Induction of the expression of miR-146 in human articular chondrocytes by IL-1β

To confirm the induction of expression of miR-146a in normal human articular chondrocytes following stimulation with IL-1β, we conducted real time PCR to investigate the expression of mature miR-146a and MMP13 (Figure 3). The expression of miR-146 was markedly increased in human chondrocytes after stimulation with IL-1β (Figure 3A). There was a significant difference between cells treated with and without IL-1β. MMP-13 expression was also induced after IL-1β stimulation, in the same way as miR-146 (Figure 3B).

Discussion

Recently, it has been well established that miRNAs play a crucial role in the pathogenesis of human diseases [9-11,13]. Marcucci *et al.* identified that the microRNA-181 family plays an important role in acute myeloid leukemia (AML) and encodes proteins involved in pathways of innate immunity mediated by toll-like receptors and interleukin-1β [10]. Hébert *et al.* reported that increased expression of proteins like APP or BACE1 beta-secretase may also be associated with genetic Alzheimer's disease, and that miR-29a, -29b-1, and -9 can regulate BACE1 expression *in vitro* [18]. Bruneau *et al.* recognized that dysregulation of miR-1 or other developmentally important miRNAs might result in congenital heart disease in humans [19]

miR-146a/b has been described as one of the key molecules in the inflammatory response and oncogenesis [12,13,20-24]. Taganov *et al.* reported that miR-146a/b is an NF-κB dependent gene which inhibits the expression of IRAK 1 and TRAF 6 by binding to the 3' UTR of their mRNAs, and its expression is induced by inflammatory cytokines [12]. They proposed that miR-146a/b might regulate cytokine signaling in the immune response through a negative feedback regulation loop involving down-regulation of IRAK 1 and TRAF 6. Monticelli *et al.* demonstrated that miR-146 expression is higher in Th1 cell than in Th2 or naïve T cells [25], while Stanczyk *et al.* reported that miR-155 and 146a were intensely expressed in rheumatoid arthritis synovial fibroblasts and synovial tissues [24]. Nakasa *et al.* demonstrated that miR-146a is expressed in RA synovial tissues and showed that the miR-146a

expressing cells were primarily CD68+ macrophages, but also included several CD3+ T cell subsets and CD79a+ B cells [13].

Inflammatory cytokines also play a key role in OA cartilage degeneration. One of the most prominent catabolic cytokines playing a crucial role in OA is IL-1β. IL-1β not only promotes the release of degenerative enzymes such as MMPs and aggrecanases, but also inhibits the synthesis of extracellular matrix proteins by chondrocytes [26]. In the current study, we confirm that miR-146 is expressed following stimulation by IL-1β in chondrocytes isolated from normal cartilage. This strongly supports the hypothesis that miR-146a expression is induced in OA pathogenesis.

In our preliminary studies, miR-146a in OA cartilage was expressed at a significantly greater level than in normal cartilage when analyzed using real time PCR (data not shown). In the present study, we divided OA cartilage into three grades according to a modified Mankin score. MiR-146 was expressed intensely in low grade OA cartilage, and decreased with increasing cartilage degeneration. In each case, miR-146a was likely to be expressed at a higher level in the cartilage with lower expression of MMP13, and the expression of miR-146a decreased with increasing expression of MMP13. MiR-146a induced by inflammatory cytokines might play a role in repression of catabolic factors such as MMP13 through miR-146 negative feedback including down regulation of IRAK1 and TRAF6 in early OA cartilage. In late stage OA cartilage with low expression levels of miR-146a, cartilage degradation might progress due to loss of miR-146a acting as a repressor of catabolic signals. *In situ* hybridization of miR-146a in our study revealed that a greater number of miR-146a expressing chondrocytes were observed in the superficial zone with matrix degenerative changes. Proinflammatory cytokines such as IL-1β and TNFα were reported to be expressed by chondrocytes in the superficial zone, and these cells are sparsely distributed in the deep zone in OA specimens [27]. Far fewer miR-146a expressing chondrocytes were observed in the deep zone where the matrix appeared normal. In contrast, clustered chondrocytes surrounded by normal matrix expressed miR-146a. These results suggest that miR-146a is not expressed in normal chondrocytes, but starts to be expressed in chondrocytes which begin to undergo degenerative changes. We were unable to clarify the reason why miR-146a is expressed abundantly in early OA

cartilage, and its expression is decreased as the cartilage degrades. The target genes for miRNAs are estimated to range from one to hundreds based on target predictions using bioinformatics approaches [28]. Mir-146a might therefore have other target genes apart from IRAK1 and TRAF6 in cartilage, and play a role in the progression of OA.

To our knowledge, the present study is the first report to focus on miRNA expression in OA cartilage. Our results revealed that miR-146a is expressed intensely in low grade OA cartilage, and is induced by IL-1β. Our study shows that miRNA could be a novel player in the anabolic and catabolic signals of cartilage homeostasis. However, the function of miR-146 in OA pathogenesis still remains unclear. MiR-146 is reported to be a negative regulator in the inflammatory response, its expression induced by inflammatory cytokines [12, 21, 22]. There are several studies showing that chondrocytes in OA cartilage secrete MMP13 in response to IL-1 [26, 29, 30]. These reports support our speculation that miR-146 is a negative feedback regulator of MMP13 in OA cartilage. However, our results also raise the possibility that miR-146 may be an activator in early OA cartilage because of the high degree of chondrocyte activation at local sites in the early stages of OA. Direct proof is necessary to substantiate our speculation. This might be difficult to prove in human cartilage samples from individual patients that are sampled at one point in time, which is the limitation of our study. Quite recently, several therapeutic trials to regulate the endogenous miRNAs related to various diseases were conducted [31,32]. Further functional analysis of miR-146 in OA pathogenesis could provide a novel and reasonable system for OA treatment.

