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GRIP1 enhances estrogen receptor α-dependent extracellular matrix gene expression in chondrogenic cells

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SUMMARY

Objective: The role of postmenopause on the pathogenesis of cartilage degeneration has been an open question. We assessed cartilage degeneration in estrogen receptor (ER)α null mice and examined the role of glucocorticoid receptor-interacting protein 1 (GRIP1) in the ERα-dependent transcription of a type II collagen gene (*col2a1*) with special reference to a crosstalk with the transforming growth factor (TGF)-β signaling pathway.

Methods: The vertebral cartilaginous endplate from female ERα null mice was subjected to histological analyses. *Col2a1* expression of primary chondrocytes (PCs) obtained from ERα null mice after 17β-estradiol (E_2) and TGF-β1 stimulation was examined by reverse transcription polymerase chain reaction (RT-PCR). Estrogen response element (ERE) or *col2a1* promoter–enhancer luciferase reporter system was used to investigate the crosstalk among ERα, GRIP1, and MKK6. *Col2a1* expression and glycosaminoglycan (GAG) content were measured in ATDC5 cells treated with GRIP1 small interfering RNA (siRNA).

Results: ERα deficiency clearly accelerated impairment of the vertebral cartilaginous endplate. E_2 and TGF-β1 stimulation increased *col2a1* expression in PC from wild-type mice, but not that from ERα null mice. The same stimulation increased the *col2a1* promoter–enhancer reporter activity, and the elevated activity was decreased by dominant-negative ERα and p38 mitogen-activated protein kinase (MAPK) inhibitor. GRIP1 increased the E_2-dependent ERE activation in the presence of ERα and constitutive-active MKK6. GRIP1 siRNA repressed *col2a1* expression and GAG production in ATDC5 cells.

Conclusions: Crosstalks between ERα/GRIP1 and TGF-β/MKK6/p38 MAPK pathway have protective roles on cartilage metabolism *via* regulating the extracellular matrices expression. The finding may lead to the development of a novel therapeutic approach for cartilage degeneration.

Introduction

Endogenous estrogen depletion is believed to contribute to several health problems through women's life. Several epidemiological studies suggested that the postmenopausal state is involved with the development of some gender-specific diseases such as osteoarthritis and lumbar degenerative spondylolisthesis[1,2]. These diseases are attributed to cartilage damage in the knee joint, the vertebral endplate, and the intervertebral disc of the lumbar spine. The epidemiological gap of the incidence of diseases between females and males continues to increase with aging[3]. Although the presence of estrogen receptors (ERs) in chondrocytes raises a possibility that cartilaginous tissues are estrogen-sensitive, the precise mechanism of cartilage homeostasis by estrogen is not fully understood[4].

Estrogen, especially 17β-estradiol (E_2) has versatile effects on physiology of chondrocytes from proliferation to differentiation[5,6]. In fact, aged ERα null mice show an early closure of the epiphyseal growth plate in long bones[7]. ERα exerts its function by binding to the estrogen response elements (EREs) in the promoter region of the target genes, recruiting many cofactors to form a large multi-subunit complex, which defines the transcription of the genes[8]. Glucocorticoid receptor-interacting protein 1 (GRIP1) is one of the

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p160 steroid receptor coactivator (SRC) gene family that interacts with nuclear receptors[9]. Interaction of GRIP1 with E_2-bound ERα recruits histone acetyl-transferase and cAMP response element-binding (CREB) binding protein (CBP)/p300, and leads to form a large coactivator complex. Through phosphorylation of the coactivator complex, some of the signaling cascades are known to promote target gene expressions. p38 mitogen-activated protein kinase (MAPK), a member of MAPK signaling pathway, is reported to stimulate the ERα-dependent ERE transcription by phosphorylation of S736 of GRIP1 in breast and endometrial carcinoma cells[10].

A crosstalk between ERα and p38 MAPK enhances ERα-dependent tumor growth in MCF-7 cells[11]. In ATDC5 cells, a transcriptional crosstalk among Sma and mothers against decapentaplegic (MAD)-related protein 2 (Smad2), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 MAPK regulates aggrecan gene expression[12]. One of the signal transduction modulators in the transforming growth factor (TGF)-β signaling, p38 MAPK, plays an important role for proliferation of chondrocytes[13]. TGF-β rapidly activates p38 MAPK and its direct activator mitogen-activated protein kinase kinase (MKK) 6 in its transcriptional activation in mouse mammary gland cell[14]. However, it remains to be determined whether estrogen affects MKK6-p38 MAPK pathways in chondrocytic cells.

Type II collagen is a major extracellular matrix component of the articular cartilage and has crucial roles in cartilaginous homeostasis. Transcription of *col2a1* is mainly controlled by sex-determining region Y (SRY)-type high mobility group box9 (Sox9)[15,16]. Sox9 binds to the enhancer region of *col2a1* gene, which is located in the first intron, and forms a large transcriptional complex with cofactors such as CBP/p300[17]. The transcriptional complex is believed to bend backward and make a contact with the general transcriptional machinery, especially RNA polymerase at a translation initiation site leading to chondrocyte-specific expression of *col2a1*[18]. TGF-β1 controls the Sox9-mediated *col2a1* transcription by Smad-dependent[19] and -independent pathways[20]. Both the TGF-β/Smad3 and TGF-β/MKK6/p38 MAPK pathways result in the formation of Sox9-containing transcriptional complex. In addition, Sox9 is a post-transcriptional target of the p38 MAPK pathway in chondrocytes. Indeed, p38 MAPK is involved in both superinduction of Sox9 by cycloheximide and stabilization of Sox9 mRNA[21].

