are emerging as key regulators of cytokine and growth factor action in chronic inflammatory diseases. In this study, we address the role of these receptors in cartilage homeostasis during inflammatory joint disease. We demonstrate that PGE2 represses interleukin-1β-induced matrix metalloproteinase (MMP)-1 and that this receptor targets a critical region of the MMP-1 promoter (−1772 to −1546 bp) and that repression does not require consensus binding sites for NURR1. We confirm that NURR1 targets a 40-bp promoter sequence that is also positively regulated by ETS transcription factors. Finally, functional studies indicate that transcriptional antagonism exists between NURR1 and ETS1 on the MMP-1 promoter. We propose a protective function for NURR1 in cartilage homeostasis by selectively repressing MMP gene expression during inflammation.

Nuclear hormone receptors comprise a large family of tightly regulated transcription factors that play critical roles in normal physiology and disease processes (1). Several of these receptors are activated by ligands such as vitamins and hormones, which cause allosteric changes in the receptors and alter the transcription of target genes. Modulation of these receptors with synthetic ligands has shown great promise in the treatment of cancer and inflammatory diseases (2). In contrast, orphan nuclear receptors control gene expression in the absence of ligands, and elucidating the functions and transcriptional targets of these receptors may also lead to therapeutic advances.

The NR4A receptors are a subfamily of orphan nuclear receptors consisting of nerve growth factor-induced clone B (Nur77, NR4A1), NURR1 (nuclear receptor related-1; NR4A2), and NOR-1 (neuron-derived orphan receptor-1; NR4A3) (3). As ligand-independent receptors (4), the activity of these transcription factors is tightly controlled at the level of expression. Nur77, NURR1, and NOR-1 exhibit distinct and overlapping expression patterns in a number of cell and tissue types (3), indicating that these receptors have unique and partially redundant functions. A number of NR4A target genes have been elucidated in the central nervous system (5, 6), providing models for the transcriptional mechanisms employed by these receptors. All three receptors can activate transcription by binding to consensus nerve growth factor-induced clone B response elements (NBREs) in the promoters of target genes (3). Nur77 and NURR1 can also heterodimerize with the retinoid X receptor (RXR) and regulate distinct transcriptional pathways (7–10). In addition, NR4A receptors can interact with other transcription factors and co-regulatory proteins to modulate gene expression (11–15).

The NR4A receptors are emerging as key regulators of cytokine and growth factor action in diseases affecting our aging population. NURR1 plays a critical role in the development of dopaminergic neurons (16), and mutations in this receptor are associated with Parkinson disease (17). The NR4A receptors contribute to cellular transformation (18) and promote tumor growth by enhancing proliferation (19) and cell survival (20).

* This work was supported by grants from the Health Research Board (to K. S. M.) and the Science Foundation Ireland (to E. P. M.) and by National Institutes of Heath Grant AR-26599 (to C. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

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Nur77 and NOR-1 modulate endothelial cell proliferation (21, 22), and Nur77 induces expression of plasminogen activator inhibitor-1 in response to tumor necrosis factor-α (23). In addition, Nur77 can promote vascular endothelial growth factor-induced angiogenesis in vivo (24). In atherosclerosis, these receptors appear to have a protective role, as they suppress smooth muscle cell proliferation and lesion formation (21, 25–27). NR4A receptors are potently induced in activated macrophages (28, 29), and here, they regulate the transcription of genes in inflammatory pathways (26, 30). As these immediate-early genes are induced by inflammation and can, in turn, enhance or attenuate inflammation by modulating transcription, the NR4A receptors may have diverse roles in a number of inflammatory diseases.

Chronic inflammation is a hallmark of rheumatoid arthritis and osteoarthritis (OA). Inflammatory cytokines and prostaglandins are released from infiltrating immune cells and resident joint cells. These soluble mediators induce changes in gene expression that modulate the tightly regulated program of inflammation and the subsequent degradation of extracellular matrix components (31). We have demonstrated elevated levels of NR4A receptors in synovial tissue from patients with rheumatoid arthritis (32–34), suggesting that these transcription factors play a pivotal role in inflammatory joint disease. The cytokines interleukin (IL)-1β and tumor necrosis factor-α and the growth factors vascular endothelial growth factor and basic fibroblast growth factor potently induce NURR1 in synoviocytes and endothelial cells derived from inflamed joints (32–34). Cyclooxygenase-2 (COX-2)-generated prostaglandin E₂ (PGE₂) is the most robust regulator of NURR1 expression in these cell types (32–34). The NURR1 promoter is activated by NFκB and cAMP-responsive element-binding protein (CREB) in response to inflammatory signals (33). NURR1 induces expression of the chemokine IL-8 in synoviocytes (35), which may enhance cell migration and resolution during the early stages of inflammatory joint disease. NURR1 has also been documented in bone (36, 37), and here, this receptor regulates the expression of genes involved in bone homeostasis, osteopontin and osteocalcin (38, 39). Interestingly, osteopontin differentially modulates inflammation in synovial tissue and cartilage (40–42), and regulation of this gene may have downstream effects on matrix degradation. Taken together, the expression patterns of the NR4A receptors and the target genes identified in joint tissues suggest a role for these transcription factors in modulating inflammation and tissue homeostasis.

