Estrogen-related receptor γ causes osteoarthritis by upregulating extracellular matrix-degrading enzymes

Young-Ok Son, Seulki Park, Ji-Sun Kwak, Yoonkyung Won, Wan-Su Choi, Jinseol Rhee, Churl-Hong Chun, Je-Hwang Ryu, Don-Kyu Kim, Hueng-Sik Choi & Jang-Soo Chun

The estrogen-related receptor (ERR) family of orphan nuclear receptor is composed of ERRα, ERRβ, and ERRγ, which are known to regulate various isoform-specific functions under normal and pathophysiological conditions. Here, we investigate the involvement of ERRs in the pathogenesis of osteoarthritis (OA) in mice. Among ERR family members, ERRγ is markedly upregulated in cartilage from human OA patients and various mouse models of OA. Adenovirus-mediated overexpression of ERRγ in mouse knee joint or transgenic expression of ERRγ in cartilage leads to OA. ERRγ overexpression in chondrocytes directly upregulates matrix metalloproteinase (MMP)-3 and MMP13, which are known to play crucial roles in cartilage destruction in OA. In contrast, genetic ablation of Esrrg or shRNA-mediated downregulation of Esrrg in joint tissues abrogates experimental OA in mice. Our results collectively indicate that ERRγ is a novel catabolic regulator of OA pathogenesis.
Osteoarthritis (OA), the most common form of arthritis, is a leading cause of disability and incurs a large socioeconomic cost. OA is a whole-joint disease characterized by cartilage destruction, synovial inflammation, osteophyte formation, and subchondral bone sclerosis. However, no effective disease-modifying therapy for OA has been developed to date. OA is caused by an anabolic/catabolic factor imbalance that can be induced by various etiologic risk factors and pathophysiological processes. Important among the potential OA-causing mechanisms are mechanical stresses, including joint instability and injury, and factors that predispose toward OA, such as aging. These factors alter biochemical pathways in chondrocytes, resulting in degradation of the extracellular matrix (ECM). Among the matrix-degrading enzymes, matrix metalloproteinase 3 (MMP3), MMP13, and ADAMTS5 (a disintegrin-like and metallopeptidase with thrombospondin type 1 motif 5; aggreganase-2) are known to play crucial roles in OA cartilage destruction. Various catabolic regulators, including the proinflammatory cytokine, interleukin (IL)-1β, can upregulate MMP3, MMP13, and ADAMTS5. We previously demonstrated that hypoxia-inducible factor (HIF)-2α (encoded by Epas1), which is transcriptionally upregulated in chondrocytes by proinflammatory cytokines or mechanical stress, causes OA pathogenesis through upregulation of matrix-degrading enzymes. We also recently demonstrated that the zinc-ZIP8-MTF1 axis—reflecting induction of the zinc importer ZIP8 (encoded by Slc39a8), zinc influx, and subsequent activation of the zinc-dependent transcription factor MTF1—acts as a crucial catabolic regulator of OA pathogenesis by upregulating matrix-degrading enzymes in joint articular chondrocytes.

OA is currently considered as a disease associated with metabolic disorders. Although this is still a matter of controversy, a number of studies suggest an association of metabolic syndrome with OA pathogenesis. Among the various regulatory molecules involved in metabolism, isoforms of estrogen-related receptors (ERRs) are currently known to regulate various metabolic processes.
Fig. 2 Signaling upstream of ERRγ expression. a qRT-PCR analysis (n ≥ 8) of IL-6 in chondrocytes treated with IL-1β and infected with 800 MOI of control virus (Ad-C) or the indicated MOI of Ad-Epas1 or Ad-Slc3918. b, c Detection of IL-6 in chondrocytes treated with IL-1β (1 ng ml⁻¹) by immunofluorescence microscopy (b) and Western blotting (c). d, e Western blot (d) and qRT-PCR (e) analysis of ERRγ, MMP3, and MMP13 in chondrocytes treated with IL-6 (n = 10). MMP3 and MMP13 proteins in conditioned medium of chondrocyte cultures were detected by Western blotting. f qRT-PCR analysis (n ≥ 8) of ERRγ mRNA levels in chondrocytes treated with IL-1β (left panel), qRT-PCR analysis (n ≥ 8) of ERRγ mRNA levels in chondrocytes treated with AG490 and exposed to IL-1β (middle panel). qRT-PCR analysis (n ≥ 8) of ERRγ mRNA levels in chondrocytes treated with AG490 and exposed to IL-6 (right panel). Values are presented as means ± s.e.m. (*P < 0.05, **P < 0.005, and ***P < 0.0005. One-way ANOVA)

processes, including metabolic disorders and energy metabolism16,17. The ERR family consists of the orphan nuclear receptors, ERRα (NR3B1), ERRβ (NR3B2), and ERRγ (NR3B3). Unlike other nuclear orphan receptors, ERRs are constitutively active owing to the active conformation of their ligand-binding domain, even in the absence of a ligand19. Because ERRs share nearly identical DNA-binding domains, their transcriptional regulatory functions are similar to each other; thus, they regulate overlapping common target genes. For instance, ERRα and ERRγ regulate energy metabolism and promote oxidative capacity in skeletal and cardiac muscle16,20,21. However, individual ERRs also exhibit isof orm-specific functions. For example, ERRα regulates iron homeostasis in various tissues22,23, ERRβ regulates embryonic stem cell pluripotency and self-renewal24,25, and ERRα acts as a determinant of breast cancer etiology24,25.