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Legends

Table 1

Clinical features of the patients in this study.

Figure 1

Quantitative reverse transcription-polymerase chain reaction analysis of the expression of mature miR-146a, Col2a1 and MMP13. The expression level of mature miR-146a, Col2a1, and MMP13 in each patient assessed using the modified Mankin scale (**A**), and in the 3 grades according to a modified Mankin scale ; Grade I: 0 - 5, grade II: 6 - 10, grade III: 11 - 14 (**B**). The expression of miR-146a and Col2a1 in grade I was significantly higher than in the other groups. In grades II and III, the expression of miR-146a and Col2a1 decreased with increasing MMP13 expression.

Figure 2

Safranin O staining and *in situ* hybridization of OA cartilage from patient 4 (**A**, **B**, **C**) with a lower expression level of mature miR-146a, patient 5 (**D**, **E**, **F**, **G**, **H**), with a higher expression level of mature miR-146a, and patient 6 with high Mankin score (**I**, **J**). In cartilage samples from patients 4 and 5, miR-146a-expressing chondrocytes can be observed in all layers (**A**, **B**). The miR-146a expressing cells in the cartilage of patient 4 are sparsely distributed compared to that of patient 5 (**B**). miR-146a is expressed in clustered chondrocytes (arrows indicate chondrocytes in **a** cluster)(**C**). The chondrocytes expressing miR-146a are likely to be frequently distributed in the superficial zone, where proteoglycans are depleted from the matrix (**F**; superficial, **G**; middle, **H**; deep layer in the cartilage from

patient 5). In the deep layer, chondrocytes surrounded by apparently normal matrix do not express miR-146a (arrows indicate chondrocytes without expression of miR-146a). In fibrillation site of cartilage from patient 6, clusterd chondrocytes expressed miR-146a (arrows), but there are few miR-146a expressing cells (**J**).Original magnification (**A**, **D**, **I**) X 40 ;bar: 50 μm, (**B**, **E**, **F**, **G**, **H**) X100 ;bar: 25 μm , (**C**) X 200 ; bar: 10 μm.

Figure 3

Induction of mature miR-146a expression in chondrocytes from normal cartilage samples stimulated with IL-1β. Mature miR-146a (**A**) and MMP13 (**B**) expression, as determined by real time PCR analysis. The expression of miR-146 was significantly upregulated in human chondrocytes after stimulation with IL-1β. MMP-13 expression was also induced after IL-1β stimulation, in the same way as miR-146. *p* values were determined by the Mann-Whitney U test.

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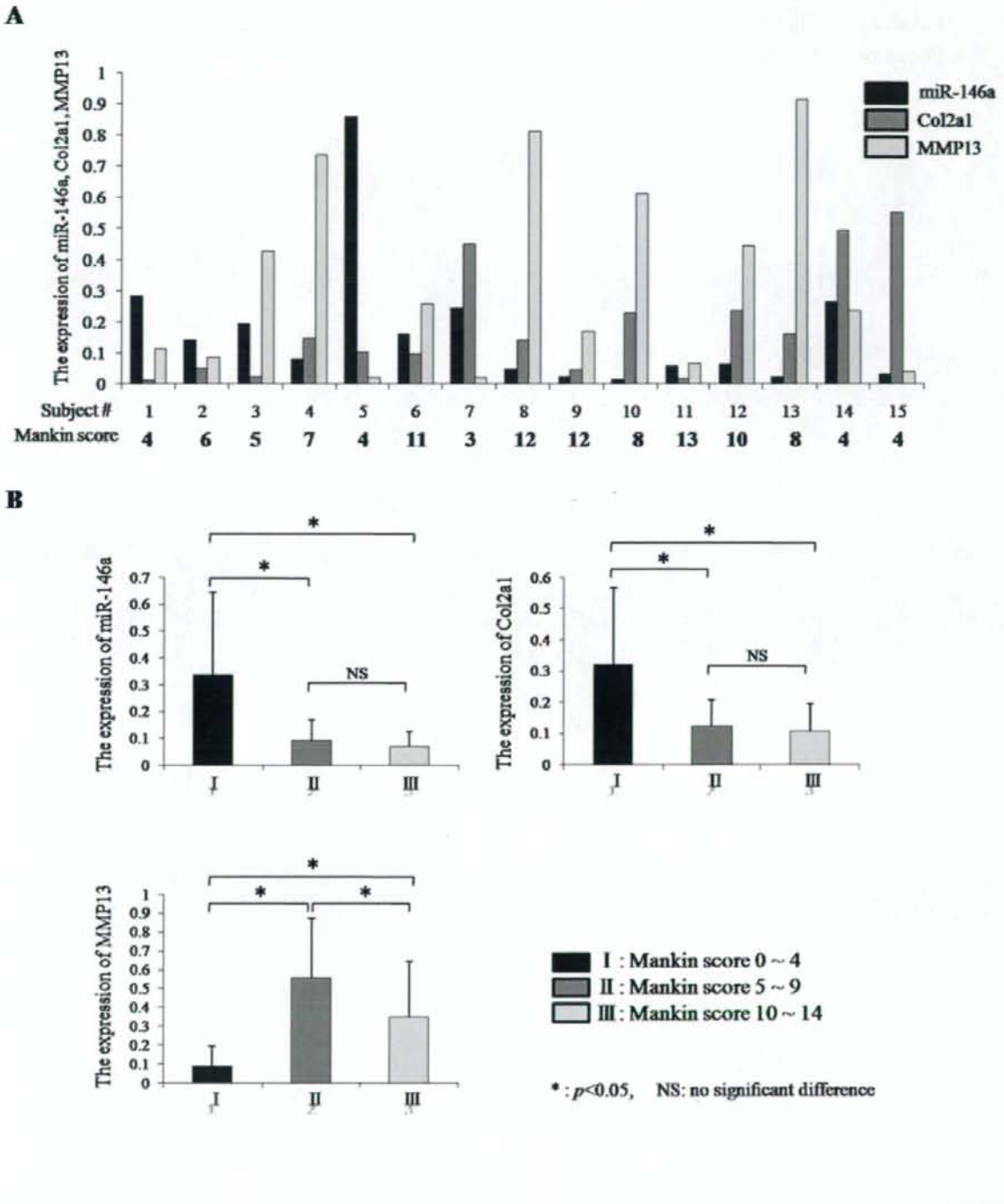
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The expression of miR-146a, Col2a1, MMP13
miR-146a
Col2a1
MMP13
Subject #
Mankin score
B
The expression of miR-146a
The expression of Col2a1
The expression of MMP13
NS
I : Mankin score 0 ~ 4
II : Mankin score 5 ~ 9
III : Mankin score 10 ~ 14
* : p<0.05, NS: no significant difference