Chondrocytes are another important cell type involved in the pathogenesis of inflammatory joint disease (43). These cells are embedded within articular cartilage, where they function to maintain homeostasis by balancing the synthesis of matrix components with the expression of catabolic factors such as matrix metalloproteinases (MMPs). During the progression of inflammatory joint disease, chondrocytes secrete elevated levels of MMPs, which digest components of the extracellular matrix, ultimately leading to the degradation of cartilage, tendon, and bone (31). Expression of these enzymes is largely controlled by critical transcription factors such as activator protein-1 (AP-1), ETS (E26 transformation-specific sequence), and NFκB, which function through defined promoter elements (31). Interestingly, PGE₂ can intersect with inflammatory signals and differentially modulate the expression of MMPs and matrix components (44–47), suggesting that transcriptional pathways regulated by this prostaglandin may serve as novel therapeutic targets in inflammatory joint diseases. In addition, MMP transcription can be altered by nuclear hormone receptors in response to steroid hormones, vitamins, and a number of synthetic compounds (48–52). Targeting specific members of the nuclear receptor family may be a viable strategy to suppress MMP transcription in inflamed joints.

The functional role of the NR4A orphan receptors has not been investigated in chondrocytes, and we postulate that these receptors may be important regulators of cartilage homeostasis and MMP transcription. In this study, we document elevated levels of NURR1 mRNA in cartilage derived from patients with OA. Relative to Nur77 and NOR-1, NURR1 is expressed at the highest level in chondrocytes, and PGE₂ rapidly and potently induces receptor expression. IL-1β-induced MMP-1 expression is dramatically antagonized by PGE₂, and because NURR1 is robustly activated by PGE₂, this orphan receptor may directly inhibit MMP gene expression. Our results indicate that NURR1 selectively antagonizes IL-1β-induced MMP-1, -3, and -9 gene expression in chondrocytes. We further elucidate MMP-1 as a transcriptional target of NURR1 and characterize novel interactions between NURR1 and ETS transcription factors that mediate repression of the MMP-1 promoter. In conclusion, our findings implicate NURR1 as a novel transcriptional repressor of MMPs, and we propose a protective function for NURR1 in maintaining cartilage homeostasis during inflammation.

**EXPERIMENTAL PROCEDURES**

**Procurement of Cartilage**—Human cartilage slices were obtained from the knees of patients with advanced OA (50–70 years old) who were undergoing knee replacement surgery. Non-arthritic knee cartilage was obtained from patients with fractures or from patients undergoing amputation because of trauma.

**Isolation of Primary Chondrocytes**—Human cartilage slices were cut into small pieces and digested with Pronase (0.1%) for 30 min in phosphate-buffered saline, followed by digestion with collagenase P (0.1%) for 12–16 h in Ham’s F-12 medium. This cell suspension was used to establish cell cultures and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Human chondrocytes were maintained in chondrocyte growth medium (Cell Applications) as monolayer cultures for not more than 3 days to maintain the chondrocyte phenotype.

**Tissue Culture and Reagents**—SW1353 human chondrosarcoma cells (American Type Culture Collection) were maintained in RPMI 1640 medium containing 10% fetal calf serum, HEPES, and penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO₂. Mouse embryonic fibroblasts (peroxisome proliferator-activated receptor-α⁺/⁺; generous gift from Dr. Evan Rosen, Beth Israel Deaconess Medical Center) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, glutamine, and penicillin/streptomycin. Cells were passaged with 1× Trypsin/EDTA and seeded into 6- or 24-well plates for experiments. For treatments, cells were washed with Hanks’ balanced salt solution (Sigma), and PGE₂ (Sigma and
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Cayman Chemical) or IL-1β (R&D Systems) was added to cells in serum-free medium. In initial experiments, we determined that the vehicles used for these treatments (ethanol, acetone, and phosphate-buffered saline) had no effects on gene expression (supplemental figure). Untreated controls were cells treated in an identical manner with serum-free medium alone.

**RNA Extraction**—RNA was isolated from SW1353 cells using TRIzol solution (Sigma) according to the manufacturer’s protocol. For primary human chondrocytes, micro-RNeasy columns (Qiagen Inc.) were used after TRIzol extraction for further purification. Cartilage slices were milled into fine powder in liquid nitrogen and extracted with 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sodium dodecyl sarcosine, and 0.1 M 2-mercaptoethanol for 4 h on a rocker. RNA was then extracted with water-saturated phenol, followed by phenol and chloroform. The RNA in the aqueous phase was precipitated with an equal volume of isopropyl alcohol. The RNA pellet was further purified using a Qiagen RNaseasy mini kit according to the manufacturer’s RNA cleanup protocol. RNA concentrations were determined by A260 and the integrity of RNA was checked by gel electrophoresis.

**Real-time Reverse Transcription (RT)-PCR**—Two micrograms of RNA were reverse-transcribed into cDNA using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR was conducted using SYBR Green Master Mix and an ABI PRISM 7900HT thermocycler (Applied Biosystems). Duplicate reactions were analyzed in 10-μl volumes containing 40 ng of cDNA and 0.2 μM primers. Real-time primers have been described previously: NURR1 (38); MMP-1 (53); MMP-2, -3, -9, and -13 and COX-2. Relative expression levels were calculated using the 2^-ΔΔCt method with GAPDH as a control.

**Western Blotting**—Nuclear extracts from SW1353 cells were generated using NE-PER protein extraction reagents (Pierce). Five micrograms of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-NURR1 antibody (clone E-20 (catalog no. sc-990), Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology) were used, followed by detection with ECL Western substrate (Amersham).Five micrograms of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-NURR1 antibody (clone E-20 (catalog no. sc-990), Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology) were used, followed by detection with ECL Western substrate (Amersham). Five micrograms of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-NURR1 antibody (clone E-20 (catalog no. sc-990), Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology) were used, followed by detection with ECL Western substrate (Amersham). Five micrograms of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-NURR1 antibody (clone E-20 (catalog no. sc-990), Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology) were used, followed by detection with ECL Western substrate (Amersham). Five micrograms of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-NURR1 antibody (clone E-20 (catalog no. sc-990), Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology) were used, followed by detection with ECL Western substrate (Amersham).Five micrograms of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-NURR1 antibody (clone E-20 (catalog no. sc-990), Santa Cruz Biotechnology, Inc.) and horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology) were used, followed by detection with ECL Western substrate (Amersham).

