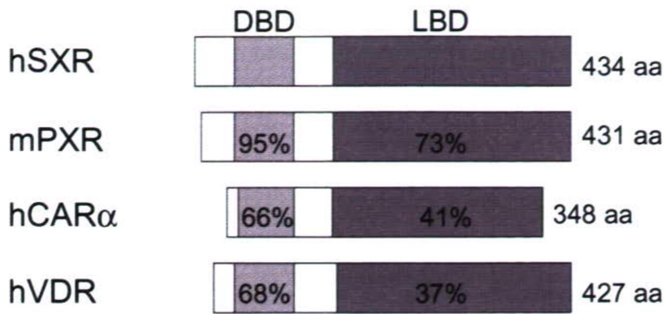

Fig. 1. Schematic comparisons among the nuclear receptor steroid and xenobiotic receptor (SXR) and its related receptors. All the receptors belong to nuclear receptor subfamily 1, group I (NR1I), and form heterodimers with their common partner retinoid X receptor (RXR). The similarity between SXR and other receptors is expressed as percent amino acid identity [1]. *DBD*, DNA-binding domain; *LBD*, ligand-binding domain; *hSXR*, human SXR; *mPXR*, mouse pregnane X receptor; *hCARα*, human constitutive androstane receptor-α; *hVDR*, human vitamin D receptor

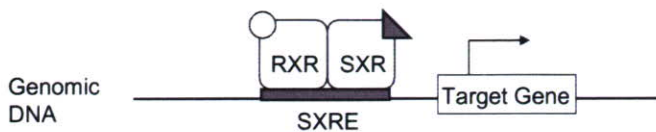

Fig. 2. Transcriptional regulatory mechanism of SXR. The ligand-activated SXR forms heterodimers with RXR and regulates the transcription of adjacent target genes by binding to SXR response elements (SXREs) in the genome

(UGT) 1A gene, and phase II drug transporters including multidrug resistance protein 1 are SXR-targeted drug-metabolizing enzymes, and they coordinately function in the catabolic processes [3].

Regarding SXR agonists, the antibiotic rifampicin, the calcium blocker nifedipine, and a constituent of St. John's wort, hyperforin, are known as prototypic human SXR ligands. As already described, there is a difference in ligand specificity between human SXR and its orthologue mouse PXR. For example, a potent agonist for human SXR rifampicin is not reactive to mouse PXR, whereas a synthetic steroid pregnenolone 16α-carbonitrile is an effective agonist for mouse PXR [2].

Physiological roles of SXR and vitamin K in bone metabolism

Recently, our group and collaborators have demonstrated that SXR is expressed in osteoblastic cells and that vitamin K_2 modulates the expression of bone-related genes via the SXR signaling pathway [4]. It has been revealed that vitamin K_2 activates SXR by binding to the LBD of SXR and that the active form of SXR promotes the transcription of a prototypic SXR target gene CYP3A4 by conjugating to the SXR in the regulatory region of CYP3A4 gene. Furthermore, it has been also shown that osteoprotegerin (OPG) and alkaline phosphatese (ALP), which are related to the maintenance of bone-building function, are induced in osteoblastic cells by vitamin K_2 and other SXR ligands such as rifampicin and hyperfolin. Because vitamin K_2-dependent induction of the OPG gene was not observed in osteoblasts derived from PXR-deficient mice, it has been suggested that this vitamin K_2-induced gene expression is mediated through SXR-dependent transcriptional regulation.

Vitamin K has been known as a critical cofactor of γ-glutamyl carboxylase (GGCX) as well as a potent stimulator of the bone-building process. Vitamin K_2, or menaquinones, is one of the natural forms of vitamin K derived from animal sources and the Japanese fermented soybean food, *natto*, as well as being produced by intestinal bacteria. Vitamin K_2 is particularly more active in both promoting bone formation and reducing bone loss compared with vitamin K_1, which is also a natural vitamin K originated from plants [5–9]. Thus, vitamin K_2 is clinically important for bone tissues and it is shown to be effective for osteoporosis treatment as well as the prevention of bone fractures [10,11]. Interestingly, we have previously shown that there was a significant inverse correlation between the incidence of hip fracture and *natto* intake in Japanese postmenopausal women. Serum MK-7 levels were much higher in Japanese women compared with British women and were significantly associated with *natto* consumption, suggesting that *natto* is a good resource of vitamin K_2 that reduces the risk of bone fracture [12].

In regard to the functions of vitamin K, the posttranslational modification of vitamin K-dependent proteins containing γ-carboxyglutamic acid (Gla) residues is the best known action, which is particularly important in blood coagulation [13]. In vitamin K-dependent carboxylation reactions, the reduced form of vitamin K deprotonates glutamate via the GGCX and the reduced vitamin K is converted to vitamin K epoxide. Osteocalcin (bone Gla protein, BGP) and matrix Gla protein (MGP) are known as bone-related Gla proteins that play roles in bone homeostasis. Osteocalcin is a protein that is only synthesized in osteoblasts and odontoblasts and thus it serves as a good biochemical marker of the metabolic turnover of bone. The expression of human and rat osteocalcins is positively regulated by 1,25-dihydroxyvitamin D_3 [14–17], whereas mouse osteocalcin genes OG1 and OG2 are indirectly downregulated by 1,25-dihydroxyvitamin D_3 [18]. Osteocalcin contains three Gla residues at positions 17, 21, and 24 of the amino acid sequence, which are responsible for its high affinity for hydroxyapatite, one of the major components of bone matrix [19,20]. Thus, the level of undercarboxylated osteocalcin reflects vitamin K function, and it has been reported to increase during aging and to significantly correlate with fracture risk [21]. On the other hand, MGP is predominantly expressed in chondrocytes and vascular smooth muscle cells. *Mgp*-deficient mice exhibited inappropriate calcification of various cartilages as well as arterial walls, indicating that MGP is a modulator of extracellular matrix mineralization [22,23]. As considered by the functions of these bone-related Gla proteins, it is plausible that one of the major vitamin K actions is the modulation of bone matrix mineralization. Yet, it has been speculated that vitamin K may

have another critical mechanism that is involved in the modulation of bone quality.

We have recently found a novel mechanism of vitamin K action in bone cells. Based on microarray analysis, we showed that vitamin K_2 regulated the transcription of genes encoding extracellular matrix (ECM) proteins in an SXR-dependent manner and increased the accumulation of collagen in osteoblastic cells [24]. In the experimental system of the human osteoblastic cell line MG63 that stably expresses SXR, ECM-related genes tsukushi (TSK) and matrilin-2 (MATN2) have been identified as genes inducible by vitamin K_2 and rifampicin. Tsukushi is a novel gene that has been found to belong to the small leucine-rich repeat (SLRR) proteoglycan family [25]. It has been shown that the SLRR family plays a critical role in bone formation, as significant bone phenotypes have been observed in mice deficient for biglycan and decorin, other known members of this family [26,27]. The matrilins form a four-member family of modular, multisubunit matrix proteins that are expressed in cartilage but also in many other forms of extracellular matrix. They participate in the formation of fibrillar or filamentous structures and are often associated with collagens [28]. The interaction between the SLRR proteins and matrilins has been reported previously as biglycan and decorin form complexes with collagen fibrils together with the matrilin-1 [29]. We generated MG63 cells stably expressing TSK and showed that collagen accumulation was significantly increased in the TSK-expressing cells compared with the control MG63 cells expressing empty vector. Vitamin K_2 itself augments collagen accumulation in MG63 cells, and this reaction can be repressed by gene knockdown using a small interfering RNA specific to TSK or SXR [24]. Moreover, we confirmed, by performing immunoprecipitation and immunohistochemistry studies, that TSK and MATN2 could colocalize and interact with each other on the cell surface. Taken together, our results demonstrate that SXR has a collagen-accumulating action in osteoblastic cells by regulating the transcription of ECM-related genes and suggest that vitamin K_2 potentially contributes to the improvement of bone quality using the SXR-dependent pathway (Fig. 3).