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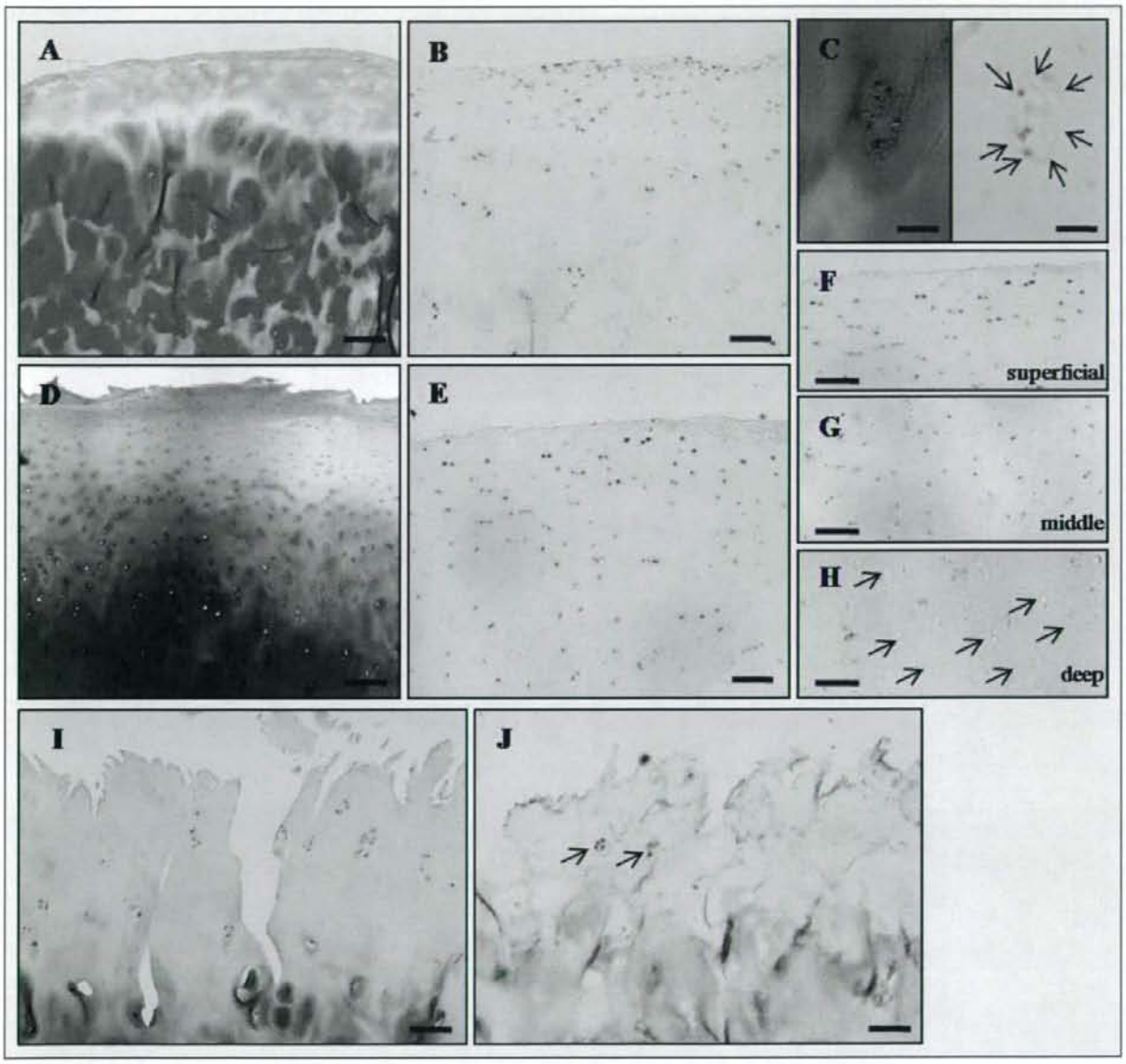
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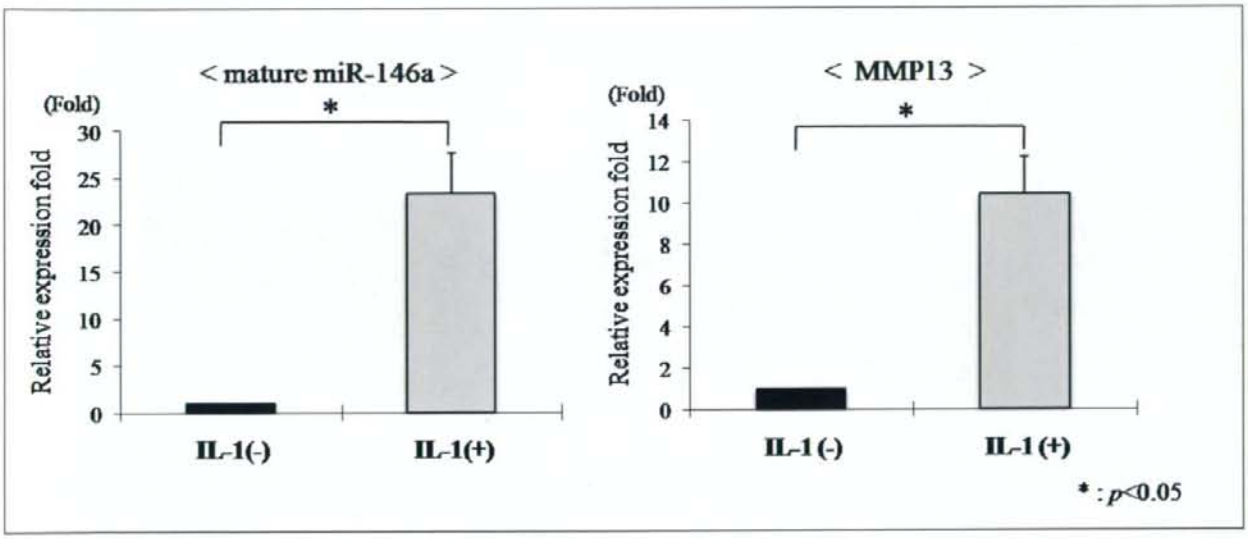
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25
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IL-1(+)
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Relative expression fold
14
12
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8
6
4
2
0
*
IL-1 (-)
IL-1 (+)
* : p<0.05