Here, we verified a hypothesis that the postmenopausal estrogen depletion accelerates cartilage degradation by examining the phenotypes of ERα null mice. We present evidence that estrogen increases *col2a1* transcription through the TGF−MKK6 pathway in primary chondrocytes (PCs) and chondrogenic ATDC5 cells. Furthermore, we revealed the function of GRIP1 in ERα-dependent *col2a1* expression and glycosaminoglycan (GAG) production in ATDC5 cells. The results obtained in this study manifest the role of estrogen in the maintenance of cartilage homeostasis.

Materials and methods

Plasmids

A DNA fragment containing 1 kb human *col2a1* promoter or a *col2a1*-4 × 48 bp enhancer and basal promoter (−164 to 37 bp) was generated by PCR using genomic DNA from HeLa cells. The fragments were cloned into pGL3-basic (Promega, Madison, WI). The constructs were verified by DNA sequencing. Human ERα and dominant-negative (DN) ERα plasmids were gifts from Dr. D.P. McDonnell[22]. ERE-TATA-luc was from Dr. J. Yanagisawa[23]. MKK6CA was from Dr. T. Sudo[24]. Sox9 and p300 were gifts from Dr. H. Asahara[17]. GRIP1 and GRIP1-ΔAD1 (full-length GRIP1 without amino acids 1057–1109) plasmids were from Dr. M. Stallcup[25]. Enhanced green fluorescent protein (EGFP)-GRIP1 was from Dr. G.L. Hager[26].

Histological stainings

Heterozygous ERα null mice in C57BL/6 background were purchased from Taconic (Hudson, NY). The animals were kept under pathogen-free conditions and cared for in accordance with the Laboratory Animal Care and Use Committee of School of Medicine Keio University. The dissected lumbar units of wild-type (WT) and ERα null female mice were fixed in 10% neutral buffered formalin. The samples were decalcified by 5% formic acid, paraffin-embedded, and sectioned to 4 μm thickness. The decalcified sections were stained with alcian blue and type II collagen with rabbit anti-mouse polyclonal antibody (Millipore, Billerica, MA) as previously described[27].

Cell culture and treatments

PCs of WT and ERα null mice were prepared from newborn and E18.5 embryos in pregnant heterozygous ERα null mice as described[28]. PCs were plated in 24-well dish at a density of 1×10^5 cells in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM)/F12 and 10% fetal bovine serum (FBS). Next day, the medium was replaced by a fresh medium supplemented with 2% Charcoal/Dextran treated FBS (HyClone, Waltham, MA), 10^{-7} M E_2 and 10 ng/ml TGF-β1. After 16 h, RNA was recovered from chondrocytes with RNAiso (Takara Bio, Shiga, Japan) for reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR

Total RNA was used for the first strand cDNA synthesis with SuperScript II (Invitrogen, Carlsbad, CA). Real-time RT-PCR amplification was carried out using a SYBR Premix Ex Taq (Takara Bio) in a Light Cycler Quick System (Roche Diagnostics, Mannheim, Germany). Relative mRNA expression levels were obtained by normalization against 18S rRNA. The experiments were repeated at least three times to confirm the reproducibility. The primers for real-time RT-PCR used were: mouse 18S rRNA 5′-ATCAGATACCG TCGTAGTTCC-3′ and 5′-TGCCCTTCCGTCAATTCC-3′; mouse *col2a1* 5′-ACTGGTAAGTGGGGCAAGAC-3′ and 5′-CCACACCAAATTCCTGT TCA-3′. RT-PCR was performed with TaKaRa Ex Taq (Takara Bio). The primers used were: mouse β-actin 5′-GGCCCAGAGCAAGAGA GGTATCC-3′ and 5′-ACGCACGATTTCCCTCTCAGC-3′; mouse *col2a1* 5′-GCCAAGACCTGAAACTCTGC-3′ and 5′-GCCATAGCTGAAGTGGA AGC-3′; mouse GRIP1 5′-CCCATGCAAGATCCAAACTT-3′ and 5′-AGG CCTCAGAGTCAGGTTCA-3′.

Immunofluorescence

ATDC5 cells (5×10^4 cells) were plated on a glass coverslip in a 24-well plate and cultured in DMEM/F12 with 10% FBS. Thirty minutes later, the cells were transfected with 800 ng of EGFP-GRIP1 using FuGENE HD (Roche Diagnostics). Next day, the cells were cultured with serum-free DMEM/F12 for 24 h. Then, cells were incubated with 10^{-7} M E_2 in 2% Charcoal/Dextran treated FBS for 16 h. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.5% NP-40 in PBS and incubated with rabbit anti-rat ERα. Confocal images were obtained by Olympus IX71 (Olympus Corp., Tokyo, Japan).