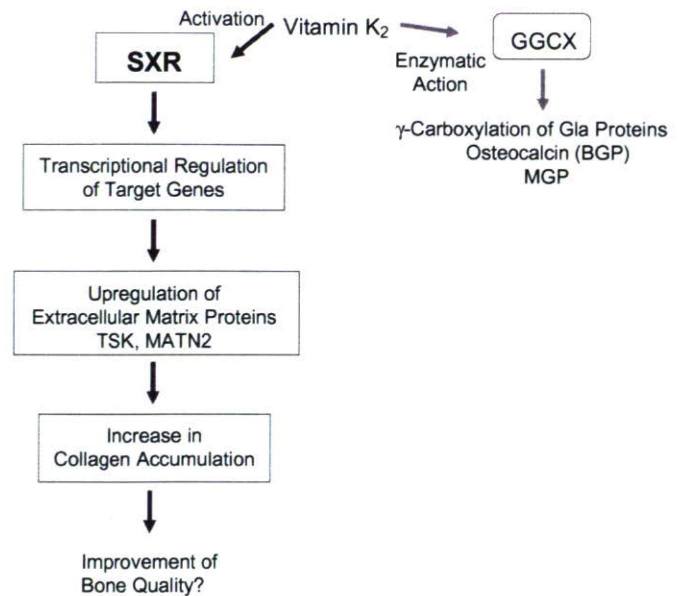

Fig. 3. SXR- and vitamin K_2-dependent regulatory mechanisms of bone metabolism in osteoblastic cells. SXR promotes collagen accumulation in osteoblastic cells by regulating the transcription of its target genes including those encode extracellular matrix proteins. Vitamin K_2 plays a role in the posttranslational modification of Gla proteins by functioning as a coenzyme of γ-glutamyl carboxylase (GGCX) and also acts as a potent SXR ligand in bone metabolism

SXR and its surrounding molecules

It remains to be studied how SXR exerts its physiological functions, including osteogenic differentiation and collagen accumulation in bone tissues. Although SXR seems to have a relatively low specificity for ligands compared with other nuclear receptors such as steroid hormone receptors and vitamin D receptor, it has also been reported that the types of SXR target genes as well as the cofactors involved in the transcriptional regulation may vary depending on the ligands [30]. In hepatocytes, it has been shown that the transcription of CYP3A4 gene is activated by SXR in collaboration with another transcriptional factor, hepatocyte nuclear factor 4α [31], suggesting that SXR signaling may be modulated by the interaction with other surrounding molecules such as nuclear receptors, transcriptional factors, and cofactors. Interestingly, SXR has been recently revealed to compete with the NF-κB signaling pathways that can be activated by inflammatory status [32]. This reciprocal repression between SXR and NF-κB is physiologically important as it is known that inflammatory stimuli suppress the expression of hepatic CYP genes and that various xenobiotics and SXR-responsive drugs exert immunosuppressant effects. Whether this mutual inhibitory mechanism of SXR and NF-κB is critical also in the bone system remains to be investigated. If the activation of SXR represses the NF-κB pathways in bones, some of the SXR agonists could be beneficial to suppress inflammation caused by bone fracture or osteoarthritis.

In conclusion, the effectiveness of vitamin K in bone metabolism has been known for years, but its molecular mechanisms have not been precisely clarified except the function related to γ-carboxylation. Recent advances in vitamin K study has revealed the novel signaling pathway of vitamin K that activates the transcriptional factor SXR and regulates its target genes including collagen-interacting genes. Further investigations of SXR and vitamin K mechanisms will provide new therapeutic options for the treatment of bone disorders including osteoporosis, osteoarthritis, and bone fracture.

Acknowledgments We thank Drs. Bruce Blumberg, Tomoe Ichikawa, and Kazuhiro Ikeda for their thoughtful discussion and collaboration. This work was supported in part by grants-in-aid from the Ministry of Health, Labor and Welfare; of the Genome Network Project and the Nuclear System to Decipher Operation Code Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan;

and from The Promotion and Mutual Aid Corporation for Private Schools of Japan.

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Spine (in press)

Association of a single nucleotide polymorphism in the insulin-like growth factor-1 receptor gene with spinal osteoarthritis in postmenopausal Japanese women

Tomohiko Urano, MD, PhD,*† Ken'ichiro Narusawa MD, PhD,‡ Masataka Shiraki MD, PhD,§ Takahiko Usui MD, PhD,*† Noriko Sasaki BS,† Takayuki Hosoi MD, PhD,¶ Yasuyoshi Ouchi MD, PhD,* Toshitaka Nakamura MD, PhD,‡ Satoshi Inoue MD, PhD, *†ʃ

From the *Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, †Department of Coca-Cola Anti-Aging Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, ‡Department of Orthopedic Surgery, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan, §Research Institute and Practice for Involutional Diseases, Nagano, Japan ¶Department of Advanced Medicine, National Center for Geriatrics and Gerontology, Aichi, Japan, and ʃResearch Center for Genomic Medicine, Saitama Medical School, Saitama, Japan

Address correspondence and reprint requests to Satoshi Inoue, M.D., Ph.D., Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1, Hongo, Bumkyo-ku, Tokyo, Japan. Phone: +81-3-5800-8652, Fax: +81-3-5800-6530, E-mail: INOUE-GER@h.u-tokyo.ac.jp

Acknowledgments

This work was partly supported by grants from the Japanese Ministry of Health, Labor, Welfare and the Ministry of Culture, Education, Sports, Science and Technology of Japan. This work was also partly supported by a grant of the Japan Osteoporosis Foundation.

STUDY DESIGN. An association study investigating the genetic etiology for spinal osteoarthritis.

OBJECTIVE. To determine the association of single-nucleotide polymorphism (SNP) in the insulin-like growth factor-1 receptor (IGF1R) with spinal osteoarthritis.

SUMMARY OF BACKGROUND DATA. Insulin-like growth factor-1 (IGF-1) signaling pathway is involved in cartilage development and homeostasis, suggesting that genetic variations of genes involved in this pathway may affect the pathogenesis of cartilage-related diseases, such as osteoarthritis.

METHODS. We evaluated the presence of endplate sclerosis, osteophytes, and narrowing of disc spaces in 434 Japanese postmenopausal women. A SNP in the IGF1R gene at intron 1 was determined using TaqMan polymerase chain reaction (PCR) method.

RESULTS. We compared those who carried the G allele (GG or GC, n=290) with those who did not (CC, n=144). We found that the subjects with the G allele (GG or GC) were significantly over-represented in the subjects having higher disc narrowing score (P=0.0033; odds ratio, 2.04; 95% confidence interval, 1.27-3.29 by logistic regression analysis).

CONCLUSIONS. We suggest that a genetic variation at the *IGF1R* gene locus is associated with spinal osteoarthritis, in line with the involvement of the *IGF1R* gene in the cartilage metabolism.

Key Words: Single-nucleotide polymorphism (SNP), insulin-like growth factor 1 receptor, spinal osteoarthritis, disc narrowing

Key Points

Insulin-like growth factor 1 (IGF-1) signaling pathway regulates cartilage metabolism.

The single-nucleotide polymorphism in insulin-like growth factor-1 receptor (IGF1R) gene at intron 1 was associated with spinal disc degeneration in Japanese postmenopausal women.

We suggest that a genetic variation at the IGF1R gene locus is associated with spinal osteoarthritis.

Mini Abstract

Insulin-like growth factor 1 (IGF-1) and its receptor regulate cartilage metabolism. The single nucleotide polymorphism in insulin-like growth factor 1 receptor gene at intorn 1 was associated with spinal disc narrowing in Japanese postmenopausal women. We suggest that a genetic variation at the IGF1R gene locus is associated with spinal osteoarthritis.

Introduction

Osteoarthritis of the spine is a very common condition in the axial skeletons of aged people [1]. Vertebral osteophytes, endplate sclerosis and intervertebral disc narrowing are recognized as characteristic features of spinal degeneration. Recent studies indicate that the appearance of these radiographical features is influenced by genetic factors, physical loading and other environmental factors [2,3]. Genetic association studies using various definitions of osteoarthritis have been performed, mainly investigating genes encoding structural proteins of the extracellular matrix of cartilage (e.g. collagen type II α1, cartilage matrix protein, and aminoguanidine) or genes playing a role in the regulation of bone density and mass (e.g. vitamin D receptor and estrogen receptor α) [4,5]. Many studies have shown that the Tt and the tt genotypes of Taq I polymorphism of the vitamin D receptor gene was associated with disc degeneration in various population [5 citation].

