ROCK inhibition stimulates SOX9/Smad3-dependent COL2A1 expression in inner meniscus cells

ABSTRACT

Background: Proper functioning of the meniscus depends on the composition and organization of its fibrocartilaginous extracellular matrix. We previously demonstrated that the avascular inner meniscus has a more chondrocytic phenotype compared with the outer meniscus. Inhibition of the Rho family GTPase ROCK, the major regulator of the actin cytoskeleton, stimulates the chondrogenic transcription factor Sry-type HMG box (SOX) 9-dependent α1(II) collagen (COL2A1) expression in inner meniscus cells. However, the crosstalk between ROCK inhibition, SOX9, and other transcription modulators on COL2A1 upregulation remains unclear in meniscus cells. The aim of this study was to investigate the role of SOX9-related transcriptional complex on COL2A1 expression under the inhibition of ROCK in human meniscus cells.

Methods: Human inner and outer meniscus cells were prepared from macroscopically intact lateral menisci. Cells were cultured in the presence or absence of ROCK inhibitor (ROCKi, Y27632). Gene expression, collagen synthesis, and nuclear translocation of SOX9 and Smad2/3 were analyzed.

Results: Treatment of ROCKi increased the ratio of type I/II collagen double positive cells derived from the inner meniscus. In real-time PCR analyses, expression of SOX9 and COL2A1 genes was stimulated by ROCKi treatment in inner meniscus cells. ROCKi treatment also induced nuclear translocation of SOX9 and phosphorylated Smad2/3 in immunohistological analyses. Complex formation between SOX9 and Smad3 was increased by ROCKi treatment in inner meniscus cells. Chromatin immunoprecipitation analyses revealed that association between SOX9/Smad3 transcriptional complex with the COL2A1 enhancer region was increased by ROCKi treatment.

Conclusions: This study demonstrated that ROCK inhibition stimulated SOX9/Smad3-dependent COL2A1 expression through the immediate nuclear translocation of Smad3 in inner meniscus cells. Our results suggest that ROCK inhibition can stimulates type II collagen synthesis through the

cooperative activation of Smad3 in inner meniscus cells. ROCKi treatment may be useful to promote the fibrochondrocytic healing of the injured inner meniscus.

Introduction

The meniscus is a fibrocartilaginous tissue that plays an important role in controlling complex biomechanics of the knee [1]. Proper functioning of the meniscus depends on the composition and organization of its extracellular matrix (ECM) [2]. Type I collagen accounts for more than 90% of the total meniscal collagen and is present throughout the entire meniscus. On the other hand, type II collagen is restricted to the inner avascular region of the meniscus [2-4]. However, the distributions of types I and II collagen in human menisci are different from those in rabbit and sheep menisci [5]. We previously demonstrated that the avascular inner meniscus has a more chondrocytic phenotype compared with the outer meniscus [6, 7]. In addition, human inner meniscus cells have chondrocytic morphology and an ability to produce type II collagen, a cartilage-specific ECM component [6-9]. Physiological biomechanical stress induces α1(II) collagen (COL2A1) expression through the activation of the chondrogenic transcription factor Sry-type HMG box (SOX) 9 in inner meniscus cells [8]. Inhibition of the Rho family GTPase ROCK, the major regulator of the actin cytoskeleton, also stimulates SOX9-dependent COL2A1 transactivation in inner meniscus cells [9]. Several transcription factors and coactivators, such as transforming growth factor-β receptor-regulated Smad3, Scleraxis, E47, and p300, cooperatively modulate SOX9-dependent transcription by interacting with SOX9 in chondrocytic cells [10]. However, the crosstalk between SOX9 and other transcription modulators on COL2A1 upregulation remains unclear in meniscus cells.

Meniscal injury located in the avascular inner zone has poor healing potential even after meniscal repair compared with that in the outer zone [11, 12]. Trials using the major angiogenic factor, vascular endothelial growth factor (VEGF), have failed to promote the healing of meniscal injuries in the avascular inner zone [13-15]. These angiogenic factor-based treatments for enhancing meniscal healing may be inhibited by the deposition of endogenous anti-angiogenic factors such as chondromodulin-I and endostatin in the inner region of the meniscus [7, 16]. Based on these findings, we considered that the activation of chondrocytic gene expression may have a key role in promoting the healing of inner meniscus injury. In this study, we analyzed the epigenetic regulation of COL2A1

transactivation in chondrocytic inner meniscus cells. Furthermore, we investigated the role of SOX9-related transcriptional complex on COL2A1 expression under the inhibition of ROCK.