Luciferase reporter assay

ATDC5 cells were maintained in DMEM/F12 containing 5% FBS and antibiotics. Cells were plated at 7500 cells in a 48-well plate and transfected with a 50 ng luciferase reporter plasmid containing a human *col2a1* promoter, which harbors a 4 × 48 enhancer element,

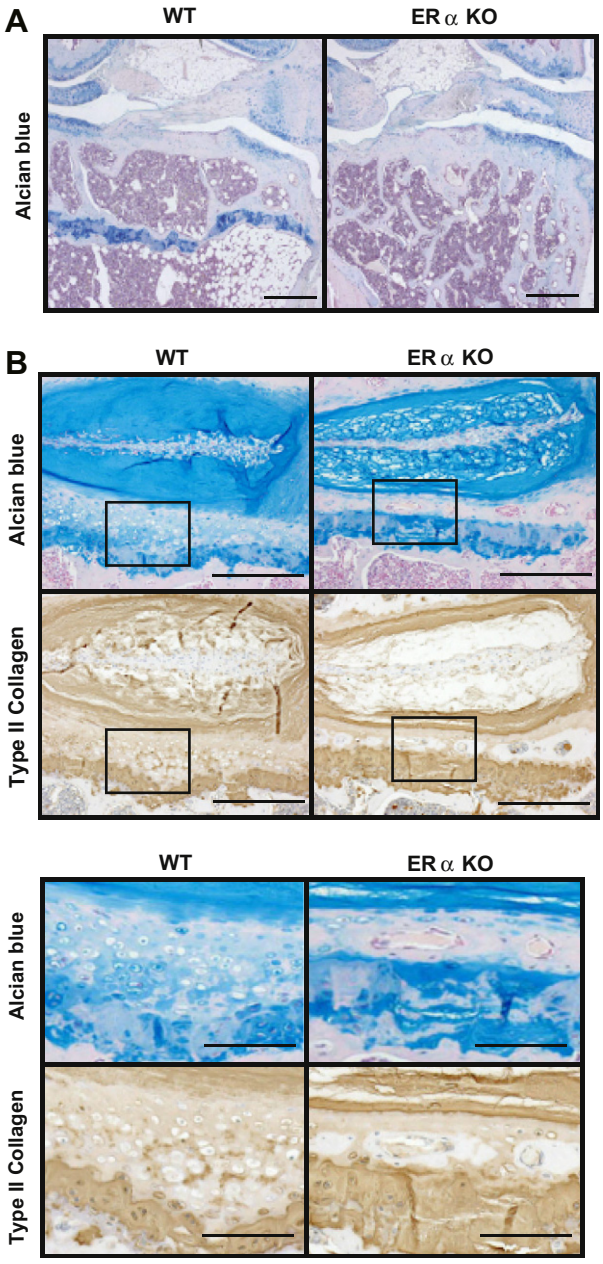

Fig. 1. Impaired development of cartilage in ERα null mice. (A) Alcian blue staining of the knee joint of 15-month-old WT and ERα null mice. Scale bar, 250 μm. (B) Alcian blue (upper panel) staining and type II collagen immunostaining (lower panel) of the intervertebral disc of 15-month-old WT and ERα null mice. Scale bar, 500 μm. Boxed areas in the disorganized endplate are displayed in higher magnification underneath. Scale bar, 100 μm.

a *col2a1* promoter without the enhancer element, or a three consensus EREs TATA luciferase reporter with Lipofectamine 2000, according to the protocol provided (Invitrogen). We also transfected 5-100 ng of a DN ERα expression vector[22]. One day after transfection, cells were incubated with 2% Charcoal/Dextran treated FBS for 4 h and were stimulated by 10^{-7} M or 10^{-9} M E_2 in the presence of 10^{-7} M ICI182780 (E_2 antagonist) (Tocris, St. Louis, MO) or 10^{-5} M SB202190 (p38 MAPK inhibitor) (Calbiochem, La Jolla, CA). After 16 h, cell lysates were recovered for luciferase assay. Luciferase activity was measured with a Dual Luciferase Reporter Assay System according to the manufacturer's instruction (Promega). The results shown were as relative luciferase activities (RLA), the ratio of the firefly activities to the Renilla (pRL-TK, Promega) activities.

For luciferase assays using ON-TARGET plus small interfering RNA (siRNA) (Dharmacon, Lafayette, CO), we adopted a different protocol. Twenty-four hours prior to transfection of the luciferase reporters, ATDC5 cells were seeded at a density of 1×10^4 cells per well in a 48-well plate. Fifty nanograms of the luciferase reporter vector per well was transfected in combination with 5 ng of pRL-TK, using FuGENE HD (Roche Diagnostics). On the next day, various expression vectors (50 ng/well) and non-targeting siRNA or the ON-TARGET plus smart pool of mouse GRIP1 siRNA (12 pmoles/well) were also transfected with FuGENE HD (for expression vectors), or Lipofectamine RNAiMAX (Invitrogen; for siRNAs).

DMB assay

ATDC5 cells were plated at a density of 1×10^4 cells per well in a 24-well plate and maintained in DMEM/F12 medium containing 5% of charcoal-filtrated FBS and 10^{-9} M of E_2. Next day, non-targeting siRNA or GRIP1 siRNA (24 pmoles/well) was transfected with Lipofectamine RNAiMAX in the presence of 10^{-9} M E_2. The transfection was repeated every 3 days. Culture media and cell lysates were harvested for GAG measurements at 14 and 21 days after plating. Cells were harvested with ice-cold saline solution containing 0.2% of Triton X-100 (Wako, Osaka, Japan). Cell lysates were centrifuged at 2×10^3 *g* for 5 min, and the supernatant was used for determination of DNA content and sulfated GAG, as described previously[29,30].

Statistical analysis

Statistical analysis was performed by Student's *t* test. *P* values less than 0.05 were considered significant.