The insulin-like growth factor 1 (IGF-1) is a small 70-amino acid polypeptide mediator with a potent anabolic impact on cartilage homeostasis [6]. IGF-1 is expressed in cartilage, where it can act in a paracrine and autocrine manner to stimulate cartilage matrix synthesis as well as inhibit matrix degradation [7]. IGF-1 stimulates the production of both proteoglycans and collagen, the two major constituents of the cartilage matrix, and it also stimulates production of integrins, which are cell receptors that bind extracellular matrix proteins and are important for tissue repair [8, 9]. An age related decline in the ability of IGF-1 to stimulate chondrocytes to produce articular matrix components has also been demonstrated [10]. Recent study reported that chronic growth hormone and IGF-1 deficiency caused an increased severity of articular cartilage lesions of osteoarthritis [11]. Moreover, two reports have shown that a polymorphism in the promoter region of the IGF-1 gene associated with an increased prevalence of radiographical osteoarthritis in the subset data on the Rotterdam study [12, 13]. These reports have highlighted the importance of IGF-1 in promoting cartilage growth and development, implying a potential role of IGF-1 in the etiology of osteoarthritis. The first step in IGF-1 activation of chondrocytes is interaction with insulin-like growth factor 1 receptor (IGF1R), which cause auto-phosphorylation of the receptor intracellular β-subunit, triggering phosphorylation of downstream signal molecules, and initiating an array of anabolic effects. Thus, it is assumed that IGF1R modulates IGF-1 signaling pathway in the cartilage homeostasis. In the present study, we examine an association between a polymorphism in the IGF1R gene and radiographic feautures of spinal osteoarthritis including osteophyte formation, endplate sclerosis and disc space narrowing number to investigate a possible contribution of IGF1R to human cartilage metabolism.

Materials and methods

Subjects.

Genotypes were analyzed in DNA sample obtained from 434 healthy postmenopausal Japanese women (mean age + SD; 66.5 + 8.4) living in central area of Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin sodium), or unusual gynecologic history. Patients with severe hip and knee arthritis were excluded from the present study. The eligibility of subjects was determined by taking history-physical examination. All were non-related volunteers and provided informed consent before this study. Ethical approval for the study was obtained from appropriate ethics committees.

Radiographic grading of osteoarthritis of the spine.

Conventional thoracic and lumbar spinal plain roentgenograms in lateral and anteroposterior projection were obtained from all participants. The severities of spinal degeneration including osteophyte formation, endplate sclerosis and disc space narrowing were assessed semi-quantitatively from Th4/5 to L4/5 disc level or from Th4 to L4 vertebrae by using the grading scale of Genant [14]. Then we assessed radiographical spinal osteoarthritis using scoring system as previously reported [15]. Briefly, osteophyte formation at a given disc was graded 0-3 degrees, endplate sclerosis at given vertebra was graded 0-2 degrees, and disc space narrowing was graded 0-1 degrees. Then we defined sum of each degree from Th4/5 to L4/5 disc level for osteophyte formation on anteroposterior radiographs as a score of osteophyte formation. We also defined sum of each degree from Th4 to L4 vertebra for endplate sclerosis and that from Th4/5 to L4/5 disc level for disc space narrowing on lateral radiographs as a score of endplate sclerosis and disc narrowing, respectively.

Determination of a single nucleotide polymorphism in the IGF1R gene.

We extracted a polymorphic variation in the IGF1R gene intron 1 region from the Assays-on-Demand SNP Genotyping Products database (Applied Biosystems, Foster City, CA) and, according to its localization on the gene, denoted it IVS1+14488C>G (rs11247361). We determined the IVS1+14488C>G polymorphism of the IGF1R gene using the TaqMan (Applied Biosystems) polymerase chain reaction (PCR) method [16]. To determine the IGF1R SNP, we used Assays-on-Demand SNP Genotyping Products C_11527385_10 (Applied BioSystems), which contains sequence-specific forward and reverse primers and two TaqMan MGB probes for detecting alleles. TaqMan PCR method utilizes two kinds of TaqMan probes that correspond to a DNA fragment including the target SNP site with different alleles and the 5'-3' nuclease activity of Taq polymerase that is essential for PCR. TaqMan probes include fluorescence dyes at their 5' ends and a quencher at their 3' ends. During PCR cycles, TaqMan probes will anneal

to target DNA and will be excised by the 5'-3' nuclease activity of Taq polymerase if there is no mismatch between the probes and target sequences. Then the fluorescence dyes will be released from the probes and the intensity of fluorescence can be monitered by using ABI PRISM 7000 (Applied Biosystems) as a fluorescence detector. The allele frequencies of IVS1+14488C>G polymorphism were confirmed as they were not significantly deviated from Hardy-Weinberg equilibrium. Since Hardy-Weinberg equilibrium is based on the following assumptions including no genetic drift, no gene flow, no natural selection, negligible mutations, and random mating, the population under the equilibrium is not evolving and its genotype and allele frequencies are predicted to remain unchanged over successive generations. Thus, we considered that our subjects were eligible for the correlation study.

Statistical analysis.

Age, height, body weight, body mass index (BMI) and osteoarthritis parameters (number of osteophyte, endoplate sclerosis and disc narrowing) in the groups of subjects classified by the IGF1R SNP genotypes were compared by ANOVA and Kruscal-Wallis test. Stepwise regression analysis was carried out to assess the independent effect of 4 variables (age, height, body weight, IGF1R SNP genotypes) on disc narrowing score. We also divided subjects into those having one or two allele(s) of the minor G allele (GC+GG) and those with only the major C allele (CC) encoded at the same locus. Multivariate logistic regression was used to estimate odds ratios and 95% confidence intervals (95% CIs) for these two groups and the risk of disc narrowing. Analyses for the association of IGF1R genotypes and radiographic spinal endplate sclerosis were performed with adjustment for age. *P* values less than 0.05 were considered significant. Analysis was performed using StatView-J 4.5 software (SAS Institute Inc., Cary, NC).

Results

We analyzed the genotypes for the SNP of IGF1R gene at intron 1 (IVS1+14488C>G, rs11247361) in 434 subjects, using the TaqMan method. Among these postmenopausal Japanese women, 144 were CC homozygotes, 229 were GC heterozygotes, and 61 were GG homozygotes (Table 1). The allelic frequencies of this SNP in the present study were in Hardy-Weinberg equilibrium.

The background data (age, height, body weight, BMI) were not statistically different among these groups (Table 1). On Kruskal-Wallis analysis, we found significant associations between IGF1R genotype and disc narrowing score (Fig 1, Table1, P=0.0051). On ANOVA analysis, we also found significant associations between IGF1R genotype and disc narrowing score (Table1, P=0.015). On the other hand, the occurrence of endplate sclerosis and osteophyte formation did not significantly differ among those SNP genotypes (Table 1).

Recent studies have shown that the physical and constitutional factors contribute to spinal osteoarthritis. Therefore, we carried out stepwise regression analysis to assess the independent effect of age, height, body weight, and IGF1R SNP genotypes on disc narrowing score. Among these factors, age and IGF1R SNP genotypes correlated significantly with spinal disc narrowing score (Table 2). The standard regression coefficients were 0.291 for age and 0.110 for IGF1R SNP genotypes.

We finally analyzed the association between the alleic frequency of IGF1R SNP genotypes and disc narrowing score after stratification by age. In these analyses, we divided subjects into two groups, those who carried the G allele (GG or GC, n=290) and with those who did not (CC, n=144). We found that the subjects with the G allele (GG or GC) were significantly over-represented in the subjects having two or more disc narrowing score compared in the subjects having no or one disc narrowing after age-adjusted (P=0.0042; odds ratio 1.84; 95% confidence interval 1.21-2.79 by logistic regression analysis). We also found that the subjects with the genotype GG or GC were significantly over-represented in the subjects having higher (three or more) disc narrowing score compared in the subjects having lower (no, one or two) disc narrowing score after age-adjusted (P=0.0033; odds ratio 2.04; 95% confidence interval 1.27-3.28 by logistic regression analysis). Thus, we suggest that a genetic variation at the IGF1R gene locus is associated with spinal osteoarthritis, especially with disc narrowing, independently with background parameters.

Discussion

The present study is the first report that shows the influence of a single-nucleotide polymorphism of IGF1R gene on spinal osteoarthritis as far as we know. Targeting the pathogenesis of low back pain, we have previously investigated associations of genetic factors with osteoporosis. Our group and several other groups have reported that the IGF-1 gene polymorphism was correlated with bone mineral density and risk of fracture [17-21]. Spinal osteoarthritis is another major reason for low back pain and IGF-1 polymorphism has been shown to be associated with osteoarthritis [12, 13]. Thus, we have extended our study to the association of a polymorphism in the IGF1R gene, the receptor of IGF-1 signaling, with spinal osteoarthritis. We demonstrated that Japanese postmenopausal women who had one or two allele(s) of G allele in the IGF1R gene at intron 1 showed significantly higher disc narrowing score of spine. These data suggest that IGF-1 signaling-related genes may affect the pathogenesis of osteoarthritis. Our finding is in line with the genome-wide scan for osteoarthritis-susceptibility loci that showed a linkage to chromosome 15q21.3-26.1 [22], which includes the IGF1R gene locus on 15q25-q26.