The roles of ERRs in cartilage biology are beginning to be investigated, with most studies focusing on the functions of ERRα26. For instance, studies have shown that ERRα activates the Sox9 promoter in a rat chondrogenic cell line27 and rat mandibular condylar chondrocytes28, and is required for cartilage development in zebra fish29. In contrast, cartilage-specific ERRγ transgenic (Tg) mice exhibit chondrodysplasia, in which repressed chondrocyte proliferation decreases the proliferative zone of the growth plate and reduces bone length30. It has also been reported that ERRα expression is reduced in bones and joints during collagen-induced inflammatory rheumatoid arthritis31. However, the functions of ERRs in regulating joint cartilage homeostasis and/or OA pathogenesis have not yet been studied, although an in vitro study by Bonney et al.32 suggested a possible association of ERRα with OA pathogenesis. They demonstrated that IL-1β in human OA chondrocytes upregulates ERRα, which directly targets genes encoding MMP3 and SOX9.

In this study, we used human OA cartilage and mouse models of OA to investigate whether ERRs are associated with OA pathogenesis. Here, we report that ERRγ is a novel catabolic regulator of OA pathogenesis. We found that, among the ERR family members, ERRγ was specifically upregulated in cartilage from human OA patients and various mouse models of OA. Our gain-of-function (adenovirus-mediated overexpression in joint tissues or Col2a1-Esrrg Tg mice) and loss-of-function (Esrrgfl/fl– mice or shRNA-mediated knockdown) approaches clearly indicated that ERRγ acts as a novel catabolic regulator of OA pathogenesis, at least in part, by the upregulating matrix-degrading enzymes, MMP3 and MMP13, in articular chondrocytes.

Results

ERRγ is upregulated in chondrocytes of OA cartilage. To explore the possible association of ERRs with OA pathogenesis, we first examined the expression levels of ERRs in OA cartilage of human patients and various mouse models. Our quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses revealed that ERRγ was significantly increased in OA-affected, damaged regions of human cartilage compared with undamaged regions of arthritic cartilage (Fig. 1a). Although the fold-increases in ERRα and ERRβ mRNAs were statistically significant, their expression levels were negligible relative to those of ERRγ (Fig. 1a). Consistent with the increased mRNA levels, immunostaining revealed that ERRγ protein levels were markedly elevated in chondrocytes of human OA cartilage (Fig. 1b, c). Similarly, ERRγ, but not ERRα or ERRβ, was significantly increased in cartilage from an experimental mouse model of OA induced by destabilization of the medial meniscus (DMM) surgery (Fig. 1d–f). ERRγ was also increased in the cartilage of mice
with OA caused by the overexpression of HIF-2α via intra-articular (IA) injection of an adenovirus-expressing HIF-2α (Ad-Epas1)\(^{10,11}\) or by IA injection of Ad-Slc39a8 to overexpress the zinc importer, ZIP8\(^{12,13}\) (Supplementary Fig. 1a, b). However, we were unable to detect ERR\(^{\gamma}\) in OA cartilage of humans or mice (Supplementary Fig. 2a, b). Additionally, IL-6, which acts as a catabolic regulator of OA cartilage destruction\(^{33}\), increased mRNA levels of ERR\(^{\gamma}\), but not ERR\(^{\alpha}\) or ERR\(^{\beta}\), in primary cultured mouse articular chondrocytes (Supplementary Fig. 2c).

Because these results collectively suggested that ERR\(^{\gamma}\) was associated with OA pathogenesis, we focused on the role of ERR\(^{\gamma}\) in OA pathogenesis.

ERR\(^{\gamma}\) expression is regulated by multiple signaling pathways, including alcohol-induced activation of the cannabinoid receptor, IL-6-mediated inflammation, fasting-induced glucagon receptor activation, feeding-induced insulin receptor activation, hypoxia, and endoplasmic reticulum stress\(^{17}\). In an attempt to identify regulators of ERR\(^{\gamma}\) expression, we examined the expression of its upstream signaling components in primary cultured chondrocytes treated with various OA-associated catabolic regulators, including IL-1β\(^{9}\), HIF-2α\(^{10,11}\), and ZIP8\(^{12,13}\), using microarray analyses. Among the known ERR\(^{\gamma}\) upstream regulators, IL-6 expression was markedly increased by IL-1β treatment and, to a lesser degree, by the overexpression of HIF-2α or ZIP8 (Supplementary Table 1). We therefore further characterized the involvement of IL-6 signaling in ERR\(^{\gamma}\) expression in chondrocytes. Similar to microarray analyses, our qRT-PCR analyses indicated upregulation of ERR\(^{\gamma}\) by IL-1β treatment and, to a lesser degree, by overexpression of HIF-2α or ZIP8, in primary cultured mouse articular chondrocytes (Fig. 2a).