Results

Cartilaginous endplate impairment in ERα null mice

We compared the skeletal growth plate morphology of female ERα null mice with that of age matched female WT mice. There was no difference in the morphology of 4-month-old mice between the two genotypes. As reported[7], the 15-month-old ERα null mice had a closure of the epiphyseal growth plate in the proximal tibia, while WT mice showed no sign of the growth plate closure [Fig. 1(A)]. We also found more prominent decrease in the thickness of the vertebral cartilaginous endplate in 15-month-old ERα null mice compared to WT. As shown in Fig. 1(B), the number of alcian blue-positive chondrocytes decreased in the cartilaginous endplate of 15-month-old ERα null mice. The disorganized cartilage was replaced by bony tissues. Immunostaining of type II collagen revealed that the collagen-negative areas increased in the vertebral cartilaginous endplate of the ERα null mice [Fig. 1(B)]. These results suggested that the inactivation of the ERα gene caused impaired development of the mouse vertebral endplate cartilage *in vivo*.

Regulation of col2a1 expression in mouse PCs

Because *in vivo* results described above indicated the possible link between the signaling elicited by ERα and the *col2a1* expression in chondrocytes, we examined the direct effect of E_2 on the *col2a1* expression using mouse PCs by RT-PCR and quantitative real-time RT-PCR. PCs precultured with 2% FBS were stimulated with 10^{-9}–10^{-7} M E_2 for 16 h. E_2 increased the *col2a1* message at all concentrations tested [Fig. 2(A)]. Real-time RT-PCR confirmed a significant increase in *col2a1* message by E_2 [Fig. 2(B)]. To confirm

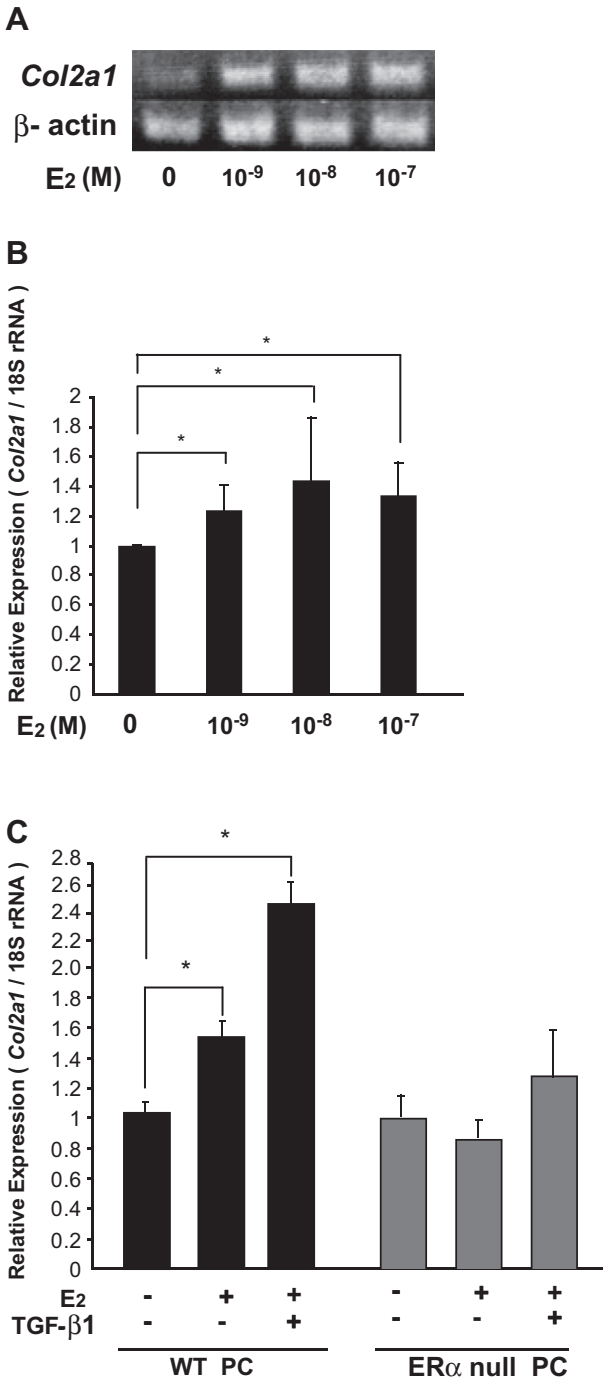

Fig. 2. Crosstalk between E_2 and TGF-β1 signaling in *col2a1* mRNA expression in PC. (A) The effect of E_2 on *col2a1* mRNA in PCs. Mouse PCs were incubated with indicated concentrations of E_2 for 16 h and processed for RT-PCR. (B) The effect of E_2 on *col2a1* mRNA in PCs. Mouse PCs were incubated with indicated concentrations of E_2 for 16 h and processed for real-time RT-PCR. $N = 3$. *Significantly different from control, $P < 0.05$. (C) Crosstalk of E_2 and TGF-β1 signal in *col2a1* expression in PCs. WT (left) and ERα null (right) PCs were treated with 10^{-7} M E_2, 10 ng/ml TGF-β1 for 16 h and processed for real-time RT-PCR. $N = 3$. *Significantly different from control, $P < 0.05$.