Recently, mutations of the IGF1R gene have been described to be associated with

both intrauterine and postnatal growth retardation [23]. This suggests that the variant of IGF1R gene may have an important role in the pathogenesis of the human diseases. Actually, some reports have shown that the SNPs in the IGF1R gene were associated with human diseases. Common polymorphism in exon16 of the IGF1R gene was reported [Abu-Amero S, Preece M, Wakeling E, et al. A common polymorphism in exon 16 of the human insulin-like growth factor-1 receptor gene. Mol. Cellular Probes. 1997,11, 381-383]. Bonafe et al. studied a polymorphism 1013G>A presented in the exon 16, codon 1013, whose variant is predicted to generate no change of amino acid, E1013E [24]. This polymorphism has been reported to be more represented among people with a long lifespan [24]. Moreover, this 1043G>A polymorphism was associated with vascular dementia [25].

Osteoarthritis occurs as results of both mechanical and biological events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix as well as subchondral bone [1, 26]. Cartilage destruction during osteoarthritis involves the loss of differentiated phenotype and apoptotic death of chondrocytes [27]. IGF-1 has been shown to regulate differentiation, mainteinance of the differentiated phenotype, and apoptosis of articular chondrocytes [28-30]. One of the essential pathomechanisms of age-related degeneration of intervertebral discs is a loss of proteoglycan, which account for the reversible resistance to the compressive force of intervertebral discs [31, 32]. IGF-1 is a peptide known to activate matrix metabolism, particularly proteoglycan synthesis [33-35]. It was also reported that the expression and downstream signaling of IGF1R in cultured nucleus pulposus cells from aged rat interval discs decreased compared with in the cells from young rat interval discs, suggesting that a loss of proteoglycan synthesis during the late stage of aging is caused by the down regulation of IGF-1 and IGF1R pathway [36]. The interaction of IGF-1 and IGF1R is modulated by a set of high-affinity binding proteins, IGF binding proteins (IGFBPs) 1-6 (Le Roith et al. 2001). IGFBPs 2-5 have been detected in articular cartilage from multiple species (Olney et al. 1993, Tardif et al. 1997). Increased levels of IGFBPs, particularly IGFBP-3, in arthritic cartilage most likely contribute to IGF-1 hyposensitiveness (Chevalier and Tyler, 1996, Olney et al. 1996, Tardif et al. 1996, Morales 2002). Taken together, our results and the recent reports suggest that the IGF-1, IGFBPs and IGF1R signaling pathway is critical role in the pathogenesis of cartilage diseases, such as osteoarthritis and disc degeneration. The intronic IVS1+14488C>G polymorphism may influence the IGF1 and IGFBPs signaling by alteration in gene transcription or mRNA stability. Further studies will be required to clarify the role of this variant of the IGF1R gene in the pathogenesis of disc space narrowing.

In this study, we have evaluated not only disc space narrowing but also endplate screlosis and osteophyte formation. Among them, only disc space narrowing was associated

with IGF1R polymorphism. We have previously shown that the LRP5 polymorphism was associated with osteophyte formation and the WISP1 polymorphism was associated with ebdplate screlosis among these parameters (Urano et al. Spine 2007, Urano et al. J. Bone Miner. Metab. 2007). These data suggest that these 3 parameters of disk degeneration may have each genetic background in some part independently. Based on our data, the IGF1R polymorphism has specific effect on the disk space homeostasis in spine. It is important to study the IGF1R polymorphism in patients with disc space narrowing in other tissues, especially with hip and knee osteoarthritis.

LRP5 is a co-receptor of Wnt signaling pathway and its polymorphism associate with spinal osteophyte formation. Why the SNP of the IGF1R gene influence the disc narrowing scores while the SNP of the LRP5 gene associate with the osteophyte formation scores? It will be important to study how signals from Wnt and IGF-1 can be delivered to the regulation of this machinery.

Conclusion

We have shown an association of the SNP in the IGF1R gene at intron 1 with a radiographic feauture of spinal disc narrowing in postmenopausal Japanese women. The women with G allele had significantly higher disc narrowing scores. The IGF1R genotyping could be beneficial in the prevention and management of spinal disc degeneration. The present findings regarding the correlation of IGF1R polymorphism with spinal osteoarthritis provide a new promising direction for the clinical medicine of the spine disease, which leads us to the development of new diagnostic markers as well as therapeutic options based on the molecular target.

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Table 1.

Comparison of background and clinical characteristics among subjects with single nucleotide polymorphism (SNP) genotypes (GG genotype, GC genotype and CC genotype) in the IGF1R gene at intron 1.

Items	Genotype (mean ± SD)			*P* value	*P* value
	CC	GC	GG	(ANOVA)	(Kruskal-Wallis)
Number of subjects	144	229	61		
Age (years)	65.0 ± 9.2	66.3 ± 8.5	67.1 ± 10.6	NS	NS
Height (cm)	150.8 ± 6.1	150.1 ± 6.3	150.3 ± 6.1	NS	NS
Body weight (kg)	50.6 ± 7.4	49.5 ± 8.1	50.6 ± 8.7	NS	NS
BMI	22.3 ± 2.8	21.8 ± 3.0	22.6 ± 3.2	NS	NS
Endplate sclerosis	0.40 ± 0.86	0.35 ± 0.83	0.43 ± 1.04	NS	NS
Osteophyte	5.69 ± 3.68	5.31 ± 3.44	6.10 ± 3.58	NS	NS
Disc narrowing	1.63 ± 1.70	2.13 ± 1.82	2.26 ± 1.89	0.015	0.0051

BMI; body mass index, NS; not significant

Table 2.

Results of stepwise regression analysis of four factors for disc narrowing score.

Factors	*F* value			s.r.c.
	Step 0	Step 1	Step 2	Step2 (R^2=0.103)
Intercept	523	11.7	13.2	-2.323
IGF1R SNP genotypes (CC=0, GC, GG=1)			5.7	0.110
Age		43.1	40.4	0.291
Weight		not selected		
Height		not selected		

s.r.c.; standard regression coefficient

Table 3.

Association of the IGF1R SNP genotype (GG and GC (n=290) vs. CC (n=144)) in the subjects with disc narrowing score after stratifying age.

Severity of disc narrowing	OR	95%CI	*P* value
One or more disc narrowing (n=342) versus no disc narrowing (n=92)	1.36	0.83-2.23	0.21
Two or more disc narrowing (n=223) versus less (≤1) disc narrowing (n=211)	1.84	1.21-2.79	0.0042
Three or more disc narrowing (n=140) versus less (≤2) disc narrowing (n=294)	2.04	1.27-3.29	0.0033

OR; odds ratio, 95%CI; 95% confidence intervals

Figure 1.

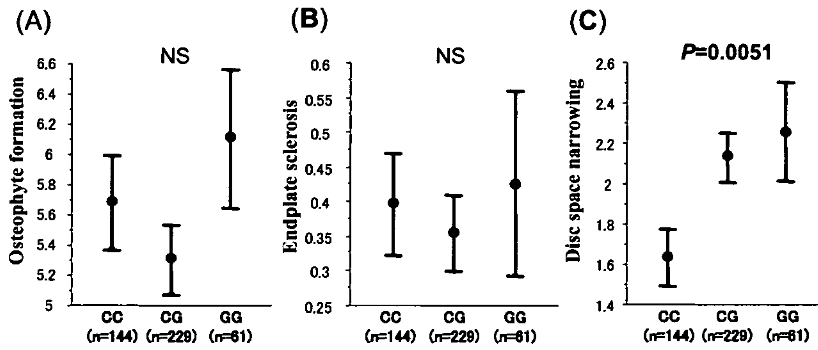

Figure 1. Scores of spinal osteoarthritis between the single nucleotide polymorphism (SNP) genotypes (CC genotype, CG genotype, GG genotype) in the IGF1R gene at intron1. A, Scores of osteophyte formation are shown for CC, CG and GG genotype. Scores are expressed as mean $\pm$ SE. Number of subjects are shown in parentheses. B, Scores of endplate sclerosis. C, Disc space narrowing scores. The association of the three genotype groups with osteoarthritis parameters was determined by Kruskal-Wallis test.