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Demographic features of the study subjects

subject	age	sex	source of cartilage	K/L score	CRP (mg/ml)	medication
1	18	female	patella	I	0	NSAIDs
2	51	female	femoral head	IV	0.3	NSAIDs
3	64	female	medial femoral condyle	IV	0	NSAIDs
4	76	female	femoral head	IV	0	NSAIDs
5	74	female	medial femoral condyle	IV	0.8	NSAIDs
6	71	male	femoral head	IV	0	NSAIDs
7	65	female	femoral head	IV	0	NSAIDs
8	79	female	femoral head	IV	0	NSAIDs
9	49	male	medial femoral condyle	III	0.7	none
10	59	female	medial femoral condyle	III	0	NSAIDs
11	75	female	medial femoral condyle	III	0	NSAIDs
12	63	female	femoral head	IV	0	NSAIDs
13	74	male	femoral head	IV	0.5	NSAIDs
14	73	female	femoral head	IV	0	NSAIDs
15	73	female	femoral head	IV	0.2	NSAIDs

K/L = Kellgren / Lawrence CRP = C-reactive protein
NSAIDs = nonsteroidal antiinflammatory drugs

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Smad3 activates the Sox9-dependent transcription on chromatin

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ABSTRACT

Transforming growth factor (TGF)-β has an essential role for the Sry-type high-mobility-group box (Sox)-regulated chondrogenesis. Chondrogenic differentiation is also controlled by chromatin-mediated transcription. We have previously reported that TGF-β-regulated Smad3 induces chondrogenesis through the activation of Sox9-dependent transcription. However, the cross-talk between TGF-β signal and Sox9 on chromatin-mediated transcription has not been elucidated. In the present study, we investigated the activity of Smad3, Sox9, and coactivator p300 using an in vitro chromatin assembly model. Luciferase reporter assays revealed that Smad3 stimulated the Sox9-mediated transcription in a TGF-β-dependent manner. Recombinant Sox9 associated with phosphorylated Smad3/4 and recognized the enhancer region of type II collagen gene. In vitro transcription and S1 nuclease assays showed that Smad3 and p300 cooperatively activated the Sox9-dependent transcription on chromatin template. The combination treatment of phosphorylated Smad3, Sox9, and p300 were necessary for the activation of chromatin-mediated transcription. These findings suggest that TGF-β signal Smad3 plays a key role for chromatin remodeling to induce chondrogenesis via its association with Sox9.