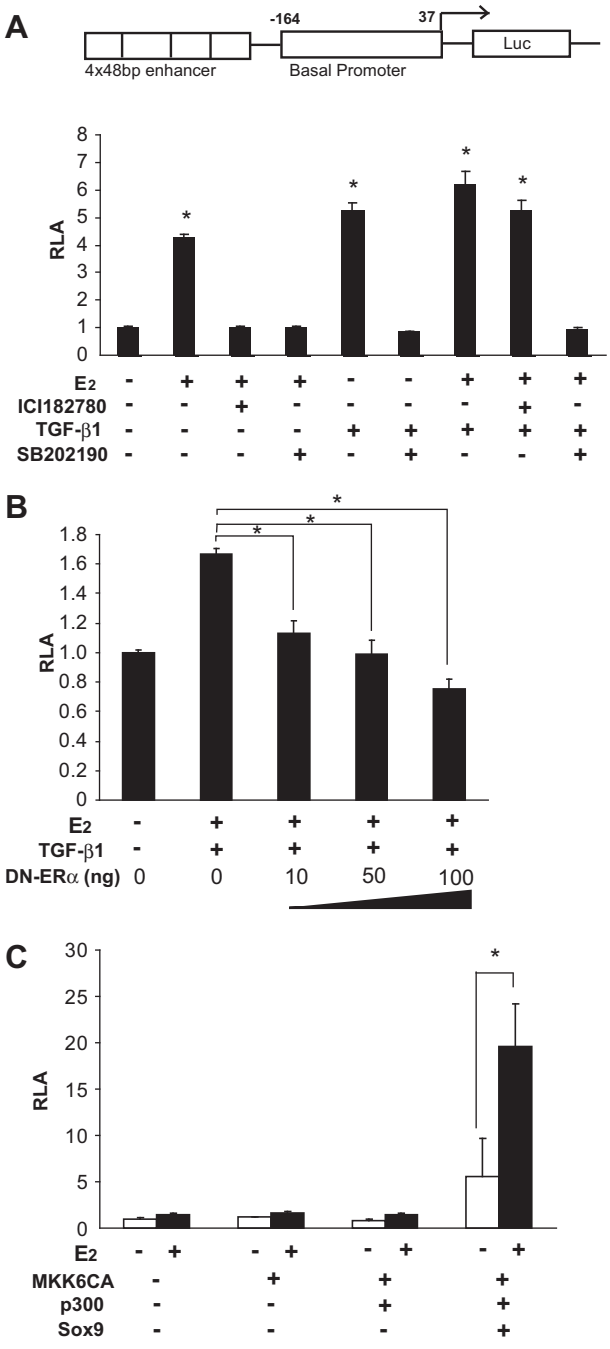

Fig. 3. Crosstalk between E_2 and TGF-β1 signaling in the *col2a1* enhancer–promoter luc reporter system. (A) ATDC5 cells were transfected with 50 ng *col2a1* luciferase reporter plasmid, which harbors *col2a1* basal promoter and 4 × 48 bp enhancer (upper panel). Transfected cells were stimulated with 10^{-7} M E_2 or 10 ng/ml TGF-β1 in presence or absence of 10^{-7} M ICI182780 and 10^{-5} M SB202190 for 16 h. *Significantly different from control, $P < 0.05$. $N = 3$. (B) ATDC5 cells were transfected with 50 ng *col2a1* luciferase reporter and indicated amount of DN ERα. Transfected cells were stimulated with 10^{-7} M E_2 in presence of 1 ng/ml TGF-β1 for 16 h. *DN ERα significantly decreased activity, $P < 0.05$. $N = 3$. (C) ATDC5 cells were transfected with 50 ng *col2a1* luciferase reporter using Lipofectamine 2000 in the presence or absence of 10^{-9} M E_2. Twenty-four hours later, 50 ng of MKK6CA, p300, and Sox9 plasmids were transfected to ATDC5 cells using FUGENE HD in the presence or absence of 10^{-9} M E_2. Twenty-four hours after the second transfection, cell lysates were prepared for luciferase assay. *Significantly different from RLA without E_2 stimulation, $P < 0.05$. $N = 3$. Results were expressed as the ratio of the firefly activities to the Renilla activities.

the involvement of ERα in the E_2-dependent *col2a1* upregulation, we determined the effect of E_2 on *col2a1* expressions using PCs prepared from ERα null mice. Real-time RT-PCR revealed that the E_2-dependent increase in the *col2a1* expression observed in WT PCs disappeared in ERα null PCs [Fig. 2(C)]. Additionally, TGF-β1 stimulation further increased the E_2-dependent *col2a1* message in WT PCs. The results suggest that the upregulation of *col2a1* expression by ERα-dependent E_2 signaling is mediated *via* TGF-β1 signaling in mouse PCs.