**Site-directed Mutagenesis**—Putative NBRE sequences in the MMP-1 promoter were identified using the nucleic acid pattern search application FUZZNUC (bioweb.pasteur.fr/seqanal/interfaces/fuzznuc.html). A site-directed mutagenesis kit (Stratagene) was used to mutate the core GG sequence in each of the putative NBRE sequences on the full-length MMP-1-luciferase construct (details shown in Fig. 5C). The mutated sequences were analyzed with TFSearch (www.cbrc.jp/research/db/TFSEARCH.html) to ensure that mutagenesis did not create or destroy any additional transcription factor-binding sites. Mutations were confirmed by DNA sequencing of purified plasmid DNA. pCMX-NURR1(C283G) was generated by site-directed mutagenesis as described previously (38).

**DNA Gel Shift Assays**—A LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce) was used to detect proteins bound to a region of the MPP-1 promoter spanning −1586 to 1626 bp. Complementary MPP-1 promoter oligonucleotides were synthesized with 3’-biotin labels (GTTAATAATTAGAAAAGGATATGACTTATC TCAAATTTGAGATAGTCA TATCCTTCTTCT-
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A biotinylated annealed probe (20 fmol) was incubated with 2–5 μg of nuclear extract, 1× binding buffer, 2.5% glycerol, 5 mM MgCl₂, 50 ng/μl poly(dI-dC), and 0.05% Nonidet P-40 for 20 min at room temperature. Where indicated, anti-NURR1 antibody (clone E-20 (catalog no. sc-990X), Santa Cruz Biotechnology, Inc.) or 100× unlabeled competitor DNA was included in binding reactions. Protein-DNA complexes were resolved on 6% DNA retardation gels containing 0.5 M NaCl and 30 mM Tris-EDTA (Invitrogen) and transferred to Biodyne B nylon membrane (Pierce). Biotinylated DNA was detected using a streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate, followed by exposure to x-ray film.

Statistics—Analysis was conducted with Prism and GraphPad software. The non-parametric Mann-Whitney test was used to compare relative gene expression levels in cartilage specimens (see Fig. 1, A–D). The non-parametric Spearman correlation was used to measure correlations between gene expression levels (Fig. 1E). Student’s t test was used for analysis of transfection data and gene expression studies in cell lines.

RESULTS

Expression of NR4A Receptors in Cartilage and Chondrocytes—Nur77, NURR1, and NOR-1 mRNA levels were measured in cartilage from normal controls (n = 8) and patients with OA (n = 18) by real-time RT-PCR. Transcripts for all three receptors were detected, and NURR1 levels significantly exceeded Nur77 and NOR-1 levels in OA cartilage (p < 0.0001) (Fig. 1A). In addition, NURR1 expression in OA cartilage was five times higher than that in normal cartilage (p < 0.01) (Fig. 1B), whereas neither Nur77 nor NOR-1 was significantly elevated (data not shown). Consistent with advanced cartilage degradation and inflammation in OA (31), MMP-1 (p < 0.05) and COX-2 (p < 0.01) levels were significantly elevated in OA cartilage (Fig. 1, C and D, respectively). We documented a positive correlation between NURR1 and COX-2 expression (r = 0.74, p < 0.0001) (Fig. 1E) and NOR-1 and COX-2 expression (r = 0.58, p < 0.01), suggesting that inflammation promotes expression of these receptors. COX-2 activity contributes to the release of PGE₂ within cartilage.
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FIGURE 2. PGE₂ is a potent inducer of NURR1 in chondrocytes. A, primary human chondrocytes derived from patients with OA were treated with PGE₂ (10 μM) for the times indicated. Nur77, NURR1, and NOR-1 mRNAs were measured by real-time RT-PCR, and levels were normalized to GAPDH. Results are representative of expression profiles from chondrocytes derived from five different patients. B, SW1353 cells were treated in triplicate with PGE₂ (1 μM) for the times indicated (hours). Nur77, NURR1, and NOR-1 mRNAs were measured by real-time RT-PCR, and levels were normalized to GAPDH. C, nuclear extracts were generated from SW1353 cells treated with PGE₂ (1 μM) for the times indicated. Western blotting was conducted with anti-NURR1 polyclonal antibody. In vitro translated NURR1 is shown as a positive control. Untr, untreated.

FIGURE 3. PGE₂ antagonizes IL-1β induction of MMP-1. A, SW1353 cells were left untreated or were treated with IL-1β (10 ng/ml) for the times indicated in triplicate. RNA was harvested, and real-time RT-PCR was conducted. MMP-1 levels were normalized to GAPDH. B, Nur77, NURR1, and NOR-1 levels were measured by RT-PCR. NURR1 was induced by 2.5-fold after 1 h (**, p < 0.01). C, SW1353 cells were left untreated or were treated with IL-1β (10 ng/ml), PGE₂ (1 μM), or IL-1β and PGE₂ for 24 h. Real-time RT-PCR was conducted, and MMP-1 levels were normalized to GAPDH. **, p < 0.005, IL-1β compared with IL-1β and PGE₂.