1. Introduction

Chondrogenesis is the fundamental process to form bones and articular surfaces. Mesenchymal condensation and the following chondrocyte differentiation are strictly regulated by several transcription factors and growth factors, such as Sry-type high-mobility-group box (Sox) genes and the transforming growth factor (TGF)-β superfamily, respectively. Sox5, 6, and 9 cooperatively regulate the sequential differentiation steps of chondrogenesis (Akiyama et al., 2002, 2004; Stricker et al., 2002). In these transcription factors, Sox9 has an essential role to initiate mesenchymal condensation and to maintain chondrogenic potential in early stages. The expression of α1 chain of type II collagen (Col2a1), a major component of cartilage extracellular matrix, is controlled by Sox9 through the Sox9-binding site on the Col2a1 enhancer region (Bell et al., 1997) and closely parallels that of Sox9 (Ng et al., 1997). The TGF-β superfamily including the two major families (TGF-β and bone morphogenetic protein) is a multifunctional growth factor for many cellular responses such as differentiation and proliferation (Heldin et al., 1997; Shi and Massagué, 2003). In chondrogenesis, TGF-β stimulation is necessary for primary chondrogenesis derived from mesenchymal stem cells (Pittenger et al., 1999). We previously described that TGF-β signal Smad3 promotes the early chondrogenesis through the activation of Sox9 (Furumatsu et al., 2005a). However, the precise mechanisms of Sox9 and TGF-β in the epigenetic regulation for initiating chondrogenesis are still unclear.

The epigenetic regulation is another dynamic system to control gene expression and other fundamental cellular processes, such as proliferation and differentiation (Li, 2002; Felsenfeld and Groudine, 2003; Jaenisch and Bird, 2003). Chromatin remodeling system including histone modification is the representative mechanism of epigenetics. The eukaryotic DNA and histones are packaged into chromatin as the nucleosome-repeated structure. Accesses of transcription factors and other regulators to DNA are highly restricted by chromatin structure. Many molecules have been revealed as important factors to form chromatin. Nucleosome assembly protein-1 (NAP-1) acts as a histone-shuttling protein (Ito

Abbreviations: AcCoA, acetyl-coenzyme A; ACF, ATP-utilizing chromatin assembly and remodeling factor; Col2a1, α1 chain of type II collagen; EMSA, electrophoretic mobility shift assay; MNase, micrococcal nuclease; MAPK, mitogen-activated protein kinase; NAP-1, nucleosome assembly protein-1; si-, small interfering; Sox, Sry-type high-mobility-group box; TβR-I(TD), constitutively active form of TβR-I; TGF, transforming growth factor.

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et al., 1996; Nakagawa et al., 2001). ACF (ATP-utilizing chromatin assembly and remodeling factor), consisting of Acf1 and ISWI subunits, assembles periodic nucleosome arrays on histone-attached DNA in an ATP-dependent process (Ito et al., 1999; Nakagawa et al., 2001). On the other hand, histone modification on chromatin, such as acetylation, enables transcription regulators to access to DNA sequences. DNA-binding transcription factors, such as CREB and MyoD, exert their transcriptional potential on histone-acetylated chromatin (Asahara et al., 2001; Dilworth et al., 2004). However, the relationship between chromatin-mediated transcription and signaling molecules is not elucidated. We previously reported that p300, which has an intrinsic histone acetyltransferase activity, directly associates with Sox9 (Tsuda et al., 2003) and activates the Sox9-dependent transcription on chromatin (Furumatsu et al., 2005b). In this study, we further analyzed the cross-talk between the Sox9-dependent transcription and TGF-β receptor-regulated Smad3 on chromatin using an in vitro chromatin assembly model.

The present study demonstrates that TGF-β-stimulated Smad3 activates the Sox9-dependent transcription on chromatin. This is the first report to explain the importance of TGF-β treatment in chromatin-mediated chondrogenesis.

2. Materials and methods

2.1. Cells, plasmids, si-RNA, and antibodies

A human chondrosarcoma cell line (SW1353) was used as an immature chondrogenic cell line. A plasmid encoding full-length of rat Sox9 and a small interfering (si-) RNA against Smad3 were used (Furumatsu et al., 2005a). p300 was a gift from Tso-Pang Yao. FLAG-tagged Smad3/4 and the constitutively active form of TβR-I [TβR-I(TD)] were generous gifts from Takeshi Imamura. pGL3-585E, which contains a mouse Col2a1 promoter and enhancer, was constructed with a pGL3-Basic (Promega) vector and used as a native Col2a1 reporter gene. 12 × 48-pGL3-P containing 12 sets of a 48-bp Col2a1 enhancer element was used as a reporter plasmid. PCR fragments of FLAG-tagged Sox9, FLAG-tagged Smad3, and Smad4 were subcloned into baculovirus expression vector pENTR3C (Invitrogen) as described (Furumatsu et al., 2005b). The following antibodies were used: FLAG M2, FLAG M2 affinity gel (Sigma), phospho-Smad2/3 (Santa Cruz), Smad2/3 (Upstate), Smad4 (Cell Signaling), and Sox9 (Chemicon).

2.2. Luciferase reporter assay

pGL3-585E and 12 × 48-pGL3-P were used as reporter genes for investigating the Sox9-dependent transcriptional activity. These reporter plasmids were different from our previous constructs (Furumatsu et al., 2005a). Appropriate plasmids (50 ng) and si-Smad3 (200 nM) were transiently transfected into SW1353 cells using FuGENE6 (Roche). pRL-CMV (10 ng, Promega) was used as an internal control. The cells were harvested for 24 h, and then the luciferase activities were analyzed using Dual-Luciferase Reporter Assay System (Promega). The assays were performed in triplicate.