IL-1β also increased IL-6 protein levels in chondrocytes, as determined by immunofluorescence microscopy and Western blotting (Fig. 2b, c and Supplementary Fig. 9). IL-6 treatment of chondrocytes increased ERR\(^{\gamma}\) mRNA levels in a concentration- and time-dependent manner (Fig. 2e). IL-6 also increased mRNA levels and secreted levels of MMP3 and MMP13 protein (Fig. 2d, e), which are encoded by target genes of IL-6 and are associated with OA cartilage destruction\(^{33}\). Additionally, IL-1β-induced
upregulation of ERRγ was abrogated by knockdown of IL-6 with specific small interfering RNA (siRNA) (Fig. 2f). IL-6-mediated inflammation is known to regulate ERRγ expression via JAK/STAT signaling. Consistent with this, inhibition of JAK/STAT signaling with AG49034 abrogated IL-6-induced ERRγ expression (Fig. 2f). These results collectively indicate that the IL-1β/IL-6 axis contributes to ERRγ expression in articular chondrocytes. This is consistent with our previous work showing that IL-6 plays an essential role in OA cartilage destruction by upregulating MMP3 and MMP13.

**Ectopic expression of ERRγ causes OA pathogenesis.** To examine the in vivo role of ERRγ in OA pathogenesis, we ectopically expressed ERRγ in knee joint tissues of 10-week-old male mice via IA injection of an adenovirus-expressing ERRγ (Ad-Esrrg). We previously demonstrated that an adenovirus system effectively delivers genes to cartilage and other joint tissues. Consistent with our prior findings, immunohistochemical staining indicated that ERRγ was effectively overexpressed in cartilage (Fig. 3a), as well as the meniscus and synovium (Supplementary Fig. 3a). ERRγ overexpression, triggered by three weekly IA injections of Ad-Esrrg, caused synovitis, as determined by H&E staining (Fig. 3b). At this stage, safranin-O staining indicated loss of glycosaminoglycans in articular cartilage above the tidemark, without apparent development of osteophyte or subchondral bone remodeling (Fig. 3b). However, after 8 weeks, joint tissues IA-injected with Ad-Esrrg showed osteophyte development and subchondral bone sclerosis, with severe erosion of cartilage (Fig. 3c, d). We also saw a loss of glycosaminoglycans in articular cartilage in both juvenile (8-week-old) and young adult (10-week-old) mice (Supplementary Fig. 3b), suggesting that developmental stage is not a critical determinant of ERRγ-induced cartilage damage. Indeed, overexpression of HIF-2α, which was previously shown to cause OA cartilage destruction, induced cartilage damage in mice at various ages from 8 to 20 weeks old (Supplementary Fig. 3c).

IA injection of Ad-Esrrg caused ectopic expression of ERRγ in all joint tissues, including the synovium and cartilage (Supplementary Fig. 3a). To examine the cartilage-specific functions of ERRγ in OA pathogenesis, we generated cartilage-specific ERRγ Tg mice (Col2a1-Esrrg) using the Col2a1 enhancer and promoter. Primary cultured chondrocytes from Col2a1-Esrrg Tg mice exhibited upregulation of ERRγ at both mRNA and protein levels (Fig. 4a and Supplementary Fig. 9). ERRγ protein levels were also elevated in the cartilage and meniscus, but not synovial tissues, of Col2a1-Esrrg Tg mice (Fig. 4b and Supplementary Fig. 4a). Because a previously generated cartilage-specific ERRγ Tg mouse was reported to exhibit chondrodysplasia, in which repressed chondrocyte proliferation decreases the proliferative zone of the growth plate and reduces bone length, we characterized skeletal development in our Tg mice. Skeletal staining of E18.5 embryos revealed that skeletal structure was similar in Tg mice and wild-type (WT) littermates (Supplementary Fig. 4b). Additionally, alcin blue staining of the metatarsal bone of 2-week-old mice revealed that the lengths of resting/proliferative and hypertrophic zones of the growth plate were similar (Supplementary Fig. 4c). Thus, in contrast to the previous report, our data collectively indicate no significant difference in skeletal development between our Tg mice and WT littermates.