Materials and methods

Cells and cell culture

Institutional Review Board approval and informed consent were obtained before all experimental studies. Macroscopically intact lateral menisci were obtained at total knee arthroplasty in patients suffering from medial compartmental osteoarthritis of the knee (n = 6). Inner and outer meniscus cells were prepared from the undegenerated meniscal samples as described [6-9]. In brief, synovial and capsular tissues were removed from the meniscus. Middle segments of the menisci were assessed for histological analyses (Supplemental Fig. 1). Inner and outer meniscal tissues were obtained by careful cut along the midpoint of meniscal width. Inner and outer meniscus cells were prepared by collagenase (Sigma, St. Louis, MO) treatment from the inner 1/2 and outer 1/2, respectively. Attached cells (passage 0) were maintained with Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (HyClone, South Logan, UT), and 1% penicillin/streptomycin (Sigma). Meniscus cells between passage 1 and 3 were used.

ROCK inhibitor (ROCKi) treatment, immunohistological, western blot, and immunoprecipitation (IP) analyses

Meniscus cells were cultured for 12 h in the presence or absence of ROCKi (Y-27632, Wako; 0, 0.1, 1, and 10 μM) [9, 17]. After the ROCKi treatment, distribution of type I and II collagen in each meniscus cell was observed using rabbit anti-collagen I antibody (1:200, Bioss, Woburn, MA) and mouse anti-collagen II antibody (1:200, MP Biomedicals, Santa Ana, CA), respectively. Positive cell ratios for type I and II collagens were calculated using a fluorescent microscope (5 experiments for each cell). Nuclear translocalization of SOX9 and Smad2/3 in inner meniscus cells was assessed using

mouse anti-SOX9 antibody (1:200, Sigma, St. Louis, MO) and goat anti-phosphorylated (P-) Smad2/3 antibody (1:200, Santa Cruz, Santa Cruz, CA) after 12-h-serum starvation followed by 2-h-ROCKi treatment [18]. Alexa Fluor 568 phalloidin for detection of F-actin (Invitrogen, Carlsbad, CA) was used under a fluorescence microscope. ROCKi-treated cellular morphologies are shown in Supplemental Fig. 2. Nuclear extracts of inner meniscus cells were prepared in the presence (1 μM) or absence of ROCKi. Protein concentrations were measured by BCA protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were applied (20 μg/lane). Western blot (WB) analyses were performed using anti-SOX9 antibody (1:1000) and rabbit anti-Smad2/3 antibody (1:1000, Millipore, Temecula, CA). A bovine serum albumin solution without the primary antibody was used as a negative control. Relative amounts of detected proteins were compared with ROCKi-free controls using Image J 1.31 [7]. Nuclear fractions of inner meniscus cells were prepared in the presence or absence of ROCKi (1 μM, 2 h) as described [8, 9, 17]. Twenty percent volume of nuclear fraction was loaded as an input fraction. Forty percent volume of extract was incubated with rabbit anti-Smad3-specific antibody (Abcam, Cambridge, UK) and protein G beads (Sigma) for 4 h at 4°C. The following western blot analysis was performed using an anti-SOX9 antibody.

Quantitative real-time PCR

RNA samples were obtained from cultured meniscus cells. Total RNAs were isolated using ISOGEN reagent (Nippon Gene, Toyama, Japan). RNA samples (500 ng) were reverse-transcribed with ReverTra Ace (Toyobo, Osaka, Japan). The following specific primer sets were used: SOX9, COL2A1, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) [18, 19]. Quantitative real-time PCR analyses were performed using FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). The cycle number crossing the signal threshold was selected in the linear part of the amplification curve. Amplification data of G3PDH were used for normalization. Relative mRNA levels were normalized with the level of ROCKi-free condition for every sample.