LETTERS

Dioxin receptor is a ligand-dependent E3 ubiquitin ligase

Fumiaki Ohtake[1,2], Atsushi Baba[2], Ichiro Takada[2], Maiko Okada[2], Kei Iwasaki[1], Hiromi Miki[2], Sayuri Takahashi[2,3], Alexander Kouzmenko[1,2], Keiko Nohara[4], Tomoki Chiba[5], Yoshiaki Fujii-Kuriyama[6,7] & Shigeaki Kato[1,2]

Fat-soluble ligands, including sex steroid hormones and environmental toxins, activate ligand-dependent DNA-sequence-specific transcriptional factors that transduce signals through target-gene-selective transcriptional regulation[1]. However, the mechanisms of cellular perception of fat-soluble ligand signals through other target-selective systems remain unclear. The ubiquitin–proteasome system regulates selective protein degradation, in which the E3 ubiquitin ligases determine target specificity[2–4]. Here we characterize a fat-soluble ligand-dependent ubiquitin ligase complex in human cell lines, in which dioxin receptor (AhR)[5–9] is integrated as a component of a novel cullin 4B ubiquitin ligase complex, CUL4B^{AhR}. Complex assembly and ubiquitin ligase activity of CUL4B^{AhR} *in vitro* and *in vivo* are dependent on the AhR ligand. In the CUL4B^{AhR} complex, ligand-activated AhR acts as a substrate-specific adaptor component that targets sex steroid receptors for degradation. Thus, our findings uncover a function for AhR as an atypical component of the ubiquitin ligase complex and demonstrate a non-genomic signalling pathway in which fat-soluble ligands regulate target-protein-selective degradation through a ubiquitin ligase complex.

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. This target selectivity depends on the recognition of specific DNA elements by sequence-specific transcription factors[1] and the recognition of degradation substrates by E3 ubiquitin ligases[2–4]. These transcription factors and ligases serve primarily as specific adaptors that subsequently recruit transcriptional co-regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. The selective biological effects of fat-soluble ligands have been reported to be mediated by two classes of sequence-specific transcription factors, nuclear receptors[1] and arylhydrocarbon receptor (AhR) belonging to the basic helix–loop–helix (bHLH)/Per-Arnt-Sim (PAS) family[5–9].

AhR ligands modulate oestrogen and sex hormone, signalling both positively and negatively[8,10–13]. Functional impairments of male and female reproductive organs in AhR-deficient mice indicate the possible importance of AhR in sex hormone signalling[10,14]. Different AhR agonists[9], including 3-methylcholanthrene (3MC) and 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), modulate oestrogen-dependent oestrogen receptor (ER)-α transactivation through the association of activated AhR/Arnt with ER-α[15]. Similarly, the transcriptional activity of nuclear androgen receptor (AR) was modulated by association with activated AhR (Supplementary Fig. S2a). However, ligand-bound AhR did not block oestrogen-induced co-activator recruitment on the oestrogen-responsive promoter (Supplementary Fig. S2b). This implies another mode of function for ligand-activated AhR beyond transcriptional regulation.

On activation of AhR by 3MC, we observed that protein levels of endogenous ER-α (in mammary tumour MCF-7 cells), ER-β (in ovarian tumour KGN cells) and AR (in prostate cancer LNCaP cells) were drastically decreased (Fig. 1a–c, and Supplementary Fig. 3a) without a change in messenger RNA levels (data not shown), irrespective of the presence of their cognate hormones. Other AhR agonists[9] (namely β-naphthoflavone (β-NF), environmental toxins such as TCDD and benzo[a]pyrene, and the endogenous metabolite indirubin) were similarly effective in protein degradation for ER-α (Fig. 1b) and ER-β/AR (data not shown), in agreement with a previous report on downregulated levels of uterine ER-α protein in rats treated with TCDD[16]. An AhR partial agonist/antagonist α-naphthoflavone (α-NF) was unable to accelerate the degradation of either AhR or ER-α (Fig. 1b, and Supplementary Fig. S3b).

AhR ligand-induced degradation (Fig. 1a–c) and functional repression (Supplementary Fig. S2c, d) of sex steroid receptors were abrogated in the presence of a proteasome inhibitor MG132. Consistently, poly-ubiquitination of ER-α was promoted by the activated AhR regardless of the presence of oestrogen (Fig. 1d, and Supplementary Fig. S3c). Pulse-chase kinetic analysis indicated that 3MC-induced degradation of ER-α was coupled to that of AhR[8,17,18] (Supplementary Fig. S3d). Moreover, the self-ubiquitination activity of the ligand-bound AhR immunocomplex was detected in an E1/E2-dependent manner (Supplementary Fig. S3e). Together with 3MC-dependent recognition of sex steroid receptors by AhR[8,12,13,15], these properties of AhR resemble those of classical adaptor components of the E3 ubiquitin ligase complexes, such as F-box proteins[3] or von Hippel–Lindau protein[19]. We therefore reasoned that activated AhR might act as an E3 ubiquitin ligase complex component.

To address this idea, AhR-containing complexes were purified from HeLa cells expressing Flag–AhR treated with 3MC or α-NF[15,20]. AhR formed large complexes in the presence of 3MC (Supplementary Fig. S4a–c). Further purification revealed five major 3MC-dependent complexes containing AhR (Fig. 1e). Complexes A and C contained well-known co-activators TRAP220/DRIP205/Med220 and p300 (ref. 1) (Supplementary Fig. S4d, e). Endogenous ER-α was detected in complexes B and C; however, ubiquitinated components were seen only in complex B (Fig. 1f, g).

Complex B was composed of the ubiquitin ligase core components cullin 4B (CUL4B)[3,21,22], damaged-DNA-binding protein 1 (DDB1)[23–27] and Rbx1 (Roc1)[3], together with subunits of the proteasomal 19S regulatory particle (19S RP), Arnt and transducin-β-like 3 (TBL3) (Fig. 1h). These components eluted with AhR in the presence of 3MC but not in the presence of α-NF (Fig. 1i, and Supplementary Fig. S4f). Neither CUL4A nor known substrate-specific adaptor components of CUL4A, such as DDB2, CSA and DET1[23,24], were present

[1]ERATO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan. [2]Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. [3]Department of Urology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, 113-8655, Japan. [4]National Institute for Environmental Studies, Tsukuba, Ibaraki 305-8506, Japan. [5]Graduate School of Life and Environmental Sciences, and [6]TARA Center, University of Tsukuba, 1-1-1 Tennodai Tsukuba, 305-8577, Japan. [7]SORST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan.

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in the AhR–CUL4B complex. As the cullin amino terminus binds adaptor components and the carboxy terminus interacts with an E2 enzyme-binding subunit Rbx1 (ref. 3), we performed tandem purification of the AhR–CUL4B complex with glutathione S-transferase (GST)-tagged CUL4B-N (N-terminal domain of CUL4B) and Flag–AhR. This led to the identification of a core complex consisting of five components: DDB1, AhR, Arnt, TBL3 and CUL4B (Fig. 1j). Together with Rbx1, this complex is denoted by $CUL4B^{AhR}$.

Immunoprecipitation of AhR together with endogenous CUL4B from MCF-7 and LNCaP cells was observed only in the presence of 3MC (Fig. 2a, b). Consistently, ligand-dependent co-localization of AhR with CUL4 was seen in MCF-7 cells (Fig. 2c). Whereas CUL4B seemed to act as a scaffold mediating DDB1–TBL3 and AhR–DDB1 interactions in $CUL4B^{AhR}$ (Fig. 2d, lane 4), ligand-activated AhR induced the assembly of complex components (Fig. 2d, lanes 1–3). DDB1 did not bridge CUL4B association with TBL3 or AhR, apparently because of the absence of the signature WDXR/DWD box[22,25–27] of either TBL3 or AhR, which is essential for DDB1 binding (Fig. 2d, lane 5, and Supplementary Fig. S5a). Consistently, specific and 3MC-dependent interaction of the conserved C-terminal acidic domain of AhR with the N-terminal region of CUL4B, but not with DDB1, was observed in a GST pulldown assay (Supplementary Figs S5b and S6). Because a constitutively active AhR mutant (AhRΔPASB)[9] interacted with CUL4B in the absence of ligand (Supplementary Fig. S5b), ligand-dependent structural alteration presumably induces AhR–CUL4B interaction. An AhR mutant lacking the CUL4B-binding