2.3. Nuclear extract and immunoprecipitation

Nuclear extracts of SW1353 cells were prepared in 2× buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT]. Protein concentrations were measured by BCA protein assay kit (Bio-Rad). Immunoprecipitation analyses using purified recombinant proteins were performed with anti-Sox9 or Smad2/3 antibody in 1× buffer D as described previously (Furumatsu et al., 2005b). Briefly, indicated amounts of recombinant proteins and/or nuclear extracts were incubated for 1 h at 25 °C. Ten percent volume of reaction mixture was loaded as an input fraction. Half of the mixture was incubated with each antibody and protein A beads (Sigma) for 1 h at 4 °C. Remaining mixture was incubated with rabbit IgG as a control.

2.4. Purification of histones and recombinant proteins

Core histones were purified from HeLa nuclear pellets and dialyzed in HEG buffer [10 mM HEPES (pH 7.6) 10% glycerol, 50 mM KCl, 0.1 mM EDTA]. Baculovirus of histidine-tagged NAP-1, FLAG-tagged ISWI, and Acf-1 were kindly gifts from Takashi Ito and used as chromatin assembling molecules (Ito et al., 1999, 2000). The baculovirus expression vectors carrying Sox9 and Smad3/4 were constructed using BaculoDirect Systems according to the manufacturer's protocol (Invitrogen). Recombinant NAP-1, recombinant ACF complex (FLAG-tagged ISWI and untagged Acf-1), FLAG-tagged p300, FLAG-tagged Sox9, and Smad3/4 complex (FLAG-tagged Smad3 and untagged Smad4) were produced in Sf9 cells (Invitrogen) and prepared as described previously (Furumatsu et al., 2005b). Recombinant Smad3/4 was purified after 30-min-treatments of TGF-β3 (R&D). Purified proteins were assessed by silver stain (BioRad) and Western blotting analyses.

2.5. Electrophoretic mobility shift assay (EMSA)

The Col2a1 enhancer probe containing the Sox9-binding site (in capital letters) was generated by annealing the following oligonucleotides: 5′-gcgcttgagaaaagcccCATTCATgagaggc-3′ and 5′-gcctctcATGAATGgggctttttctcaagcgc-3′. Probes were ^{32}P end-labeled using T4 polynucleotide kinase (Invitrogen). Purified Sox9 (30 ng) was incubated with the labeled probe (0.8 pmol). The unlabeled Col2a1 enhancer probe (16 pmol) was used as a competitor. In supershift analysis, 15 min treatment with anti-Sox9 antibody (0.2 μg) was performed before protein–DNA binding reaction.

2.6. Chromatin assembly and micrococcal nuclease (MNase) assay

Chromatin assembly and MNase digestion analyses were performed as described (Asahara et al., 2002) by using 12 × 48-pGL3-P. For chromatin reconstitution, standard reactions (20 μl) containing plasmid (150 ng), histones (100 ng), NAP-1 (500 ng), ISWI/Acf-1 (0.65 ng each), ATP (3 mM), and ATP regeneration systems (30 mM phosphocreatine and 20 ng creatine phosphokinase) were incubated at 30 °C for 4 h. In MNase assay, chromatinized plasmids (300 ng) were digested with MNase (0, 0.02, and 0.04 U/15 μl) for 5 min at 37 °C.

2.7. In vitro transcription and S1 nuclease assay

After chromatin assembly, standard reactions (12 × 48-pGL3-P, 150 ng) were incubated with Sox9 (10 ng), Smad3/4 (100 ng), p300 (40 ng), and acetyl-coenzyme A (AcCoA, 5 μM) for 30 min at 30 °C. For in vitro transcription, nuclear extracts from SW1353 cells (30 μg) were added and incubated with rNTPs at 30 °C for 40 min. In vitro-transcribed RNAs were recovered and subjected to S1 nuclease analyses using the specific primer (49 bp) against 12 × 48-pGL3-P luciferase gene as described (Furumatsu et al., 2005b). RNAs were annealed with ^{32}P end-labeled primers (0.2 pmol each) for 12 h, and then digested with 50 units of S1 nuclease (Invitrogen) for 30 min at 37 °C. The protected fragments were run on 8% denaturing polyacrylamide gels and visualized by autoradiography. Each experiment was performed at least three times.

3. Results

3.1. Smad3 stimulates the Sox9-mediated transcription in a TGF-β-dependent manner

To assess the fundamental role of Smad3 in chromatin remodeling during early chondrogenesis, we first analyzed the effect of Smad3 in the Sox9-regulated transcription using newly constructed reporter plasmids. Overexpressed Smad3 stimulated the transcriptional activity of Col2a1 reporter gene (Fig. 1A, pGL3-585E) in a Sox9-depedent manner (Fig. 1B). In addition, the effect of Smad3 was enhanced by the cotransfection of constitutively active form of TGF-β receptor I [TβR-I(TD)]. Twelve copies of the Sox9-binding fragment dramatically induced the Sox9-regulated transcription in reporter assays (Fig. 1C, 12 × 48-pGL3-P). Smad3 also activated the transcription of 12 × 48-pGL3-P in Sox9- and TGF-β-dependent manners. These findings suggest that Smad3 may act as a chromatin remodeling factor in chondrogenesis.