expression of Nur77, NURR1, and NOR-1 mRNAs in SW1353 human chondrosarcoma cells. This cell line has been used to study a number of pathways involved in cartilage biology, including the transcriptional regulation of MMPs (59, 60). Consistent with elevated NURR1 expression in OA cartilage and primary chondrocytes, we observed high levels of NURR1 mRNA in SW1353 cells (Fig. 2B). PGE₂ potently and rapidly induced NURR1 and NOR-1 expression and modestly induced Nur77 levels in these cells (Fig. 2B). The temporal expression pattern of these receptors in SW1353 cells was similar to that in primary chondrocytes, with maximal induction of NURR1 and NOR-1 after 1 h of PGE₂ treatment (Fig. 2B). Closely reflecting changes in mRNA levels, NURR1 protein was also induced by PGE₂, with levels peaking in the nucleus after 2 h of stimulation and declining by 3 h (Fig. 2C). NURR1 protein was not detected in the cytoplasm of these cells (data not shown), suggesting that NURR1 functions exclusively in the nucleus under these conditions. PGE₂ signaling leads to the phosphorylation of CREB transcription factors, which can, in turn, bind to the NURR1 promoter and activate transcription (33). Consistent with this, we observed PGE₂-induced CREB activation prior to the induction of NURR1 in SW1353 cells (30 min) (data not shown). Our results in OA cartilage, primary chondrocytes, and SW1353 cells consistently demonstrate elevated expression and potent PGE₂ modulation of NURR1. Within the NR4A receptor subfamily, NURR1 may have a dominant role in modulating transcriptional programs in chondrocytes.

NURR1 Regulation of MMP Gene Expression—MMPs are a critical set of proteases that are synthesized by chondrocytes and that contribute to the degradation of joint components (31). MMP-1 is a collagenase with high activity against collagen fibrils in articular cartilage (31). In SW1353 cells, the basal levels of MMP-1 mRNA were low, and the inflammatory cytokine IL-1β potently induced expression of this gene as early as 3 h by 10-fold (Fig. 3A). NURR1 was modestly and transiently induced by IL-1β after 1 h (Fig. 3B), whereas Nur77 and NOR-1 were not modulated. Interestingly, a decline in NURR1 levels at 3 h coincided with MMP-1 induction at this time point. Because PGE₂ can modulate cartilage homeostasis and interact with inflammatory signals (44–47, 61), we addressed the effect of PGE₂ on MMP-1 gene expression. SW1353 cells were treated with PGE₂ or IL-1β alone or in combination. PGE₂ had no effect on basal expression of MMP-1 mRNA. However, PGE₂ antagonized IL-1β-induced MMP-1 levels by 45% (p <
NURR1 paralleled that of PGE2 described above. Furthermore, IL-1β mRNA and protein (Fig. 4A) expression in control LacZ-transfected and NURR1-transfected SW1353 cells. Control-transfected cells were treated for 1 h with PGE2 as indicated. B, cells were transfected in triplicate and left untreated or were treated with IL-1β (10 ng/ml) for 24 h. Real-time RT-PCR was conducted, and relative MMP-1, -2, -3, and -9 levels normalized to GAPDH are shown. *, p < 0.005, IL-1β/control-transfected compared with IL-1β/NURR1-transfected for each gene; **, p < 0.05. C, conditioned medium was collected from transfected cells left untreated or treated with IL-1β (10 ng/ml) for 24 h. Pro-MMP-1 levels were measured by enzyme-linked immunosorbent assay. *, p < 0.05, IL-1β/control-transfected compared with IL-1β/NURR1-transfected. D, shown are TIMP-1 and -2 mRNA levels in control- and NURR1-transfected cells.

FIGURE 4. NURR1 selectively represses IL-1β-induced MMP-1, -3, and -9 expression. A, shown is relative NURR1 mRNA and protein (inset) expression in control LacZ-transfected and NURR1-transfected SW1353 cells. Control-transfected cells were treated for 1 h with PGE2 as indicated. B, cells were transfected in triplicate and left untreated or were treated with IL-1β (10 ng/ml) for 24 h. Real-time RT-PCR was conducted, and relative MMP-1, -2, -3, and -9 levels normalized to GAPDH are shown. **, p < 0.005, IL-1β/control-transfected compared with IL-1β/NURR1-transfected for each gene; *, p < 0.05. C, conditioned medium was collected from transfected cells left untreated or treated with IL-1β (10 ng/ml) for 24 h. Pro-MMP-1 levels were measured by enzyme-linked immunosorbent assay. *, p < 0.05, IL-1β/control-transfected compared with IL-1β/NURR1-transfected. D, shown are TIMP-1 and -2 mRNA levels in control- and NURR1-transfected cells.

0.005) (Fig. 3C). Because PGE2 potently induced NURR1 expression and NURR1 was elevated in OA cartilage, this nuclear receptor may play a role in down-regulating cytokine-induced genes such as MMP-1.

To further address the regulation of MMP-1 expression by NURR1, SW1353 cells were transfected with a NURR1 expression plasmid or a LacZ control plasmid, and endogenous MMP-1 mRNA levels were measured. Robust expression of NURR1 was achieved in these experiments, and the levels of ectopic mRNA and protein exceeded the endogenous levels of NURR1 induced by PGE2 (Fig. 4A). Transfection efficiencies >70% were consistently obtained in these experiments (data not shown). NURR1 did not appear to have any effect on the low levels of MMP-1 expressed basally (Fig. 4B). However, NURR1 overexpression antagonized IL-1β-induced MMP-1 levels by 50% (p < 0.005) (Fig. 4B). Significantly, the magnitude of MMP-1 repression by NURR1 paralleled that of PGE2 described above. Furthermore, this decrease in MMP-1 mRNA translated into a reduction in pro-MMP-1 protein secretion (Fig. 4C). In the presence of IL-1β, SW1353 cells secreted 18 ng/ml pro-MMP-1, and ectopically expressed NURR1 significantly reduced these levels to 10 ng/ml (p < 0.05) (Fig. 4C).