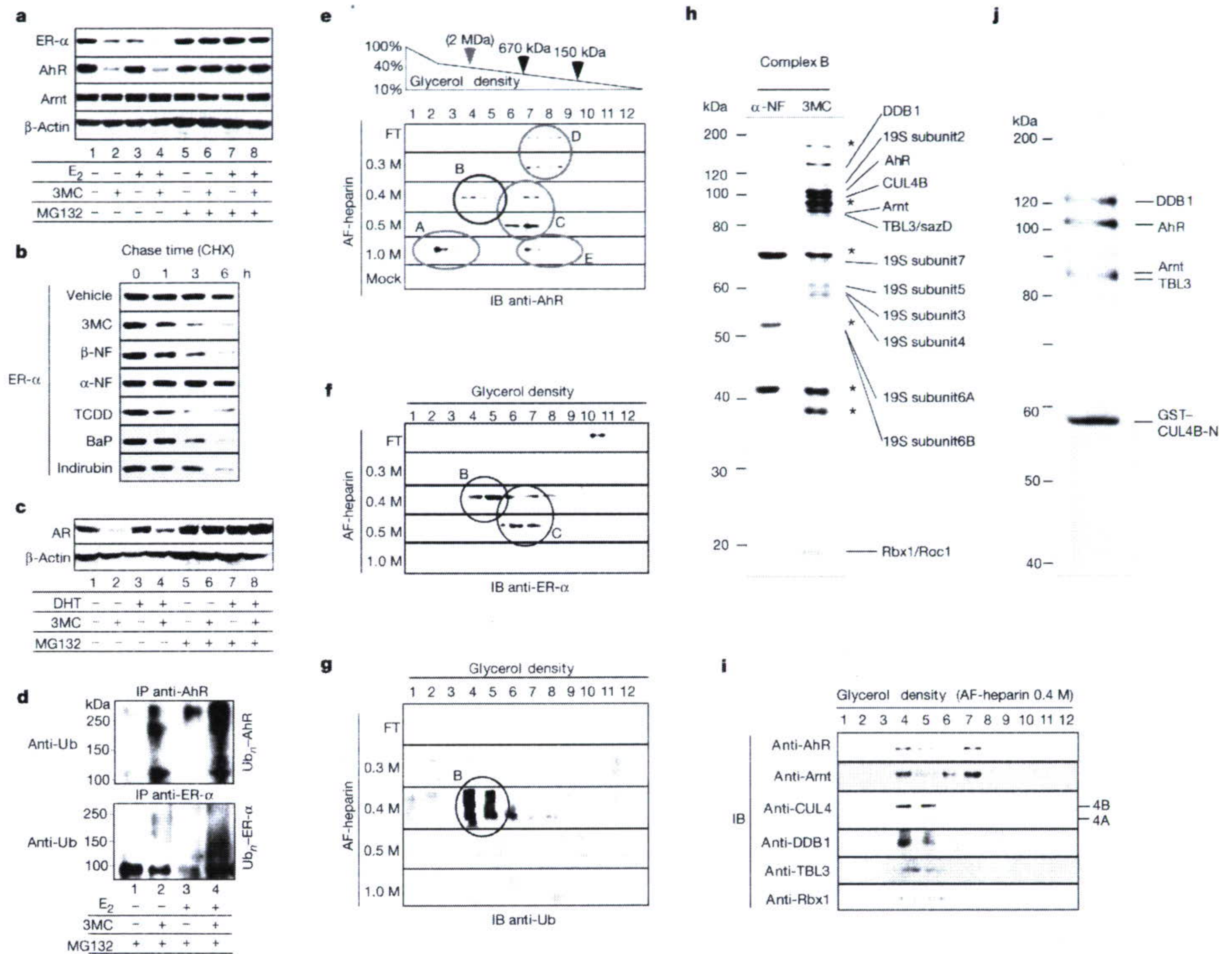

Figure 1 | Activated AhR acts as an E3 ubiquitin ligase. a–c, AhR-ligand-induced proteasomal degradation of ER-α (**a, b**) and AR (**c**). MCF-7 cells (**a, b**) and LNCaP cells (**c**) were incubated as indicated with E_2 (10 nM), DHT (10 nM) and/or 3MC (1 μM), β-NF (1 μM), benzo[a]pyrene (BaP; 100 nM), TCDD (10 nM), indirubin (10 nM) and α-NF (1 μM) in the presence or absence of MG132 (10 μM) and cycloheximide (CHX; 5 μM) for 3 h (**a, c**) or the indicated durations (**b**). Cell lysates were subjected to western blotting with specified antibodies. **d,** AhR-ligand-induced ubiquitination of ER-α. MCF-7 cells were incubated with the indicated ligands for 6 h. Western blots were subjected to dark exposure to detect poly-ubiquitinated forms of the receptors. IP, immunoprecipitation; Ub, ubiquitin. **e, f,** Biochemical separation and identification of AhR-associated complexes. Flag–AhR-associated proteins in the presence of 3MC or α-NF from HeLa cells stably expressing Flag–AhR were first fractionated by glycerol-density-gradient centrifugation (top, fractions 1–12), and then separated by Toyopearl AF-heparin column chromatography with the indicated KCl concentrations (FT, 1.0 M KCl). Samples from the 3MC-treated cells were resolved into five distinct complexes. IB, immunoblotting. **g,** Components of an AhR-associated complex are highly ubiquitinated. Western blots with anti-ubiquitin antibody. **h,** Identification of AhR-associated CUL4B ubiquitin ligase complex components. Components from complex B in **e** (fractions 4 and 5 from the glycerol-density-gradient centrifugation, eluted from an AF-heparin column at 0.4 M KCl) were resolved by SDS–PAGE, silver-stained and identified by matrix-assisted laser desorption ionization–time-of-flight MS analysis. **i,** Co-elution of the complex B components as a large complex. **j,** Association of activated AhR with the CUL4B complex. The $CUL4B^{AhR}$ complex from Flag–AhR-expressing HeLa cells treated with 3MC was affinity purified with GST-tagged N-terminal domain of CUL4B followed by anti-Flag antibody column fractionation.

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acidic domain (AhRΔacid; Supplementary Fig. S6a) was indeed unable to promote ER-α ubiquitination *in vivo*, although the mutant retained 3MC-dependent transactivation function (Supplementary Fig. S5c). This indicates that the ubiquitin ligase function of AhR is independent of its transactivation function.

With two separately prepared components of recombinant AhR and CUL4B/DDB1/Rbx1 purified from *Spodoptera frugiperda* (Sf9) cells (Supplementary Fig. S7a), complex assembly *in vitro* was also dependent on 3MC (Fig. 2e). Furthermore, by *in vitro* ubiquitination assay (Supplementary Fig. S7b), the E3 ubiquitin ligase activity of $CUL4B^{AhR}$ for ER-α was dependent on 3MC but not on 17β-oestradiol (E_2) (Fig. 2f). These data indicate that both the complex assembly and the ubiquitin ligase activity of $CUL4B^{AhR}$ may be dependent on AhR agonists.

We then examined whether the recognition of sex steroid receptors for 3MC-dependent ubiquitination is indeed mediated by AhR. Co-immunoprecipitation analyses indicated that ligand-activated AhR was required for the recruitment of ER-α (Fig. 2a, d) or AR (Fig. 2b, and data not shown) to $CUL4B^{AhR}$. TBL3 and DDB1 did not seem essential for ER-α recruitment but stabilized the association of ER-α with $CUL4B^{AhR}$ (Fig. 2d). Moreover, knockdown of $CUL4B^{AhR}$ components (Supplementary Fig. S8) impaired the 3MC-induced ubiquitination and degradation of ER-α (Fig. 3a–d, and Supplementary Fig. S9a, b) and AR (Fig. 3e, Supplementary Fig. S9c and data not shown), and abolished the AhR-ligand-induced repression of ER-α transactivation (Supplementary Fig. S10a). Recognition of ER-α by activated AhR was retained, but ubiquitination of AhR-bound ER-α was abrogated, by knockdown of the other $CUL4B^{AhR}$ components (Fig. 3d). An ER-α ΔA/B mutant[15] that lacks interaction with AhR, and an ER-α K7R mutant in which seven lysine residues had been replaced with arginine (Supplementary Fig. S6b), were resistant to AhR-dependent ubiquitination and transrepression (Fig. 3f, and Supplementary Fig. S10b). Taken together, these data suggest that ligand-activated AhR functions as a substrate-specific adaptor component of $CUL4B^{AhR}$. AhR is therefore a unique and atypical substrate-specific component of a cullin-based E3 complex, because AhR bears no known interaction motif with cullin complexes yet associates directly with CUL4B. Ubiquitination of ER-α-associated AhR was similarly abolished by the knockdown, and the overall ubiquitination and degradation of AhR[8,17,18] were partly affected (Supplementary Fig. S11a, b). This implies the existence of $CUL4B^{AhR}$-dependent (self-ubiquitination[3]) and $CUL4B^{AhR}$-independent pathways for AhR degradation.