3.2. TGF-β and Smad3 are necessary for the activation of Sox9-dependent transcription

To investigate the effect of Smad3 itself in this reporter assay system, we used a si-RNA fragment against Smad3 as an inhibitor. The activities of Sox9-regulated transcription were stimulated by the addition of Smad3 in a dose-dependent fashion (Fig. 2A and B). si-Smad3 decreased the effect of activated TGF-β receptor and overexpressed Smad3 in Sox9-regulated reporters to the basal levels. However, si-Smad3 did not inhibit the Sox9-induced transactivation. These results prompted us to analyze the function of TGF-β-stimulated Smad3 and Sox9-related transcriptional apparatus on chromatin in chondrogenesis.

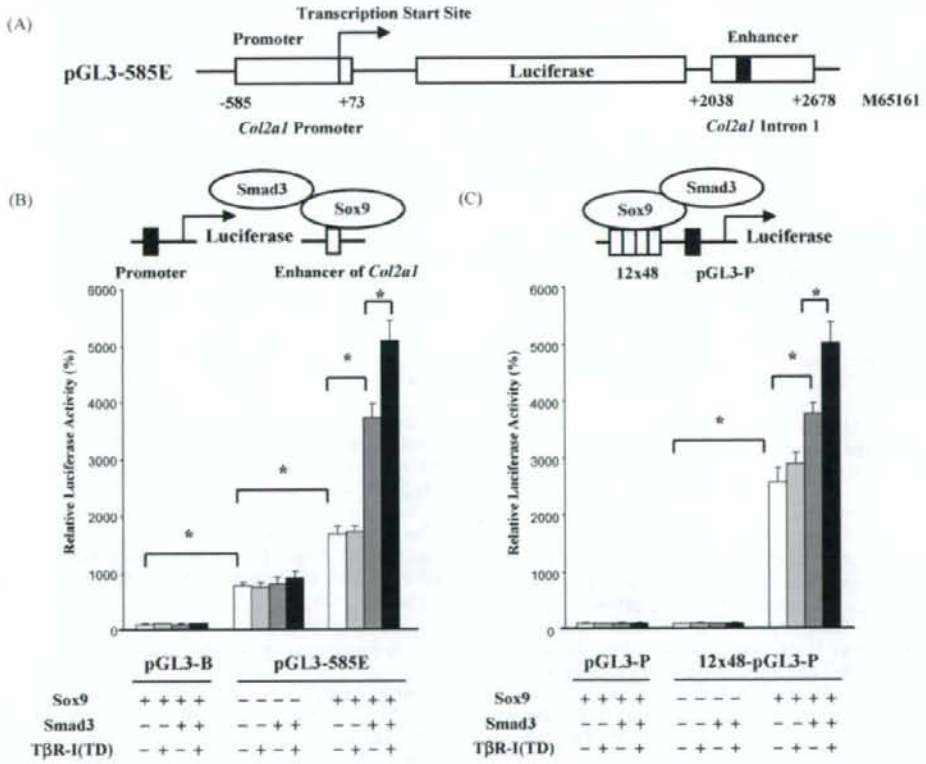

Fig. 1. Smad3 enhances the Sox9-mediated transcription in a TGF-β-dependent manner. (A) A schematic characterization of pGL3-585E which contains a native promoter and enhancer of mouse Col2a1 gene. Numbers indicate the distance from the transcription start site on mouse Col2a1 gene (National Center for Biotechnology Information, M65161). Black box denotes the SOX9-binding site on the enhancer region of Col2a1 intron 1. (B) Transient transfections of Sox9, Smad3, and TβR-I(TD) did not increase luciferase activities of pGL3-B plasmids in SW1353 cells (pGL3-B). In pGL3-585E systems, Sox9 enhanced a relative luciferase activity to a level as high as 2.2-fold over the control. Cotransfection of Smad3 augmented a luciferase activity up to 2.3-fold higher level of Sox9-transfected cells. The additional transfection of constitutively active form of TβR-I(TD) induced an approximately 36% increase of the activity in Sox9- and Smad3-transfected SW1353 cells. Luciferase activities of pGL3-585E were not increased in the absence of Sox9. Note that Smad3 and TβR-I(TD) synergistically activated the native Col2a1 reporter-mediated transcription in a Sox9-dependent manner. (C) The activity of 12 × 48 pGL3-P was enhanced by the addition of Sox9 up to 27.5-fold levels of the control. Smad3 increased the 12 × 48 pGL3-P-based luciferase activity up to 1.5-fold higher level in the presence of Sox9. TβR-I(TD) also induced 33% increase of the activity of Smad3-transfected cells in the presence of Sox9. However, the additional increase of luciferase activity was not observed in pGL3-P-transfected cells. Relative luciferase activities were calculated using the activity of pGL3-P as a control (100%). A schematic illustration of each reporter assay system is placed on the top of each figure (B and C). *Statistical significances ($p < 0.05$) were observed between the indicated bars with the Mann–Whitney *U*-test. Error bars, S.D.