Compared with WT littermates, DMM-operated Col2a1-Esrrg Tg mice exhibited significantly more cartilage destruction, as
ERRγ null mice die shortly after birth, we used heterozygous mice (Esrrg<sup>+/−</sup>) for experimental OA studies. We previously showed that mice heterozygous for HIF-2α (Epas1<sup>+/−</sup>) or ZIP8 (Slc39a8<sup>+/−</sup>) exhibited significantly reduced DMM-induced OA pathogenesis. Here, we found that Esrrg<sup>+/−</sup> mice exhibited reduced expression levels of ERRγ in joint tissues (Fig. 5a and Supplementary Fig. 6a, b) without any marked differences in skeletal staining (Supplementary Fig. 6c). Moreover, the DMM-induced manifestations of OA, including cartilage erosion, osteophyte formation, and subchondral bone sclerosis, were significantly reduced in Esrrg<sup>+/−</sup> mice (Fig. 5b, c). Consistent with this, DMM-induced upregulation of MMP3 and MMP13 proteins in damaged cartilage was markedly abrogated in Esrrg<sup>+/−</sup> mice (Supplementary Fig. 6d). Additionally, the cartilage destruction caused by overexpression of HIF-2α or ZIP8 via IA injection of Ad-Epas1<sup>+/−</sup> or Ad-Slc39a8<sup>+/−</sup>, respectively, was also significantly reduced in Esrrg<sup>+/−</sup> mice compared with WT littermates (Supplementary Fig. 6e).

The above results indicate that deletion of one allele of Esrrg is sufficient to reduce experimental OA in mice. Therefore, we further validated the function of ERRγ in OA pathogenesis by knocking down ERRγ in whole-joint tissues of mice via IA injection of adenovirus-expressing shRNA against Esrrg (Ad-shEsrrg). DMM-induced upregulation of ERRγ in the chondrocytes of cartilage tissue was effectively abrogated by IA injection of Ad-shEsrrg in DMM-operated mice (Fig. 6a). In our initial screening, we found that infection with Ad-shEsrrg effectively downregulated ERRγ expression in primary cultured mouse articular chondrocytes. Consistent with this, knockdown of cartilage ERRγ significantly suppressed all examined OA manifestations, including cartilage erosion, subchondral bone sclerosis, and osteophyte formation, in DMM-operated mice (Fig. 6b, c). These results further support the conclusion that ERRγ functions as a catabolic regulator in mouse models of OA pathogenesis caused by DMM surgery.
Inhibition of ERRγ blocks experimental OA in mice. Next, we examined whether ERRγ could be a therapeutic target for OA. Because IL-6, an upstream signaling component involved in regulating ERRγ expression, upregulates MMP3 and MMP13 in chondrocytes23, we first examined whether IL-6-induced upregulation of MMP3 and MMP13 requires the transcriptional activity of ERRγ. We found that treatment with GSK5182, an inverse agonist of ERRγ22, significantly inhibited IL-6-induced upregulation of MMP3 and MMP13 in primary cultured chondrocytes (Fig. 7e). To validate ERRγ as a potential therapeutic target for OA, we intraperitoneally administrated sham- and DMM-operated mice with GSK5182 (20 mg kg\(^{-1}\) body weight). We found that GSK5182 significantly reduced all DMM-induced manifestations of OA, including cartilage destruction, osteophyte development, and subchondral bone sclerosis (Fig. 7f). These results collectively suggest the possibility that ERRγ could serve as a therapeutic target for OA.

Finally, although mRNA and protein levels of ERRα were unchanged in OA cartilage of human patients and mouse models, we examined whether overexpression of ERRα via IA injection of Ad-Esrra in joint tissues causes cartilage damage. IA injection of Ad-Esrra caused overexpression of ERRα in cartilage (Supplementary Fig. 8a). However, this overexpression of ERRα did not cause any detectable cartilage damage or synovial inflammation (Supplementary Fig. 8b, c), clearly indicating that ERRα, but not ERRγ, functions as a catabolic regulator of OA in mice.

**Discussion**

The ERR isoforms, ERRα, ERRβ, and ERRγ, regulate various physiological functions, either in common or in an isoform-specific manner. However, whether ERRs regulate OA pathogenesis in vivo has not been investigated to date. Here, we demonstrated that, among ERR isoforms, ERRγ regulates OA pathogenesis in mice by causing cartilage destruction, osteophyte formation, and subchondral bone sclerosis. The results of both gain-of-function (adenovirus-mediated overexpression of ERRγ in mouse joint tissues or Col2a1-Esrrγ Tg mice) and loss-of-function (Esrrγ\(^{-/-}\) mice or shRNA-mediated silencing of Esrrγ) experiments clearly indicate that ERRγ functions as a critical catabolic regulator of OA pathogenesis.