Chromatin immunoprecipitation (Chromatin IP)

Chromatin IP assays were performed as described [8, 9]. Inner meniscus cells were maintained in ROCKi (1 μM)-supplemented conditions for 2 h after serum-free starvation. After crosslinking step, nuclear fraction was collected. The sonicated cell lysates were suspended in nuclear lysis buffer. The 10% volume of supernatant was stocked as an input sample. The half of each remaining sample was incubated for 12 h with mouse anti-SOX9 antibody (Sigma) or rabbit anti-Smad3-specific antibody (Abcam). The remaining supernatant was incubated with mouse (or rabbit) IgG and protein G beads as a control. Input fraction DNAs, DNA fragments immunoprecipitated with endogenous SOX9 (or Smad3), and DNAs in IgG controls were purified. PCR reactions were performed using the primer set to amplify specific regions of human COL2A1 promoter and enhancer [8], and allowed to proceed for 30 cycles.

Statistical analysis

All experiments were repeated three times independently. The expression of several genes and proteins in response to ROCKi treatments were similar among the meniscus cells from all the donors. Data were expressed as means with standard deviations. Mean values were compared with Mann-Whitney U test. Significance was set at $p < 0.05$.

Results

Collagen synthesis in ROCKi-treated human meniscus cells

Type I collagen (COL1) deposition was observed around the nucleus of inner meniscus cells (Fig. 1A, red). Type II collagen (COL2) distributed in the cytoplasm of inner cells (Fig. 1A, green). In outer meniscus cells, intracellular COL1 was more detected than in inner cells (Fig. 1A). However, COL2 signal was weak in outer cells (Fig. 1A, yellow). COL1-COL2 double positive cell ratio was increased by 1 μM of ROCKi treatment (12 h) in inner cells (Fig. 1B). ROCKi did not affect the ratio of

COL2-positive outer cells (Fig. 1C).

Chondrocytic gene expression in meniscus cells

ROCKi treatment increased expression of chondrogenic transcription factor SOX9 in inner meniscus cells (Fig. 2A). SOX9 gene expression was increased to 2.1-, 12.5-, and 2.7-fold levels of untreated cells by ROCKi (0.1, 1, and 10 μM, respectively) in inner meniscus cells. In outer meniscus cells, SOX9 expression was enhanced to 3.9- and 2.5-fold levels of control in the presence of 1 and 10 μM ROCKi, respectively (Fig. 2A). Expression of chondrocytic ECM molecule COL2A1 was increased to 13.8-fold level of control by 1 μM of ROCKi in inner cells (Fig. 2B). In addition, ROCKi treatment (1 and 10 μM) increased COL2A1 expression in outer cells (Fig. 2B). The highest expression of SOX9 and COL2A1 was induced by 1 μM of ROCKi treatment in inner meniscus cells.

Nuclear translocalization of SOX9 and Smad2/3 in ROCKi-treated inner meniscus cells

ROCK inhibition affected cellular morphology of inner meniscus cells (Fig. 3A, red). In addition, ROCKi treatment (2 h) induced nuclear translocalization of SOX9 and phosphorylated Smad2/3 (Fig. 3A, green). SOX9 accumulated in the nuclear fraction was slightly increased by ROCKi (Fig. 3B). Nuclear translocalization of Smad3 was also enhanced by 1 μM of ROCKi (Fig. 3C). IP analyses revealed that the association between SOX9 and Smad3 was increased by ROCKi treatment in inner meniscus cells (Fig. 3D).

ROCKi-mediated transcriptional regulation on the COL2A1 enhancer region

ROCKi treatment (2 h) did not show a remarkable increase of the association between SOX9 and the conserved SOX9-binding site on the COL2A1 enhancer in inner meniscus cells (Fig. 4, A and B). On the other hand, chromatin IP analysis revealed that the COL2A1 enhancer region immunoprecipitated with Smad3 increased by ROCKi treatments (Fig. 4C).