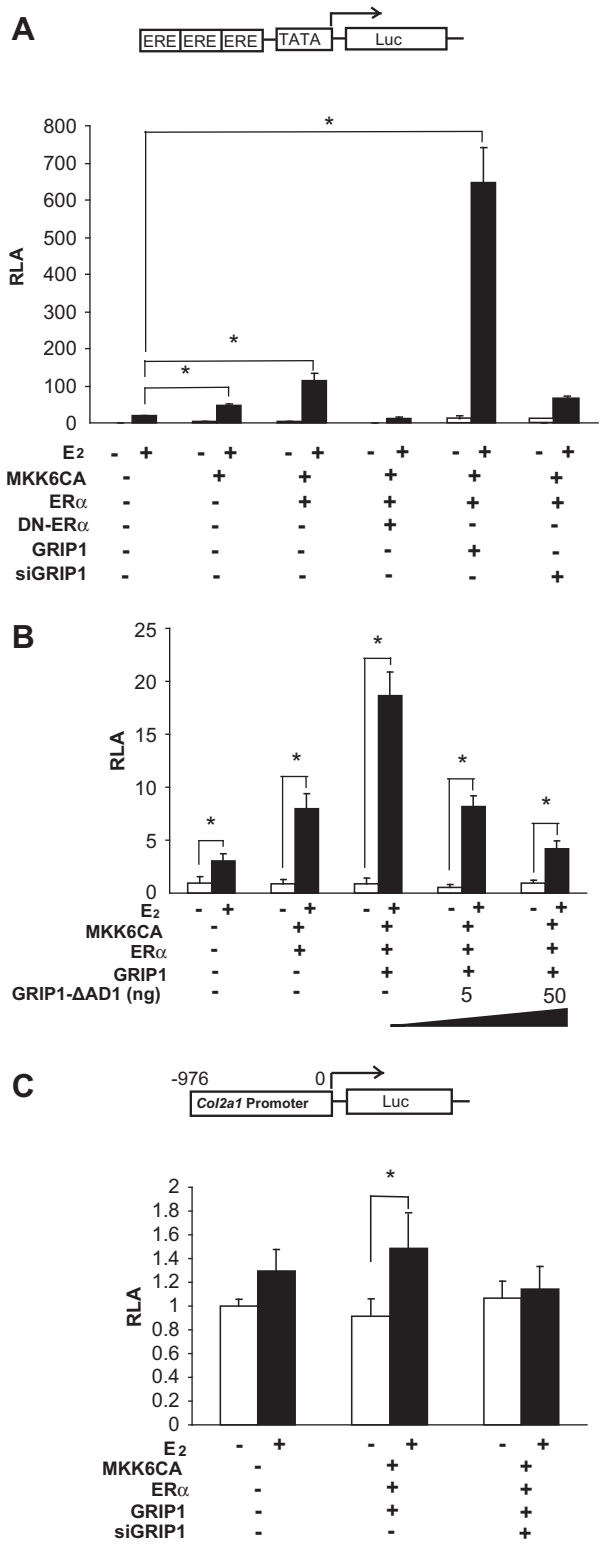

Fig. 4. GRIP1 as a coactivator of ERE-dependent transcriptional regulation in ATDC5. (A) ATDC5 cells were transfected with 50 ng ERE-TATA luciferase reporter plasmid using FUGENE HD in presence or absence of 10^{-9} M E_2. Twenty-four hours later, ERα, MKK6CA, GRIP1, control siRNA, or GRIP1 siRNA were transfected with Lipofectamine RNAiMAX in the presence or absence of 10^{-9} M E_2. Cell lysates were prepared for luciferase assay 24 h after second transfection. *Significantly different from control, $N = 3$. A schematic model of ERE-TATA-luc was shown in the upper panel. (B) The luciferase activity was measured in ATDC5 cells transfected with ERE-TATA-luc, ERα, MKK6CA, GRIP1, or GRIP1-ΔAD1. *Significantly different from RLA without E_2 stimulation, $N = 3$. (C) The luciferase activity was measured in ATDC5 cells transfected with *col2a1* promoter-luc, ERα, MKK6CA, GRIP1, control siRNA, or GRIP1 siRNA as in (A). *Significantly different from RLA without E_2 stimulation, $N = 3$. A schematic model of *col2a1* promoter-luc was shown in the upper panel. Results were expressed as the ratio of the firefly activities to the Renilla activities.

E_2-dependent regulation of col2a1 transcription via TGF-β/P38MAPK signaling

Because the histological findings of ERα null mice accorded with the *in vitro* E_2-dependent increase in *col2a1* expression of the isolated mouse PCs, we examined the mechanism of E_2-dependent *col2a1* transcription using the luciferase reporter system in ATDC5 cells, a chondrogenic cell line. ATDC5 cells were transfected with the *col2a1* luciferase reporter, which harbors a human *col2a1* promoter and a 4×48 bp enhancer [Fig. 3(A)]. E_2 at 10^{-7} M increased the reporter activity by fourfold. This increase was completely blocked by 10^{-7} M ICI182780, an E_2 antagonist. On the other hand, TGF-β1 at 10 ng/ml also increased the *col2a1* reporter activity by fivefold. The increase was cancelled by 10^{-5} M SB202190, a p38 MAPK inhibitor. A combination of E_2 and TGF-β1 increased the reporter activity by sixfold. Although the elevated transcriptional activity was partially blocked by ICI182780, SB202190 completely inhibited the activation of *col2a1* reporter activity. Importantly, SB202190 attenuated both the E_2-dependent and TGF-β1-dependent *col2a1* reporter activity to the basal level. The E_2 and TGF-β1-induced activation of *col2a1* transcription was inhibited by DN ERα in a dose-dependent manner, suggesting that E_2-bound ERα participated in the p38 MAPK pathway to activate the *col2a1* transcription in ATDC5 cells [Fig. 3(B)]. The luciferase reporter activity activated by cotransfection of Sox9, p300, and MKK6CA (constitutive-active MKK6) was also responsive to the E_2 stimulation [Fig. 3(C)]. The results accord with the notion that E_2 upregulated the Sox9-mediated activation of *col2a1* transcription, which depends on the TGF-β1/p38 MAPK pathway. Future works are clearly needed to define the exact mechanism of interaction between the E_2-mediated pathway and the TGF-β1/p38 MAPK pathway in the Sox9-mediated activation of *col2a1* transcription before reaching this conclusion.