The role of ERRs in cartilage biology has not been extensively studied, and most existing studies have focused on ERRα26. Among the earliest evidence supporting a possible functional association of ERRα with cartilage development was the spatial and temporal correlation of ERRα expression with cartilage development41. Although a relatively large number of studies have reported on the role of ERRα in chondrogenesis and cartilage development26–28, only a few reported the possible role of
ERRα in cartilage homeostasis and OA pathogenesis. Bonnelye et al.\textsuperscript{32} identified possible in vitro OA-related functions of ERRα, reporting that ERRα expression, which is lower in OA than normal cartilage, is upregulated by IL-1β in OA chondrocytes via signaling pathways involving cyclooxygenase 2, prostaglandin E2, cAMP, and protein kinase A. They also found that ERRα regulates the expression of MMP13 in primary cultured OA chondrocytes in vitro. However, ERRα function in OA pathogenesis in vivo has not been investigated. Furthermore, we found no previous report indicating a role for ERRγ in OA pathogenesis. Our results clearly revealed specific upregulation of ERRγ, among ERR isoforms, in OA cartilage of human patients and DMM-induced mouse OA cartilage. Moreover, in vivo studies using mouse models demonstrated the catabolic functions of ERRγ in OA pathogenesis. Because ERR-α and ERR-γ often display opposite effects in various physiological processes, such as epithelial-to-mesenchymal transition, Warburg effect, and value as a prognosis factor in breast tumors\textsuperscript{17,18,23}, we also examined possible ERRα functions. However, we found that ERRα expression was not modulated in OA cartilage of humans or mice. Furthermore, overexpression of ERRα in joint tissues via IA injection of Ad-Esrrα did not cause cartilage damage, indicating that ERRγ, but not ERRα, functions as a catabolic regulator of OA pathogenesis.

Among the interesting findings in the current study is the observed inhibition of IL-6-induced ERRγ upregulation in chondrocytes by treatment with the ERRγ inverse agonist GSK5182. This suggests possible autoregulation of ERRγ in chondrocytes. Indeed, we have previously demonstrated that ERRγ protein levels are decreased by GSK5182 treatment\textsuperscript{42}, which might reflect the operation of an autoregulatory feed-forward mechanism in the regulation of ERRγ gene expression\textsuperscript{43}. Similar to our observation, it has been shown that IL-1β-induced upregulation of ERRα in chondrocytes is reduced by the ERRα inverse agonist XCT790\textsuperscript{32}. This suggests that both ERRα and ERRγ expression in chondrocytes are regulated by positive feedback mechanisms. We have previously reported amplification of HIF-2α catabolic signaling in chondrocytes during OA pathogenesis. For instance, HIF-2α signaling leading to the production of the matrix-degrading enzymes, MMP3 and MMP13\textsuperscript{10}, is amplified by the HIF-2α targets, IL-6\textsuperscript{32} and NAMPT (visfatin)\textsuperscript{11}. Additionally, the NAMPT downstream target SIRT1 also stimulates HIF-2α transcriptional activity by stabilizing HIF-2α protein\textsuperscript{34}. Furthermore, two essential catabolic
signaling pathways in OA pathogenesis—HIF-2α and the zinc-ZIP8-MTF1 axis—reciprocally activate each other, resulting in amplification of catabolic signaling. Collectively, these observations suggest that catabolic signaling processes involved in OA pathogenesis are amplified upon onset of the disease.

The only study to date describing a role for ERRγ in cartilage is one by Cardelli et al.30, who reported that cartilage-specific overexpression of ERRγ results in chondroplasia and reduced chondrocyte proliferation. These authors found a reduction in the length of the femur and tibia in Tg mice, with no differences in intramembranous ossification. They additionally found that the overexpression of ERRγ impaired chondrocyte proliferation, leading to a decrease in the size of the proliferative zone in the growth plate40. However, our Tg mice exhibited no marked differences in the whole skeleton of embryos (E18.5) and length of resting/proliferative and hypertrophic zones in 2-week-old mice. Although it is not clear what accounts for these differences, they may be attributable to differences in expression systems46 and expression levels of target genes. We previously reported that cartilage-specific Dickkopf-1 Tg mice, generated using the same system as the current Tg mice, exhibit reduced experimental OA47 without differences in cartilage or bone development46. We also showed that cartilage-specific Tg mice generated by our system, including those overexpressing HIF-2α10, NAMPT11, BATF35, or ZIP812, exhibited enhanced experimental OA without detectable differences in skeletal development. In addition to the difference in expression system, we also found that the expression level of ERRγ protein in our Tg mice was less than that in mice generated by Cardelli et al.30. We postulate that these differences in the expression levels of ERRγ contribute to the observed differences. Nevertheless, the enhanced expression of matrix-degrading enzymes in cartilage of Tg mice compared with WT mice appears to be responsible for the enhanced DMM-induced OA pathogenesis in Tg mice.