Discussion

The present study demonstrated that ROCK inhibition stimulated SOX9/Smad3-dependent COL2A1 expression through the immediate nuclear translocation of Smad3 in inner meniscus cells. ROCKi treatment also increased the gene expression levels of SOX9 and COL2A1 in outer meniscus cells (Fig. 2). However, type II collagen deposition was not affected by ROCKi in outer meniscus cells (Fig. 1, A and C). This may be caused by extremely low expression levels of SOX9 and COL2A1 in outer meniscus cells. A few fibrochondrocytes that had a similar phenotype to inner meniscus cells might be contaminated in cultured cells derived from the outer meniscus. We consider that the early effect of ROCKi on COL2A1 expression would be influenced by the rapid activation of endogenous SOX9-Smad3 transcriptional complex, not by a newly-produced SOX9. In fibrochondrocytic inner meniscus cells, a small amount of SOX9 may be constantly activated even in the absence of ROCKi to maintain type II collagen synthesis (Fig. 3, A and B). ROCK inhibition slightly increased the nuclear translocalization of SOX9. On the other hand, Smad3 activation was strongly induced by ROCKi (Fig. 3, A and C). In addition, ROCKi treatment increased the complex formation between SOX9 and Smad3 (Fig. 3D). In our previous studies, activated Smad3 can promote the SOX9-dependent transcriptional activity by strengthen the association between SOX9 and co-factor p300 [10, 20, 21]. Based on these findings, ROCKi-mediated Smad3 phosphorylation may up-regulate the function of SOX9/Smad3-related transcriptional complex in inner meniscus cells. ROCKi treatment also increases the gene expression of SOX9 itself in human articular chondrocytes [17, 22], mouse chondrocytic cells [23], and human inner meniscus cells [9]. Secondary accumulation of SOX9 in the nucleus may have an important role in a sequential induction of COL2A1 expression.

Rho family small GTPases that include the Rho, Rac, and Cdc42 subfamilies regulate a variety of cellular functions such as cytoskeletal rearrangement, migration, and cell contractility [24]. RhoA/ROCK signaling, which organizes the actin cytoskeleton, modulates SOX9 expression in several chondrocytic cell types [9, 17, 23]. In this study, we could not detect a dose-dependent effect

of ROCKi on type II collagen synthesis (Fig. 1). One μM of ROCKi showed the optimal increase of SOX9/COL2A1 gene expression and type II collagen production in inner meniscus cells (Figs. 1 and 2). Several studies demonstrate that potential antagonism between the Rho/ROCK and Rac pathways [25, 26]. ROCK inhibits the Rho-mDia-Rac pathway, whereas Rac can antagonize ROCK action. In neuronal cells, the high level of Rho-GTP induces ROCK activation, whereas the low level of Rho-GTP preferentially activates mDia and induces Rac activation [27]. In colon carcinoma cells, ERK-MAPK signaling coordinately modulates the balance between the activation of Rho and Rac [26]. Ras can also activate Rho, but this signaling appears to be complex. In some cells, the signaling from Ras to Rho has been reported to be positive, in others negative [24]. These findings indicate that the degree of ROCK inhibition can modulate the activation balance between Rho and Rac in a cell-type-dependent manner. We consider that a higher concentration of ROCKi can induce severe morphological changes of the meniscus cells and may negatively regulate the gene expression of SOX9 and COL2A1. Further investigations will be required to understand the complex crosstalk among Rho small GTPases for activating chondrocytic ECM syntheses in inner meniscus cells.

Several studies show that the Rho/ROCK pathway modulates the phosphorylation status of Smad2/3 [28-30]. In human breast cancer cells, Rho/ROCK can influence Smad signaling by modulating phosphorylation of the Smad linker region [28]. ROCKi treatment (10 μM) suppresses TGF-β1-induced Smad3 phosphorylation in mouse cardiomyocytes [30]. However, ROCKi does not affect Smad2/3 phosphorylation in neural crest stem cells [29]. On the other hand, several authors have demonstrated that Smads are also involved in the function of Rho/ROCK pathway. Dominant negative Smad3 inhibits the Rho activity in human keratinocyte cells [31]. Inhibitory Smad7 that antagonizes the phosphorylation of Smad2/3 can modulate the balance of Rho GTPases by inducing the TGF-β-dependent activation of Cdc42 in human prostate cancer cells [32]. TGF-β can directly regulate Rho/ROCK activity by inducing ubiquitin-mediated RhoA degradation in epithelial cells [33]. In our study, ROCKi treatment induced the accumulation of phosphorylated Smad3 in the nucleus of inner meniscus cells (Fig. 3). These findings suggest that the crosstalk between Rho/ROCK and

Smad3 may be cell type-specific. In addition to the ROCK pathway, RhoA has another key downstream target protein kinase PKN [34]. Because the affinity of ROCKi for PKN is at least 20-30 times lower than that for ROCK [35], ROCKi treatment using Y27632 may not perfectly block the RhoA signaling. We consider that the balance between Rho/ROCK activation and Smad3 phosphorylation may be influenced by the concentration and/or treatment time of ROCKi in a cell type-specific manner. In addition, the TGF-β-mediated p38 MAPK pathway may have an additional role in modulating the transcriptional complex formation between SOX9 and Smad3. Future work will be directed toward understanding the precise mechanism of ROCKi-dependent Smad3 activation in meniscus cells.