GRIP1 as a coactivator of ERE-dependent transcription in chondrocytes

To further dissect the mechanism of E_2-mediated upregulation of *col2a1* transcription, we elucidated the roles of GRIP1 on the ERE-specific gene regulation in chondrocytes. First, we investigated the effect of GRIP1 on the interaction of ERα and MKK6 using ERE-TATA-luc in ATDC5 cells. In the presence of E_2, MKK6CA increased the reporter activity by threefold and a combination of MKK6CA and ERα increased the activity by sevenfold [Fig. 4(A)]. DN ERα completely cancelled the MKK6CA-induced reporter activity, probably indicating that the activation of luciferase activity by MKK6 required the endogenous ERα. Interestingly, GRIP1 significantly enhanced the activity induced by MKK6CA and ERα in the presence of E_2. The GRIP1-induced increase in the ERE-luc activity was abolished by the cotransfection of GRIP1 siRNA. The GRIP1-dependent activation of the ERE reporter activity was also inhibited by the cotransfection of GRIP1 mutant, GRIP1-ΔAD1 in the presence of E_2 [Fig. 4(B)], underlining the specificity of GRIP1 in the estrogen-dependent transcription activity in ATDC5 cells. Finally, we examined the role of GRIP1 in the *col2a1* transcription using the *col2a1* luciferase reporter with a 1 kb human *col2a1* promoter (−976 to 0) without the enhancer element in ATDC5 cells [Fig. 4(C)]. Although the reporter activity in the presence of E_2 was higher than that in the absence of E_2, the increase was not significant. Cotransfection of

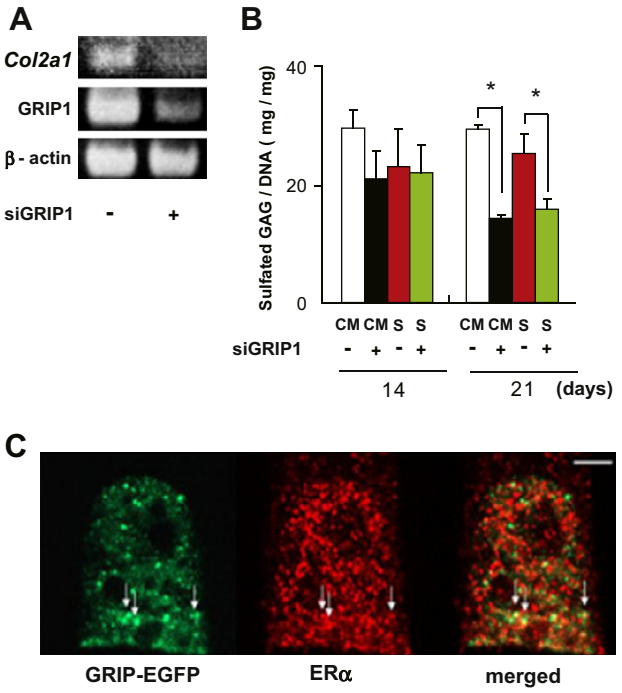

Fig. 5. Inhibition of extracellular matrix expression by GRIP1 siRNA in ATDC5 cells. (A) Inhibition of *col2a1* expression by GRIP1 siRNA. ATDC5 cells were transfected with GRIP1 siRNA and cultured for 2 days in presence or absence of 10^{-9} M E_2. Total RNA was recovered from ATDC5 cells and processed for RT-PCR. (B) GRIP1 siRNA decreased GAG contents in ATDC5 cells. ATDC5 cells were transfected with control siRNA or GRIP1 siRNA in presence of 10^{-9} M E_2. At 14 and 21 days after plating, GAG contents of cell lysate and culture medium were measured. CM, cell and matrix. S, supernatant. *Significantly different from GAG content without siGRIP1 transfection, $N = 3$. (C) Confocal images of overexpressed EGFP-GRIP1 and endogenous ERα in the nucleus of ATDC5 cells. GRIP1 partially colocalized with ERα (arrows). Scale bar, 1 μm.

GRIP1, MKK6CA and ERα conferred the significant E_2 response on the reporter activity. The E_2 responsiveness was cancelled by the cotransfection of GRIP1 siRNA. These results supported the contention that GRIP1 acted as a coactivator in the *col2a1* transcriptional machinery in ATDC5 cells and that 1 kb upstream sequence of *col2a1* gene contained the putative ERE motif.

We next confirmed the effect of GRIP1 on the endogenous extracellular matrix production by ATDC5 cells using GRIP1 siRNA. Transient transfection of GRIP1 siRNA substantially decreased the GRIP1 expression in ATDC5 cells with concomitant decrease in the *col2a1* message [Fig. 5(A)]. The effect of GRIP1 siRNA was also tested by the quantification of GAG in the cell matrix and supernatant of ATDC5 cells [Fig. 5(B)]. Transfection of GRIP1 siRNA significantly decreased the ratio of GAG/DNA at 21 days of culture, but not at 14 days. These results clearly showed that GRIP1 acted as a positive regulator not only for the *col2a1* transcription but also for the GAG production in ATDC5 cells.

We examined if ERα and GRIP1 colocalize in the nucleus using confocal microscopy. Nuclear localization of ERα detected by indirect immunofluorescence was compared with that of the transiently expressed EGFP-GRIP1 in ATDC5 cells [Fig. 5(C)]. GRIP1 expression in ATDC5 cells showed various morphologies from diffuse nucleoplasmic staining with the low intensity to discrete intranuclear foci with high intensity as described in HeLa cells[26]. ERα antibody produced numerous granular staining patterns. A few of the ERα-positive granules overlapped with some of the GRIP1-positive granules which showed the intermediate intensity. The overlapped granules might represent the active transcriptional complex that include both ERα and GRIP1[31].