The prevalence of OA is higher among women than men, and this prevalence increases after menopause. Various studies using animal models indicate that estrogen and estrogen signaling are associated with OA pathogenesis48. For instance, mice lacking estrogen receptor-α develop more and larger osteophytes and a thinner lateral subchondral plate49. A lack of estrogens also increases subchondral bone remodeling50. However, although the term “estrogen-related receptor (ERR)” reflects the molecular origin of the three family members, ERRs are functionally different from estrogen receptors. For instance, ERRs do not bind to natural estrogens, nor are they directly involved in classic estrogen-signaling pathways or biological processes18,24. Furthermore, ERRs and estrogen receptors display strict binding site specificities51, with ERRs exhibiting a marked preference for the sequence TCAAGGTCA, an ERR response element (ERRE). Furthermore, unlike estrogen receptors, ERRs do not require ligand binding for their transcriptional activity18,24. Therefore, the catabolic functions of ERRγ in OA pathogenesis might not be related to estrogen, even though estrogen is known to be associated with OA pathogenesis48.

Cartilage destruction is one of the most prominent characteristics of OA and is primarily caused by the upregulation of matrix-degrading enzymes, such as MMP3, MMP13, and ADAMTS5–8. These matrix-degrading enzymes originate from chondrocytes of cartilage tissue or from synovial cells, such as fibroblast-like synoviocytes (FLS)9. Our approaches using Esrrγ11−/− mice, shRNA-mediated silencing, and adenovirus-mediated overexpression affected ERRγ expression in all joint tissues, limiting our ability to identify specific joint tissues associated with OA pathogenesis. We observed, however, that ERRγ was upregulated in the cartilage tissue, but not the synovial tissue, of DMM-operated mice, and that ERRγ caused upregulation of MMP3 and MMP13 in primary cultured articular chondrocytes. These results strongly support the interpretation that ERRγ causes OA pathogenesis by upregulating MMP3 and MMP13 in articular chondrocytes. We also found that ERRγ downregulated SOX9, type II collagen, and aggrecan, although the degree of downregulation was small. Interestingly, compared with in vitro effects of ERRγ overexpression, ERRγ overexpression via IA injection of Ad-Esrrg in cartilage tissue caused marked decreases in the protein expression levels of SOX9 and type II collagen. The decrease in type II collagen might reflect its MMP-mediated degradation in ERRγ-overexpressing cartilage tissue. Thus, it may be possible that the reduction in SOX9 expression in Ad-Esrrg-injected cartilage is attributable to both direct and indirect actions of ERRγ. In addition to cartilage destruction, OA manifestations include osteophyte formation and subchondral bone sclerosis, reflecting the fact that OA is a whole-joint disease affecting all tissues of the joint, including cartilage, synovial tissues, and subchondral bone2. However, the relative contributions, relationships, and key tissues involved in each event that occurs during OA pathogenesis have not been elucidated2,3.

Methods

Human OA cartilage and experimental OA in mice. Human OA cartilage was sourced from individuals undergoing arthroplasty10–13. The Institutional Review Board of Wonkwang University Hospital approved the use of these materials, and all participants provided written informed consent before the operative procedure. C57Bl/6J mice were used for the experimental OA studies. Esrrγ+/- mice (B6.129P2-Esrrγtm1Dgen/Mmnc) were purchased from the Mutant Mouse Regional Resource Center (MMRRC). Col2a1-Esrrg Tg mice were generated using the Col2a1 promoter and enhancer, as previously described16–18,22. All experiments were approved by the Gwangju Institute of Science and Technology Animal Care and Use Committee. Experimental OA was induced in 10-week-old male mice by DMM surgery, or by IA injection (once weekly for 3 weeks) of adenovirus (1 × 109 plaque-forming units [PFUs] in a total volume of 10 μl) expressing ERRγ (Ad-Ergrp), ERRα (Ad-Esrα), ZIP8 (Ad-So3x/lac), or HIF-2α (Ad-Ep1a)19–22. Where indicated, male mice of various ages (8, 12, 16, and 20 weeks old) were used for experimental OA studies. Mice were killed at 8 weeks after DMM surgery or at 3 or 8 weeks after the first IA injection, and subjected to histological and biochemical analyses. Intraperitoneal injection of GSK5182 (20 mg/kg body weight) was performed every 2 days for 7 weeks, beginning 1 week after DMM surgery.

Histology and immunohistochemistry. Human OA cartilage was frozen, sectioned at a thickness of 5 μm, and fixed in paraformaldehyde. Sulfate proteoglycans were detected by alcian blue staining, and 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, and sections were deparaffinized in xylene, hydrated with graded ethanol, and stained with safranin-O. Cartilage degradation was scored by five observers under blinded conditions using the OARSI scoring system (grade 0–6)10–13,22. The results of OARSI grade scoring represent the mean of the maximum score in each mouse, and the representative safranin-O-
stained image was selected from the most advanced lesion among serial sections. The lengths of the resting/proliferative and hypertrophic zones were measured and expressed as fold-change relative to the indicated control. Significance was accepted at the 0.05 level of probability (P < 0.05).