To determine the specificity of NURR1 transcriptional activity for other genes regulating matrix degradation, we measured a panel of constitutive and cytokine-induced MMPs and TIMPs. MMP-2 is a constitutively expressed gelatinase, and NURR1 did not modulate the levels of this gene in SW1353 cells (Fig. 4B). In contrast, stromelysin, or MMP-3, is potently induced by IL-1β, and NURR1 inhibited expression of this gene by ~50% (p < 0.05) (Fig. 4B). MMP-9, or gelatinase B, is also induced by IL-1β, and NURR1 suppressed this gene to a similar extent (p < 0.05) (Fig. 4B). MMP-13 is another collagenase induced by IL-1β, although MMP-1 levels were 100 times greater than MMP-13 levels in SW1353 cells (data not shown). NURR1 preferentially suppressed the most abundant collagenase expressed by these cells, MMP-1, and did not regulate MMP-13 expression (data not shown). TIMP-1 and TIMP-2 are both constitutively expressed in SW1353 cells, and NURR1 did not regulate expression of either gene (Fig. 4D).

In summary, the selective inhibition of cytokine-induced MMP-1, -3, and -9 by NURR1 may ultimately reduce collagen degradation, suggesting a protective function for NURR1 in chondrocytes.

Transcriptional Repression of MMP-1 by NURR1—As MMP-1 activity provides a rate-limiting step in collagen degradation (31), we focused our analysis on the transcriptional regulation of this gene. A full-length human MMP-1 promoter (~4372 bp)-reporter plasmid was cotransfected into mouse embryonic fibroblasts with a NURR1 expression plasmid. These cells provide a viable system to address cytokine regulation of the MMP-1 promoter by transient transfections (62). IL-1β induced MMP-1 promoter activity by 4-fold, and NURR1 repressed this level of transcription by 50% (p < 0.005) (Fig. 5A). NURR1 also repressed basal MMP-1 transcription to a similar extent in untreated cells (p < 0.05) (Fig. 5A), indicating that NURR1 can antagonize promoter activity in the presence and absence of cytokine signaling. In SW1353 cells, NURR1 repressed MMP-1 promoter activity in a dose-dependent manner (Fig. 5B). Promoter activity was reduced by 90% with 400 ng of transfected NURR1 plasmid (p < 0.005) (Fig. 5B). In contrast, equivalent levels of NURR1 potently activated a consensus NURR1 response element, NBRE (p < 0.005) (Fig. 5B, inset), demonstrating the promoter-specific effects of NURR1. Taken together, these data indicate that NURR1 is a potent repressor of MMP-1 transcription, leading to significant decreases in MMP-1 mRNA and protein levels.

We identified four putative NBRE sequences in the MMP-1 promoter, each containing only a single nucleotide difference from the consensus sequence (AAAGGTCA). These sites are located at −296 bp (ACAGGTCA), −1852 bp (AAAAGTCA), −2312 bp (AAATGTCA), and −3678 bp (AAATGTCA) upstream from the start site of transcription (Fig. 5C). To date, NBRE sequences have been described exclusively in the pro-
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FIGURE 5. NURR1 represses MMP-1 promoter activity. A, mouse embryonic fibroblasts were cotransfected in triplicate with the full-length human MMP-1 promoter (−4372 bp) luciferase construct and either the control pUB6-LacZ or pUB6-NURR1 plasmid. Cells were treated with IL-1β (10 ng/ml) as indicated for 24 h. *, p < 0.01, control-transfected compared with NURR1-transfected; **, p < 0.005. RLUs, relative luciferase units. B, SW1353 cells were cotransfected in triplicate with the full-length human MMP-1 promoter construct (100 ng) and the control pCMV-LacZ or pCMX-NURR1 plasmid as indicated. Total DNA in each transfection was adjusted to 500 ng with pCMV-LacZ. **, p < 0.005, control-transfected compared with NURR1-transfected. C, shown is a diagram of the putative NBRE sites in the MMP-1 promoter and the specific mutations that were generated. D, SW1353 cells were transfected in triplicate with the full-length human MMP-1 promoter construct containing four putative NBRE sites (wild type) or mutations in each of these sites (NBRE mutations) and either the control pCMV-LacZ or pCMX-NURR1 plasmid. **, p < 0.005; control-transfected compared with NURR1-transfected.

FIGURE 6. NURR1 targets an ETS-responsive region of the MMP-1 promoter. A, SW1353 cells were cotransfected in triplicate with MMP-1 promoter deletion constructs (−4372, −1772, −1546, and −517 bp) and the pCMX-NURR1 or control pCMV-LacZ plasmid. **, p < 0.005; control-transfected compared with NURR1-transfected. RLUs, relative luciferase units. B, shown is the NURR1-responsive region of the MMP-1 promoter spanning −1772 to −1546 bp. AP-1 and ETS-binding sites are indicated at positions −1602 and −1607, respectively. C, SW1353 cells were transfected in triplicate with MMP-1 promoter deletion constructs (−1772 or −1546 bp) and the pCMV-ETS1 or control pCMV-LacZ plasmid as indicated. *, p < 0.05, control-transfected compared with ETS1-transfected; **, p < 0.005.
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A. 

B. 

C. 