Human ER-α (hER-α) degradation is reportedly accelerated by the binding of E_2 (ref. 1) or the phosphorylation of Ser 118 (ref. 28), whereas a partial antagonist, tamoxifen, has been shown to stabilize ER-α[1]. Nevertheless, 3MC-activated AhR efficiently induced the ubiquitination and subsequent degradation of tamoxifen-bound ER-α and ER-α-S118A mutant (Fig. 3f). Reciprocally, AhR was dispensable for E_2-dependent ER-α degradation (Supplementary Fig. S11c). These results indicate that the $CUL4B^{AhR}$ system may act independently of innate protein degradation system(s) for ER-α. XAP2/ARA9/AIP[7,8,17], a chaperone that modulates the stability of unliganded AhR, seemed unlikely to mediate the accelerated degradation of ER-α by activated AhR (Supplementary Fig. S11d).

Last, we addressed the physiological significance of $CUL4B^{AhR}$ for sex hormone signalling in intact animals. Injection with either 3MC (Fig. 4a) or β-NF (Fig. 4c) did not affect the expression of ER-α or AR mRNA (data not shown) but caused a decrease in protein levels of uterine ER-α in ovariectomized female wild-type mice and of prostate AR in castrated male wild-type mice (Fig. 4b) regardless of their treatment with cognate sex hormones. However, AhR deficiency ($AhR^{-/-}$ mice)[9,14] abolished such effects of AhR ligands but did not affect the modulation of stability of sex steroid receptors by their respective hormones (Fig. 4a, b). As a result of reduced sex steroid receptor levels after pretreatment with 3MC, E_2-dependent induction of *c-fos* in the uterus[15] and dihydrotestosterone (DHT)-dependent induction of *Probasin* in the prostate[10] were severely impaired (Fig. 4a, b). Cellular proliferation and gene induction in response to sex hormones in primary cultured epithelial cells from normal mouse uterus and prostate were consistently suppressed by 3MC (Supplementary Fig. S12a, b) and β-NF (Supplementary Fig. S12c), but no effect was detected in $AhR^{-/-}$ cells (Supplementary Fig. S12a, b). The significance of $CUL4B^{AhR}$ complex components in the AhR-mediated suppression of sex hormone effects

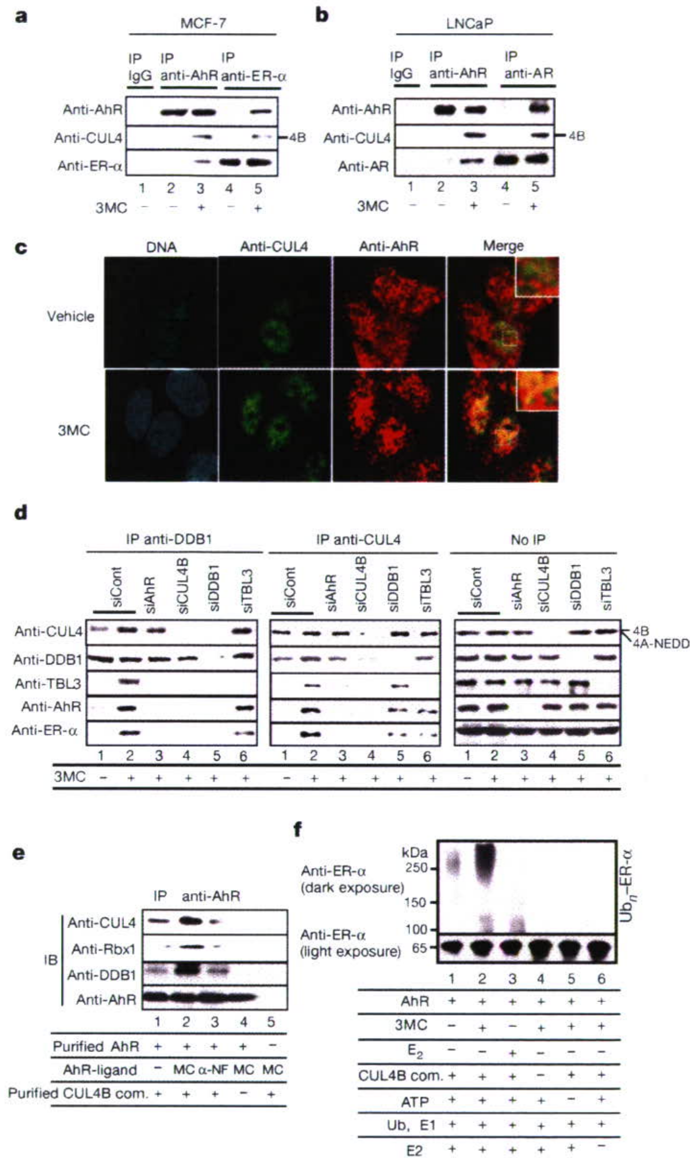

Figure 2 | AhR ligand-dependent assembly and ubiquitin ligase activity of $CUL4B^{AhR}$. a, b, 3MC-dependent association of endogenous CUL4B and AhR with ER-α and AR. Co-immunoprecipitation analyses from MCF-7 (**a**) and LNCaP (**b**) cells incubated with ligand and MG132 for 2 h. IP, immunoprecipitation. **c,** 3MC-dependent co-localization of AhR with CUL4. MCF-7 cells incubated with 3MC and MG132 for 2 h were immunostained with the indicated antibodies. **d,** Formation of the $CUL4B^{AhR}$ complex. MCF-7 cells were transfected with specified short interfering RNAs (siRNAs) for 48 h, treated with 3MC and MG132 for 2 h, and immunoprecipitated with the indicated antibodies. **e,** Assembly of the CUL4B complex components with AhR is dependent on 3MC *in vitro*. Immunoprecipitation with anti-AhR antibodies of the indicated recombinant CUL4B complex components (CUL4B com.) was observed only in the presence of 3MC. IB, immunoblotting. **f,** $CUL4B^{AhR}$ ubiquitinates ER-α *in vitro*. ER-α protein was incubated with and without recombinant $CUL4B^{AhR}$ E3 complex components, ubiquitin (Ub), ATP, E1 and E2 enzymes as indicated, then subjected to western blotting.

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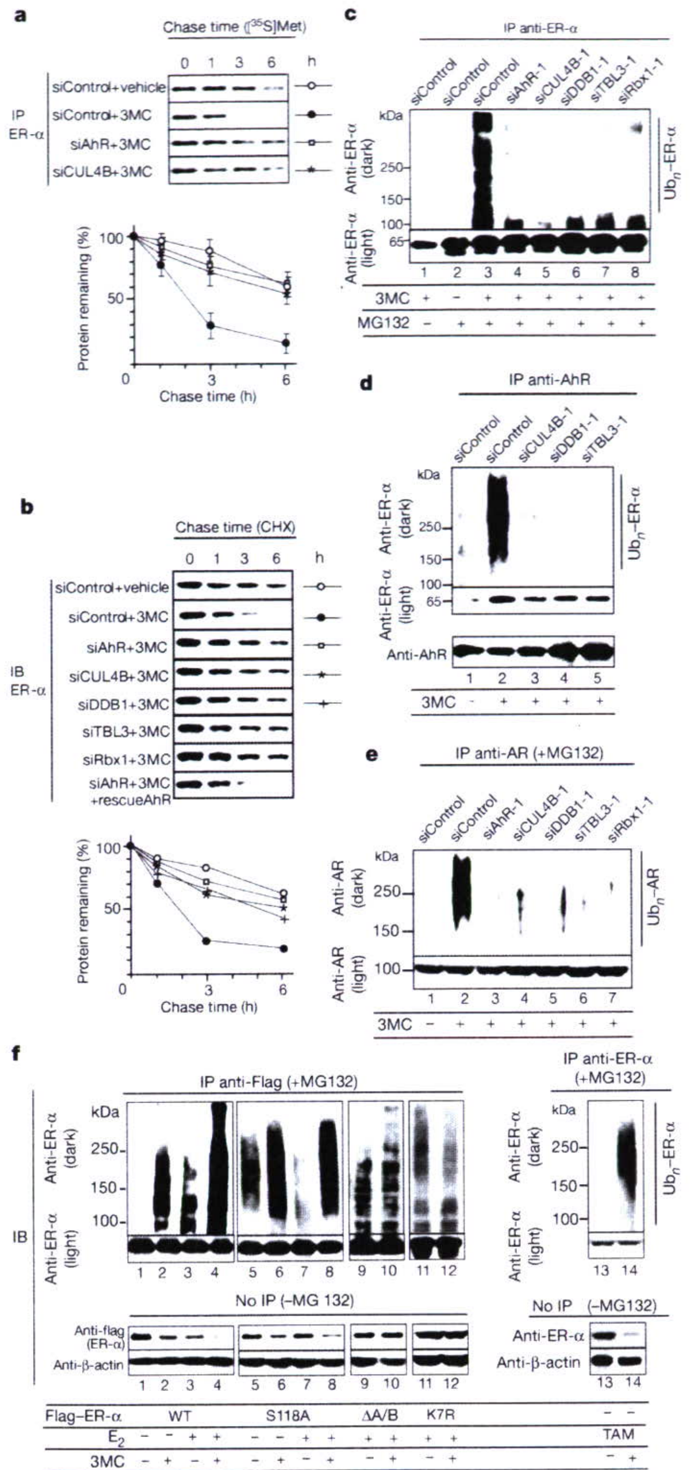