**Data availability.** Microarray data have been deposited in Gene Expression Omnibus with the accession codes GSE104794 (for HIF-2α), GSE104795 (for ZIP8), and GSE104793 (for IL-1β). All other data supporting the findings of this study are available within the paper and its supplementary information files.

Received: 10 March 2017 Accepted: 20 October 2017
Published online: 15 December 2017

**References**

1. Hunter, D. J., Schofield, D. & Callander, E. The individual and socioeconomic impact of osteoarthritis. *Nat. Rev. Rheumatol.* 17, 437–441 (2014).

2. Loeser, R. F. et al. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum.* 64, 1697–1707 (2012).

3. Moon, P. M. & Beirer, F. Novel insights into osteoarthritis joint pathology from studies in mice. *Curr. Rheumatol. Rep.* 17, 50 (2015).

4. Bion, Q. et al. Osteoarthritis: genetic factors, animal models, mechanisms, and therapies. *Front. Biosci.* E4, 74–100 (2012).

5. Troebberg, L. & Nagase, H. Proteases involved in cartilage matrix degradation in osteoarthritis. *Biochem. Biophys. Acta.* 1824, 133–145 (2012).

6. Blom, A. B. et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis involvement of matrix metalloproteinase 3. *Arthritis Rheum.* 56, 147–157 (2007).

7. Little, C. B. et al. Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritis but not chondrocyte hypertrophy or osteophyte development. *Arthritis Rheum.* 60, 3723–3733 (2009).

8. Glasson, S. S. et al. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* 434, 644–648 (2005).

9. Kapoor, M. et al. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat. Rev. Rheumatol.* 7, 33–42 (2010).

10. Yang, S. et al. Hyoxida-inducible factor-2α is a catabolic regulator of osteoarthritic cartilage degradation. *Nat. Med.* 16, 687–693 (2010).

11. Yang, S. et al. NAMPT (visfatin), a direct target of hypoxia-inducible factor-2α, is an essential catabolic regulator of osteoarthritis. *Ann. Rheum. Dis.* 74, 595–602 (2015).

12. Kim, J. H. et al. Regulation of the cascades in osteoarthritis by the zinc finger protein FLZ4. *Sci. Rep.* 4, 3028 (2014).

13. Varma, A. K. et al. NAD+ metabolism in osteoarthritis. *Nat. Rev. Rheumatol.* 13, 302–311 (2017).
15. Niu, J. et al. Metabolic syndrome: its components, and knee osteoarthritis. Arthritis Rheumatol. 69, 1194–1203 (2017).

16. Courtes, A. et al. Metabolic syndrome–associated osteoarthritis. Curr. Opin. Rheumatol. 29, 214–222 (2017).

17. Misra, J., Kim, D. K. & Choi, H. S. ERR-γ: a junior orphan with a senior role in metabolism. Trends Endocrinol. Metab. 28, 261–272 (2017).

18. Audet-Walsh, E. & Giguere, V. The multiple universes of estrogen-related receptor-α and γ in the metabolic control and related disease. Acta Pharmacol. Sinica 36, 51–61 (2015).

19. Greschil, H. et al. Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor-α. Mol. Cell 9, 303–313 (2002).

20. Alaynick, W. A. et al. ERRγ directs and maintains the transition to oxidative metabolism in the postnatal heart. Cell Metab. 6, 13–24 (2007).

21. Wang, T. et al. Estrogen-related receptor α (ERRα) and ERRγ are essential coordinators of cardiac metabolism and function. Mol. Cell Biol. 35, 1281–1298 (2015).

22. Kim, D. K. et al. Inverse agonist of estrogen-related receptor gamma controls Salmonella typhimurium infection by modulating host iron homeostasis. Nat. Med. 20, 419–424 (2014).

23. Gan, Z. et al. Nuclear receptor/microRNA circuitry links muscle metabolism. J. Clin. Invest. 123, 2564–2575 (2013).

24. Huss, J. M., Garbacz, W. G. & Xie, W. Constitutive activities of estrogen-related receptors: transcriptional regulation of metabolism by the ERR pathways in health and disease. Biochim. Biophys. Acta. 1582, 1912–1927 (2005).

25. Debios, G. & Giguere, V. Functional and physiological genomics of estrogen-related receptors (ERRs) in health and disease. Biochim. Biophys. Acta. 1812, 1032–1040 (2010).

26. Bonneye, E. & Aubin, J. E. An energetic orphan is an endocrine tissue: a revised perspective of the function of estrogen-related receptor alpha in bone and cartilage. J. Bone Miner. Res. 28, 225–233 (2013).

27. Bonneye, E. et al. The orphan nuclear estrogen-receptor-related receptor-alpha regulates cartilage formation in vitro: implication of Sox9. Endocrinology 148, 1195–1205 (2007).

28. Chen, X. et al. Impact of estrogen-related receptor-α on the biological characteristics of rat mandibular condylar chondrocytes. Mol. Med. Rep. 10, 1952–1958 (2014).