FIGURE 7. NURR1 interacts with the MMP-1 promoter element. A. SW1353 cells were cotransfected in triplicate with pNBRE-Δk-luciferase and pCMX-NURR1 (wild type (WT)) or pCMX-NURR1(C283G). RLUs, relative luciferase units. B. SW1353 cells were cotransfected in triplicate with the full-length MMP-1 promoter construct and pCMX-NURR1 (wild type) or pCMX-NURR1(C283G). **, p < 0.005, control-transfected compared with NURR1-transfected. C. DNA gel shift analysis of the MMP-1 promoter element spanning 1586 to 1626 bp. Nuclear extracts were generated from SW1353 cells left untreated or treated with 1 μM PGE2, for 2 h. Extracts were incubated with a 40-bp biotinylated double-stranded oligonucleotide. Excess unlabeled MMP-1 oligonucleotide (100X self) was added to lanes 3 and 7. Anti-NURR1 polyclonal antibodies were added to lanes 4 and 8 (anti-NURR1 antibody E-20) and lanes 5 and 9 (anti-NURR1 antibody N-20). Lane 2 contained the extract alone, and no extract was added to lane 1.

These results suggest that NURR1 does not bind directly to the MMP-1 promoter; rather, NURR1 may mediate repression by interacting with other proteins bound to the promoter.

DNA gel shift analysis was conducted next, focusing on a 40-bp region of the MMP-1 promoter centered upon the AP-1 and ETS sites at −1602 and −1607 bp. Nuclear extracts from SW1353 cells bound to this promoter region, and a specific high molecular mass complex was identified by competitions with an unlabeled oligonucleotide (Fig. 7C, lanes 2, 3, 6, and 7, Specific complex arrow). We observed a 16-fold increase in the intensity of this complex with extracts from PGE2-treated cells (Fig. 7C, lane 6), indicating that a PGE2-regulated factor can increase binding to this promoter element. This increase in binding was observed in multiple experiments (n = 7), with magnitudes ranging from 2- to 16-fold. The high molecular mass of this complex is consistent with a large protein-DNA complex potentially composed of multiple proteins. As shown above, nuclear extracts from PGE2-treated cells contained elevated levels of NURR1 protein (Fig. 2C), suggesting that NURR1 may be a component of this large complex.

Supershift analysis was conducted to evaluate the presence of NURR1 in this protein-DNA complex. We used two different anti-NURR1 polyclonal antibodies: antibody E-20, directed against the C-terminal region; and antibody N-20, directed against the N-terminal region. Anti-NURR1 antibody E-20 altered the mobility of the specific complex and caused a supershift (Fig. 7C, lanes 4 and 8, Supershift arrow). Anti-NURR1 antibody N-20 also altered the mobility of this complex, although to a lesser extent (Fig. 7C, lanes 5 and 9). In contrast, these antibodies did not affect the migration of a non-specific complex (Fig. 7C, Non-specific complex arrow). Control rabbit IgG did not alter the binding patterns of these complexes (data not shown). These results indicate that NURR1 is a component of a high molecular mass complex bound to a region of the MMP-1 promoter that is sensitive to repression by NURR1.

Functional Protein Interactions with NURR1—NURR1 may interact with other transcription factors and co-regulatory proteins to repress MMP-1 gene expression. NURR1 can heterodimerize with RXR (8–10), and RXR-specific agonists can block MMP-1 transcription (51), suggesting that RXR may cooperate with NURR1 to inhibit MMP-1 expression. However, the NURR1(P560A) mutation, which prevents heterodimerization with RXR (8), did not alter repression of the

these critical AP-1 or ETS sites result in a dramatic decrease in promoter activity (56, 65), and it was difficult to assess the effects of NURR1 in the context of these mutations, as transcriptional activity was deficient (data not shown). Alternately, we confirmed that ETS factors regulate the same region of the MMP-1 promoter that is sensitive to repression by NURR1. These results indicate that NURR1 is a component of this large complex.

NURR1 Interacts with an MMP-1 Promoter Element Important for ETS Regulation—To determine whether NURR1 binds directly to the MMP-1 promoter, we mutated a critical residue in the DNA-binding domain of the receptor. The C283G mutation prevents NURR1 from binding to a consensus NBRE sequence and thereby blocks transactivation (38). We confirmed that this residue is essential for NBRE-mediated transactivation in SW1353 cells, as activation of the NBRE reporter was ablated (Fig. 7A). However, repression of the MMP-1 promoter was not affected by this mutation (p < 0.005) (Fig. 7B).
MMP-1 promoter (Fig. 8A). Likewise, a truncated form of NURR1 (NURR1-(1–583)) that is unable to heterodimerize with RXR (10) blocked MMP-1 promoter activity as well as the wild-type receptor (Fig. 8A), indicating that repression of MMP-1 transcription does not require RXR. This truncated form of NURR1 lacks the C-terminal AF-2 activation domain, and consistent with a report in other cell types (10), the absence of this domain decreased activation of the NBRE reporter construct. On this promoter, NURR1 is a positive regulator, and ETS1 inhibited transactivation in a dose-dependent manner (Fig. 8A). Whereas the AF-2 domain contributes to NURR1 transactivation, distinct domains of NURR1 are required for transrepression.

Co-repressor proteins may be involved in MMP-1 repression, and the silencing mediator for retinoid and thyroid hormone (SMRT) is predicted to interact with the ligand-binding domain of NURR1 (11). SMRT can repress MMP-1 transcription (66), suggesting that NURR1 may recruit this co-repressor to the MMP-1 promoter. Conserved hydrophobic amino acids in NURR1 are required for SMRT binding in vitro (11), yet mutation of two of these critical residues, F592A and L593A, did not alter MMP-1 repression (Fig. 8A). Transactivation of the NBRE reporter was reduced by NURR1(F592A), whereas there was no change in transactivation by NURR1(L593A) (Fig. 8B), consistent with a previous report (10). In addition, NURR1-(1–583) lacks this entire SMRT interface region, yet retained the ability to repress MMP-1 (Fig. 8A). Taken together, the functional interaction between ETS and NURR1 in the context of the NBRE reporter construct. On this promoter, NURR1 is a positive regulator, and ETS1 inhibited transactivation in a dose-dependent manner (p < 0.005) (Fig. 9B). Similar results were obtained with ETS2 (data not shown), indicating that a functional antagonism occurs through protein-protein interactions between ETS factors and NURR1. In summary, we have identified a reciprocal antagonism between NURR1 and ETS factors, and this interaction contributes to the repression of MMP-1 transcription.