In conclusion, ROCK inhibition can stimulates SOX9/Smad3-dependent COL2A1 expression through the cooperative activation of Smad3 in inner meniscus cells. ROCKi treatment may be useful to promote the fibrochondrocytic healing of the injured meniscus.

Declaration of interest

The authors have no conflict of interest.

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Figure legends

Fig. 1. Collagen deposition in ROCKi-treated meniscus cells. (A) Type I collagen (COL1), red; Type II collagen (COL2), green or yellow; Hoechst, blue. Bars, 50 μm. (B and C) COL1-COL2 double positive cells/total cells in inner and outer meniscus cells (n = 5). * $p < 0.05$.

Fig. 2. SOX9 and COL2A1 expression in meniscus cells. (A) Real-time PCR analysis revealed that ROCKi (0.1, 1, and 10 μM) increased SOX9 gene expression (to 2.1-, 12.5-, and 2.7-fold levels of untreated cells, respectively) in inner meniscus cells (n = 4). In outer meniscus cells, SOX9 expression was enhanced to 3.9- and 2.5-fold levels of control by 1 and 10 μM ROCKi treatments, respectively. Relative expression level was normalized with the level of untreated inner meniscus cells (A and B). (B) COL2A1 expression was stimulated by ROCKi in inner cells (0.1 μM, 2.2-fold level; 1 μM, 13.8; and 10 μM, 1.6). In addition, ROCKi treatment (1 and 10 μM) increased COL2A1 expression in outer cells (7.0- and 4.8-fold levels, respectively). * $p < 0.05$.

Fig. 3. Effect of ROCKi on nuclear translocalization of SOX9 and Smad2/3. (A) Shapes of inner meniscus cells in the presence or absence of ROCKi (red, F-actin). SOX9 and phosphorylated (P)-Smad2/3 were accumulated in the nucleus by ROCKi treatment (green, SOX9 or P-Smad2/3). Bars, 50 μm. (B) Western blot (WB). ROCKi treatment (1 μM, 2 h) increased SOX9 deposition in the nuclear fraction to a 1.2-fold level of untreated control. (C) Nuclear translocalization of Smad3 was increased to a 4.8-fold level of control by ROCKi. (D) IP analysis revealed that the association between SOX9 and Smad3 was increased by ROCKi treatment (1 μM, 2 h) in inner meniscus cells.

Fig. 4. Effect of ROCKi on the association among SOX9, Smad3, and the COL2A1 enhancer. (A) A scheme involving the human COL2A1 gene and SOX9-Smad3/4 transcriptional complex is shown. Filled box denotes the conserved SOX9-binding site on the COL2A1 enhancer. Numbers indicate the

distance from the transcription start site of the COL2A1 gene (GenBank, AC004801). Arrowheads and dotted lines denote the primer sets and expected PCR fragments in chromatin IP, respectively. (B) Chromatin IP analyses revealed that endogenous SOX9 associated with the SOX9-binding site on the COL2A1 enhancer in the presence or absence of ROCKi (1 μM, 2 h treatment). (C) However, ROCKi treatment increased the COL2A1 enhancer fragments co-immunoprecipitated with nuclear-translocalized Smad3. No fragments were observed using primers for the COL2A1 promoter in chromatin IP fractions (B and C). Mouse and rabbit IgG were used as the controls for anti-SOX9 and Smad3 antibodies, respectively (IgG).

Supplemental Fig. 1. Histological phenotypes of inner and outer regions of the meniscus. (A) Preparation of the meniscal samples and cells. The middle segment of the lateral meniscus was used for histological analyses. White bar, 1 cm. (B-D) Safranin O-stained meniscal samples. (E-G) Type I collagen deposition (brown) was assessed by a rabbit anti-collagen I antibody. (H-J) Type II collagen deposition was visualized by a mouse anti-collagen II antibody. (E and H) Negative controls in the absence of primary antibodies. Bars, 100 μm.

Supplemental Fig. 2. ROCKi-treated cellular morphologies were evaluated using a phase-contrast microscope. Bars, 100 μm.