29. Kim, Y. I. et al. Cartilage development requires the function of estrogen-related receptor alpha that directly regulates Sox9 expression in zebrafish. Sci. Rep. 5, 18011 (2015).

30. Cardelli, M. et al. Cartilage-specific overexpression of ERRγ results in chondrodysplasia and reduced chondrocyte proliferation. PloS ONE 8, e81852 (2013).

31. Bonneye, E. et al. Estrogen-related receptor-alpha is dysregulated in inflammatory arthritis. Rheumatology 47, 1785–1791 (2008).

32. Bonneye, E. et al. Estrogen receptor-related receptor α regulation by interleukin-1β in prostaglandin E2– and CAMP-dependent pathways in osteoarthritic chondrocytes. Arthritis Rheum. 63, 2374–2384 (2011).

33. Rya, J. H. et al. Interleukin-6 plays an essential role in hypoxygen-inducible factor-2α-induced experimental osteoarthritic cartilage destruction in mice. Arthritis Rheum. 63, 2732–2743 (2011).

34. Meydan, N. et al. Inhibition of acute lymphoblastic leukemia by a Jak-2 inhibitor. Nature 379, 645–648 (1996).

35. Rhee, J. et al. Inhibition of BATF/JUN transcriptional activity protects against osteoarthritic cartilage destruction. Ann. Rheum. Dis. 76, 427–434 (2017).

36. Glasson, S. S., Blanchet, T. J. & Morris, E. A. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SveEv mouse. Osteoarthritis Cartilage 15, 1061–1069 (2007).

37. Poole, R. et al. Recommendations for the use of preclinical models in the study and treatment of osteoarthritis. Osteoarthritis Cartilage 18, S10–S16 (2010).

38. van der Kraan, P. M. et al. Factors that influence outcome in experimental osteoarthritis. Osteoarthritis Cartilage 25, 369–375 (2017).

39. Cardelli, M. & Aubin, J. E. ERRγ is not required for skeletal development but is a RUNX2-dependent negative regulator of postnatal bone formation in mice. PloS ONE 9, e109592 (2014).

40. Dufour, C. R. et al. Genome-wide orchestration of cardiac functions by the orphan nuclear receptors ERRα and γ. Cell Metab. 5, 345–356 (2007).

41. Bonneye, E. et al. The orphan nuclear estrogen-receptor-related receptor α regulates cartilage formation in vitro: implication of Sox9. Endocrinology 148, 1195–1205 (2007).

42. Misra, J. et al. Orphan nuclear receptor ERRα induces CRE-active protein gene expression through induction of ER-bound Rиз transmembrane transcription factor CREBH. PLoS ONE 9, e86342 (2014).

43. Misra, J. et al. Transcriptional cross talk between orphan nuclear receptor ERRα and transmembrane transcription factor ATF6α coordinates endoplasmic reticulum stress response. Nucleic Acids Res. 41, 6960–6974 (2013).

44. Oh, H. et al. Reciprocal regulation by hypoxia-inducible factor-2α and the NAMPT-NAD⁺-SIRT axis in articular chondrocytes is involved in osteoarthritis. Osteoarthritis Cartilage 23, 2288–2296 (2015).

45. Lee, M. et al. Reciprocal activation of hypoxia-inducible factor (HIF)-2α and the zinc-ZIP-8-MTF1 axis amplifies catalytic signaling in osteoarthritis. Osteoarthritis Cartilage 24, 134–145 (2016).

46. Oh, H. et al. Misexpression of Dickkopf-1 in endothelial cells, but not in chondrocytes or hypertrophic chondrocytes, causes defects in endochondral ossification. J. Bone Miner. Res. 27, 1335–1344 (2012).

47. Oh, H. et al. Dlk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. Arthritis Rheum. 64, 2588–2578 (2012).

48. Martin-Millan, M. & Castaneda, S. Estrogen, osteoarthritis and inflammation. Joint Bone Spine 80, 368–373 (2013).

49. Snoekers, Y. H. et al. Development of osteoarthritic features in estrogen receptor knockout mice. Osteoarthritis Cartilage 17, 1356–1361 (2009).

50. Bellido, M. et al. Subchondral bone microstructural damage by increased remodeling aggravates experimental osteoarthritis preceded by osteoporosis. Arthritis Rheumatol. 12, R152 (2010).

51. Debios, G. et al. Genome-wide identification of direct target genes implicates estrogen-receptor-related receptor α as a determinant of breast cancer heterogeneity. Cancer Res. 69, 6149–6157 (2009).

52. Lotz, M. et al. Value of biomarkers in osteoarthritis: current status and perspectives. Ann. Rheum. Dis. 72, 1756–1763 (2013).

53. Glasson, S. S. et al. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis Cartilage 18, S17–S23 (2010).

54. Gosset, M. et al. Primary culture and phenotyping of murine chondrocytes. Nat. Protoc. 3, 1253–1260 (2008).