**DISCUSSION**

The NR4A receptors have recently emerged as key regulators of cytokine and growth factor action in chronic inflammatory diseases. These orphan receptors function independently of ligands, and their activity is tightly controlled at the level of expression. Previous studies have suggested a role for the NR4A receptors in inflammatory joint disease, and these receptors have been documented in synovial tissue and bone (32–37). However, few target genes have been identified in these tissues (35, 38, 39), and the function of these receptors within joints remains elusive. Our study is the first to document NR4A receptor expression in cartilage and chondrocytes, the principal cell type present in articular cartilage.
Here, we have described the repression of MMP gene expression by NURR1, suggesting that this orphan receptor may have a protective role in maintaining cartilage homeostasis. NURR1 levels exceed Nur77 and NOR-1 levels in OA cartilage and chondrocytes (Figs. 1 and 2), suggesting a dominant role for NURR1 in this system. Consistent with a positive correlation between NURR1 and COX-2 expression in cartilage, PGE₂ potently and rapidly induces NURR1 expression in chondrocytes, whereas NOR-1 and Nur77 are modulated to a lesser extent (Fig. 2). Given the elevated levels of NURR1 in OA cartilage and the robust modulation of this receptor in chondrocytes, we focused on elucidating transcriptional targets of this receptor. However, because Nur77 and NOR-1 are also present in cartilage, these receptors may have overlapping transcriptional activities. In contrast to other nuclear receptors such as the estrogen and vitamin D receptors, which are present in cartilage and controlled by endogenous ligands (67, 68), NURR1 does not appear to have a ligand, and the activity of this transcription factor is controlled largely at the level of its expression (4). A recent study profiling expression of the entire nuclear receptor superfamily in OA cartilage reported expression of several nuclear receptors (69). Because COX-2 and PGE₂ levels are elevated in OA cartilage, it is possible that a subset of orphan nuclear receptors detected in this study are regulated by PGE₂. It remains to be seen whether additional orphan receptors intersect with inflammatory pathways to modulate cartilage homeostasis.

COX-2-derived prostaglandins such as PGE₂ play differential roles in inflammation and tissue homeostasis, in part through their effects on immediate-early transcription factors (45, 70, 71). During chronic inflammation, this COX-2-derived molecule is thought to enhance inflammation and to contribute to tissue destruction. However, PGE₂ may also promote the resolution of inflammation and protect joint tissues from degradation by blocking NFκB activity (70), inducing collagen expression (47), and suppressing MMPs (44). PGE₂ can impinge upon ERK (extracellular signal-regulated kinase) activity to reduce MMP-1 expression (44). In addition, this prostaglandin blocks expression of the inflammatory cytokine tumor necrosis factor-α through mechanisms involving the immediate-early gene egr-1 (early growth response factor-1) (71). Selective COX-2 inhibitors, which decrease PGE₂ production, have been shown to increase MMP gene expression in acute inflammation (72), potentially contributing to the adverse effects of these inhibitors.

We have demonstrated that IL-1β is a potent inducer of MMP-1 (140-fold) in chondrocytes and that PGE₂ antagonizes expression of this gene by 45% (Fig. 3). Overexpression of NURR1 reduces MMP-1 expression to a similar extent (Fig. 4), suggesting that PGE₂-induced NURR1 may antagonize some of the effects of IL-1β. We confirmed that NURR1 is a potent repressor of MMP-1 transcription (Fig. 5), and the extent of this repression (50–90%) is comparable with the effects of other transcriptional inhibitors such as glucocorticoids and retinoids (31). In addition to repressing MMP-1, NURR1 also reduces the levels of MMP-3 and -9 (Fig. 4). Collectively, the repression of these MMPs may reduce collagen degradation by lowering the levels of pro-MMP-1 secreted and by decreasing the activation of this latent enzyme mediated by MMP-3 (31). In addition, blocking MMP-9 expression would inhibit the breakdown of denatured collagen (31). Consistent with our observations, a recent expression profiling study documented an inverse correlation between NURR1 and MMP-1 and -3 expression in patients with active rheumatoid arthritis (73), suggesting that NURR1 may also regulate the expression of these critical MMPs in vivo.

NURR1 targets a region of the MMP-1 promoter spanning −1772 to −1546 bp (Fig. 6), and this region contains adjacent ETS and AP-1 sites (∼−1607 and −1602 bp) that are important for high levels of MMP-1 transcription (55, 56). A single nucleotide polymorphism exists at −1607 bp, where insertion of a guanine nucleotide creates an ETS-binding site (56). This ETS site increases MMP-1 gene expression and has been associated with a number of cancers (74–76). PGE₂ enhances the binding of nuclear proteins to a 40-bp promoter region containing these critical ETS and AP-1 sites, and NURR1 is a component of a large protein complex bound here (Fig. 7). We have demonstrated that ETS1 activates this region of the promoter in chondrocytes (Fig. 6C), and previous reports have shown that ETS and AP-1 factors bind to this site as part of a large protein complex (56, 65, 74, 77). Our results indicate a functional antagonism between NURR1 and ETS1, where NURR1 reduces ETS1 induction of the MMP-1 promoter, and ETS1 blocks transactivation of the NBRE reporter (Fig. 9). These results reveal interactions between these transcription factors occurring in the context of different promoters. NURR1 may repress MMP-3 and -9 transcription through similar mechanisms, as these genes are also positively regulated by ETS factors (31). ETS1 is a principal activator of MMP-3 transcription through an inverted tandem of ETS-binding sites in the proximal promoter (78, 79), and MMP-9 transcription is regulated through an ETS-binding site at −540 bp in the promoter (80). Furthermore, ETS factors have been implicated in bone and cartilage development (81), and NURR1 may provide an additional level of transcriptional regulation during these processes.