Figure 3 | Activated AhR is a substrate-specific adaptor component of the CUL4B^{AhR} complex. a–c, Components of CUL4B^{AhR} are required for 3MC-dependent ubiquitination and degradation of ER-α. MCF-7 cells were transfected with indicated siRNAs for 48 h, then used in pulse-chase analysis as in Supplementary Fig. S3d (**a**), in cycloheximide (CHX) chasing (**b**) and in the *in vivo* ubiquitination assay with ligand incubation for 6 h (**c**). All values are shown as means ± s.d. ($n = 3$) (**a**) or as means ($n = 3$) (**b**). The knockdown efficiency in the same lysates was confirmed in Supplementary Fig. S9a. IB, immunoblotting; IP, immunoprecipitation. **d**, AhR is the substrate-specific adaptor in the targeting of ER-α by CUL4B^{AhR}. MCF-7 cells transfected with the indicated siRNAs were lysed in TNE buffer and immunoprecipitated with anti-AhR antibody in the presence of MG132. Ubiquitination of the ER-α co-immunoprecipitated with AhR was detected by western blotting. **e**, LNCaP cells were subjected to the same analysis as in **a–c**. **f**, AhR-ligand-induced ER-α ubiquitination requires intact lysine residues and is independent of oestrogen binding or S118 phosphorylation of hER-α. Intact MCF-7 cells (right) or cells transfected with Flag–hER-α, AhR and their derivatives (left) were treated with the indicated ligands in the presence (top) or absence (bottom) of MG132 for 6 h, then subjected to western blotting. TAM, tamoxifen; WT, wild type.

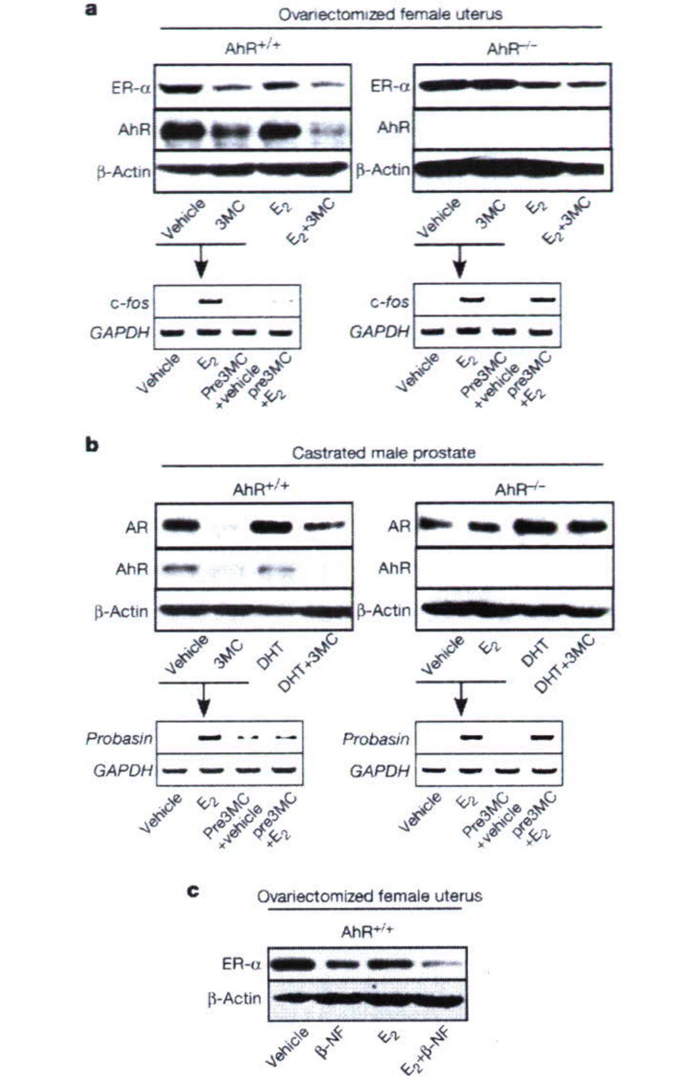

Figure 4 | Ligand-dependent ubiquitin ligase function of AhR *in vivo*. a, b, AhR activation enhances the degradation of ER-α and AR *in vivo*. Top: nine-week-old ovariectomized female mice (**a**) or castrated male mice (**b**) of the indicated genotypes were injected with vehicle or indicated ligands. After 4 h, uterus (**a**) or ventral prostate (**b**) was isolated and subjected to western blotting. Bottom: mice pretreated with vehicle or 3MC for 8 h were injected with either vehicle or E_2 (**a**), or DHT (**b**). After 4 h, the uterus or prostate was isolated for reverse transcriptase PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **c**, Other AhR agonists produce a similar effect on oestrogen signalling to that of 3MC.

(Supplementary Fig. S12a, b) and the promotion of ER-α degradation in uterine cells (Supplementary Fig. S12d) was verified by knockdown of the components.

Here we have shown that a known sequence-specific transcription factor AhR acts as a ligand-dependent CUL4B-based E3 ubiquitin ligase for selectively targeting sex steroid receptors to bring about accelerated protein degradation. The transcription and ubiquitination functions of AhR seem to be responsible for a distinct set of biological events caused by endogenous and exogenous AhR ligands. In ubiquitin ligase complexes, substrate recognition by known

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substrate-specific components is generally evoked by substrate modifications[2–4]. However, the recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type compounds as ligands but does not require the phosphorylation or ligand binding of sex steroid receptors. We have therefore shown that fat-soluble ligands directly control the function of a ubiquitin ligase complex for targeted protein destruction in animals (see Supplementary Fig. S1). In plants, auxin was recently found to control protein destruction through the auxin receptor SCF^{TIR1} (refs 29, 30). However, whereas SCF^{TIR1} is regulated by ligand-dependent substrate recognition by TIR1, $CUL4B^{AhR}$ is primarily regulated by the assembly of a ligand-dependent complex as well as substrate recognition. Considered together, ubiquitin-ligase-based perception mechanisms of fat-soluble ligands may be diverse in different species. It is possible that other nuclear receptors and binding proteins for fat-soluble ligands also serve as key components of ubiquitin ligases to mediate a non-genomic pathway of fat-soluble ligands to regulate target-protein-selective destruction.

METHODS

More detailed descriptions of all materials and methods are supplied in the Supplementary Information.

Biochemical purification and separation of AhR-associated complexes. The nuclear extracts preparation, anti-Flag affinity purification and mass spectrometry were performed as described previously[15,20]. For purification of the core $CUL4B^{AhR}$ complex, the nuclear extracts were first bound to the GST–CUL4B-N (amino acid residues 1–318) columns before being loaded on anti-Flag columns[20].

***In vitro* ubiquitination assay.** The *in vitro* ubiquitination assay was performed as described previously[23]. Purified Flag–AhR (0.2 µg) was incubated either with 3MC (10 µM) or vehicle (dimethylsulphoxide) for 30 min at 25 °C, then mixed with Flag–CUL4B/DDB1/Rbx1 complex (0.2 µg), and after further incubation for 30 min at 25 °C the substrate, ER-α (Calbiochem), was added.

Plasmids, antibodies, immunoprecipitation, *in vivo* ubiquitination, pulse-chasing, ligand responses in mice, and RNA-mediated interference experiments. Detailed methods used in this study can be found in the Supplementary Information.

Received 13 December 2006; accepted 16 February 2007.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank K. Tanaka, C. K. Glass, J. Yanagisawa, Y. Gotoh and J. Mimura for comments; S. Murata, T. Matsuda, T. Suzuki and Y. Tateishi for providing materials; T. Matsumoto, M. Igarashi and S. Fujiyama for technical assistance; and H. Higuchi for manuscript preparation. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and priority areas from the Ministry of Education, Culture, Sports, Science and Technology (to Y.F.-K. and S.K.).

Author Contributions F.O., T.C., Y.F.-K. and S.K. designed the experiments. F.O., A B., M.O., K I., H.M., S.T. and I. T. performed the experiments. F.O., A.K. and S.K. wrote the paper.

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