Discussion

The aim of the present study is to resolve the relationship between estrogen and cartilage metabolism. It has been reported that 6-month-old ERα null female mice show no sign of cartilage damage in the knee joints[32]. Consistent with this, we could not observe an impaired development of the cartilaginous endplate in 4-month-old ERα null female mice (data not shown). In contrast, ERα null female mice at 15-month-old showed an obvious impaired development of the cartilaginous endplate with a reduced number of chondrocytes [Fig. 1(B)]. Similar phenotypes were also observed in ovariectomized rats at 3 and 7 months after surgery (Supplementary data). Because the depletion of estrogen and functional loss of ERα exhibited the similar phenotypes, it is reasonable to speculate that estrogen has a positive role on the homeostasis of the cartilaginous vertebral endplate in the aged animals. Especially, the function of ERα seemed to be critical for the maintenance of the cartilage matrix. This notion was supported by *in vitro* experimental results using the ERα null PC [Fig. 2(C)] and the *col2a1* reporter assay [Fig. 3(B)].

Here we showed that the E_2 induced *col2a1* expression is mediated by ERα in PCs (Fig. 2). The E_2-dependent activation

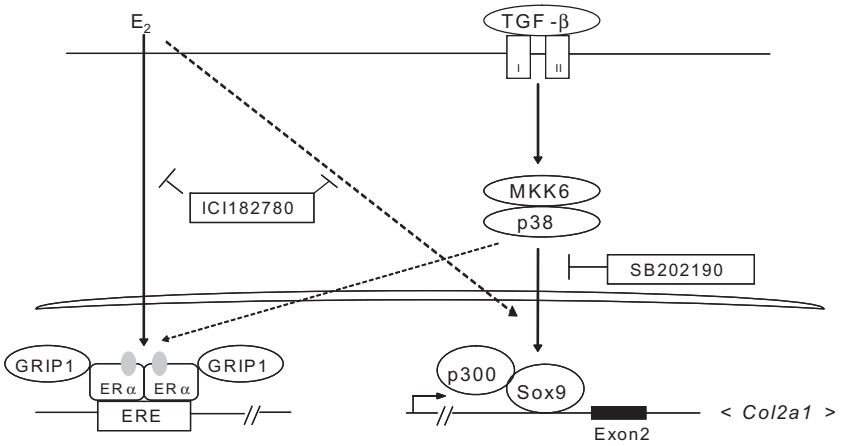

Fig. 6. Scheme of E_2-dependent regulation of *col2a1* gene expression in chondrocytes. TGF-β binds to TGF-β type I receptor on plasma membranes. Signal from TGF-β type I receptor activates *col2a1* transcription through MKK6/p38/Sox9 pathway[18]. ERα bound with E_2 interacts with TGF-β signaling *via* p38 resulting in upregulation of *col2a1* transcription. In addition, GRIP1-ERα multisubunit complex[8] might interact with Sox9-p300 transcriptional complex in the *col2a1* enhancer region. Arrows indicate pathways we examined in this study. A broken line indicates a putative pathway. Points of action of various antagonists used in this study are shown in respective pathways.

utilized the TGF-β signaling, because an addition of TGF-β1 synergistically increased the *col2a1* expression and the reporter activity [Figs. 2(C) and 3(A)]. In other cells, it has already been shown that the interaction between estrogen and the TGF-β signaling pathways occurs in multiple intracellular signaling steps to impact downstream gene transcriptions[33]. Estrogen and tamoxifen can increase TGF-β production by osteoblasts[34]. Thus, our results coincided with previous reports showing a crosstalk between estrogen and TGF-β signaling in mesenchymal lineage cells.

Results obtained from the reporter assays and various inhibitors indicated that E_2 transmits its signal through p38 MAPK in the *col2a1* transcription in ATDC5 cells [Fig. 3(A)]. It has already been well established that the Sox9-dependent *col2a1* enhancer activity is regulated through MKK6 and p38 MAPK in PCs[20]. Thus, E_2 appears to be one of the upstream signals of the *col2a1* transcriptional regulation through MKK6 and p38 MAPK. Because TGF-β1 causes p38 MAPK activation without Smad in mouse mammary epithelial cells[14], we speculate that the Smad-independent TGF-β signaling elicits p38 MAPK activation in the E_2 induced the *col2a1* expression (see Fig. 6 for details). Future studies are needed to clarify the complexity of such crosstalks.

Because GRIP1 is widely recognized as a transcriptional coactivator of the nuclear receptors including ERα, we examined the role of GRIP1 in the E_2 induced *col2a1* expression in ATDC5 cells. As expected, the experiments using GRIP1 siRNA clearly showed the involvement of GRIP1 in the E_2 induced *col2a1* expression [Fig. 5(A)]. Moreover, GRIP1 siRNA inhibited the production of sulfated GAGs in ATDC5 cells, implying a wider range of estrogen-dependent transcriptional controls of extracellular matrix proteins in chondrocytes.

Taken together, the results obtained in this study accord with a notion that GRIP1-bound ERα induces the *col2a1* gene expression through an interaction with the TGF/MKK6 pathway. This study sheds a light on the apparent controversies about the relationship between the role of estrogen and cartilage degeneration in postmenopausal women. A novel functional cooperation between GRIP1/ERα and TGF/MKK6 signaling, shown in our study, may therefore lead to a better understanding of transcriptional networks and the development of a novel therapeutics approach against postmenopausal cartilage degeneration in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.joca.2010.03.008.

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