Consistent with proposed interactions between NURR1 and ETS, our results indicate that NURR1 does not bind directly to the MMP-1 promoter. Mutation of a critical amino acid in the DNA-binding domain does not affect MMP-1 repression, whereas NBRE activation, which requires DNA binding, is ablated (Fig. 7). Our supershift analysis with two different anti-NURR1 antibodies confirmed that NURR1 is a component of a large protein complex bound to the MMP-1 promoter (Fig. 7). Deletion of the C terminus of NURR1 (NURR1-(1–583)) does not alter repression of MMP-1 transcription (Fig. 8A), suggesting that NURR1 may interact with other proteins through its N terminus. ETS factors may interact with the N terminus of NURR1 either directly or indirectly through cofactors. Another nuclear receptor, the androgen receptor, utilizes a similar mechanism for repression of MMP-1 transcription in response to androgens (52). The androgen receptor does not require DNA binding and utilizes N-terminal sequences for...
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interactions with the ETS transcription factor ETS-related molecule on the MMP-1 promoter (52). In the absence of ligands, the vitamin D receptor, estrogen receptor, and peroxisome proliferator-activated receptor-α are allosterically activated through direct interactions with ETS1 (82). Along with our results, these studies suggest conserved regulatory mechanisms between nuclear receptors and ETS transcription factors.

Although a number of positively regulated target genes have been identified for the NR4A receptors, the mechanisms of transcriptional repression are only beginning to emerge. Induced promoters are dependent on interactions with NBRE sites (5, 38), yet repression of the MMP-1 promoter does not require NBRE sequences (Fig. 5D). Likewise, repression of aromatase transcription by NURR1 is not mediated through an NBRE sequence (83), but through sequences in the proximal cAMP-regulated region of this promoter. The NURR1-responsive regions of the MMP-1 sequences in the proximal cAMP-regulated region of this mediator through an NBRE sequence (83), but through mechanisms between nuclear receptors and ETS transcription factors. Similarly, Nur77 can interact with the p65 subunit of NFκB and confer repression to genes positively regulated by this transcription factor (12, 14, 15). In addition to antagonizing positive regulatory factors, NR4A transrepression may also require co-repressor proteins (11, 13, 84). Although SMRT has been proposed to interact with amino acids in the C terminus of NURR1 in vitro (11), this putative interface domain is not required for repression of MMP-1 (Fig. 8A). The co-repressor CR6-interacting factor binds to the N terminus of Nur77 and blocks transactivation of this receptor (84), suggesting that similar co-repressor interactions may regulate NURR1 activity.

The ability of NR4A receptors to function as both positive and negative transcriptional regulators is consistent with the diverse roles ascribed to these receptors in disease processes. NR4A receptors appear to have pathogenic functions in cancer (18–20, 23). In contrast, these receptors may have protective functions in atherosclerosis, where they suppress smooth muscle cell proliferation and lesion formation (21, 25–27). The induction of these receptors in activated macrophages suggests involvement in other inflammatory diseases (28, 29). Recent studies demonstrate both pro- and anti-inflammatory effects of Nur77 in macrophages (26, 30), suggesting that chronic or acute expression levels may permit differential effects on inflammatory gene expression. NR4A receptors may serve as novel therapeutic targets in inflammatory joint disease. Our results suggest that NURR1 may have a protective function in cartilage by blocking the expression of MMP-1, -3, and -9. Furthermore, osteopontin is induced by NURR1 in osteoblasts (38), and osteopontin may inhibit inflammation in cartilage by blocking some of the effects of IL-1β (41). Within synoviocytes, NURR1 induces expression of IL-8 (35), and this chemokine acts to recruit inflammatory cells and to promote resolution early in synovitis. As additional transcriptional targets of NURR1 are elucidated, therapeutic strategies to modulate this receptor in a tissue- and promoter-selective manner may evolve. Although NURR1 does not appear to be regulated by an endogenous ligand (4), pharmacological modulation of NURR1 activity can be achieved with the anti-neoplastic agent 6-mercaptopurine (85). In addition, altering expression of this constitutively active receptor may be another viable approach to modulate NURR1 target genes. Dexamethasone and methotrexate, which are used for the treatment of inflammatory diseases and cancer, can regulate NURR1 expression (32, 34). Some of the clinical benefits of these agents may in fact be mediated through NURR1. Like other members of the nuclear receptor superfamily, NURR1 may prove to be a viable molecular target for blocking inflammation and tissue destruction associated with chronic inflammatory diseases.

Acknowledgments—We are grateful to all members of the Murphy laboratory for helpful suggestions and discussions. We thank Kevin McMahon and Ciaraan Lowen for assistance with site-directed mutagenesis; Evan Rosen, Thomas Perlmann, and Leonie Young for providing cells and plasmids; and Catherine Moss and Janet McCormack for assistance with real-time PCR. We are grateful to Charlie Coon and Ramgopal Mettu for critical discussions throughout the course of this study.

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