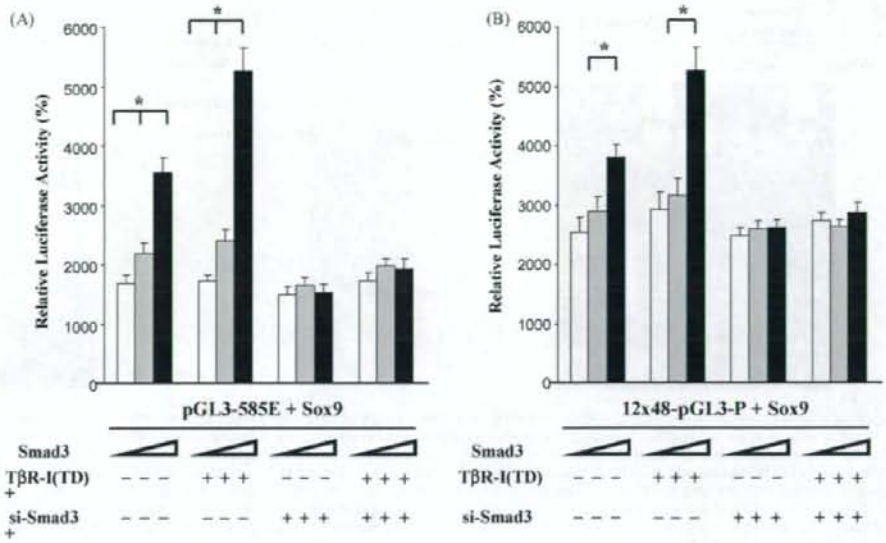

Fig. 2. Smad3 has an essential role for TGF-β-stimulated transactivation in the Sox9-regulated gene expression. (A) Smad3 enhanced the Sox9-dependent transcription in a dose-dependent manner. Smad3 and TβR-I(TD) synergistically increased the luciferase activity in pGL3-585E reporter systems. si-RNA against Smad3 (si-Smad3) totally inhibited the synergistic effects of Smad3 and TβR-I(TD). Note that si-Smad3 did not inhibit the Sox9-induced transactivation of reporter genes. (B) In 12 × 48 pGL3-P reporter systems, Smad3 and TβR-I(TD) cooperatively stimulated the relative luciferase activity up to 1.7-fold higher level in the presence of Sox9. A dose-dependent effect of Smad3 was observed. However, the increase of luciferase activity was suppressed by si-Smad3 in Smad3-transfected cells. Relative luciferase activities were calculated using the activity of pGL3-B (A) or pGL3-P (B) as a control (100%). Triangular boxes denote the transfection volume of Smad3 expression plasmid (0, 25, and 50 ng). *Statistical significances ($p < 0.05$) were observed between the indicated bars with the Mann–Whitney *U*-test. Error bars, S.D.

3.3. Recombinant Sox9 associates with p300 and binds to the Col2a1 enhancer in vitro

To examine the role of Sox9-associated transcriptional complex (Sox9, p300, and Smad3) on chromatin, we purified histones from HeLa cells, chromatin assembly-related molecules (NAP-1 and ACF complex), Sox9, p300, and Smad3 as described in Section 2. Purified NAP-1 and ACF sufficiently assembled chromatin under histone-containing conditions. Chromatin assembling abilities of these molecules were estimated by MNase digestion assays (Fig. 3A). Recombinant Sox9 purified from Sf9 cells associated with recombinant p300 in vitro (Fig. 3B). Recombinant Sox9 also bound with high affinity to the Col2a1 enhancer probe, which contains the Sox9-binding sequence, in EMSA (Fig. 3C).

3.4. TGF-β-stimulated Smad3 and p300 cooperatively activate the Sox9-dependent transcription on chromatin

For in vitro transcription analyses after chromatin assembly (Fig. 4A), we assessed the complex formation of Smad3 and Smad4. Smad3 purified from the nuclear fraction of TGF-β-treated Sf9 cells was a phosphorylated form of Smad3 (Fig. 4B). Smad4 was also detected in the same coimmunoprecipitated fraction using anti-FLAG M2 affinity gel (Fig. 4B). This result demonstrated that phosphorylated Smad3 was transferred into the nucleus with Smad4 by TGF-β treatment. In addition, purified Smad3/4 associated with recombinant Sox9 and p300 in vitro (Fig. 4C). Here we investigated the effect of phosphorylated Smad3 in the Sox9-dependent transcription on chromatin. In vitro transcription analyses on chromatinized templates revealed that the combination of Sox9, Smad3/4, and p300 were necessary for the activation of chromatin-mediated transcription (Fig. 4D). These findings suggest that the Sox9-dependent chondrogenesis might be strictly controlled by TGF-β signal Smad3 and chromatin remodeling factor p300.

4. Discussion

The present study indicates that TGF-β receptor-regulated Smad3 and p300 cooperatively activate the Sox9-dependent transcription on chromatin. The TGF-β signal plays an essential role to induce primary chondrogenesis (Pittenger et al., 1999; Heng et al., 2004). However, the differentiation of chondrocyte is regulated by the conflictive effects of TGF-β. TGF-β3 enhances the early chondrogenesis derived from mesenchymal stem cells (Fan et al., 2008). The short-term treatment with TGF-β3 has been reported to maintain a chondrogenic phenotype (Mehlhorn et al., 2006). On the other hand, TGF-β inhibits chondrocyte maturation at the late stage (Ballock et al., 1993; Ferguson et al., 2000). We previously described that TGF-β signal Smad3 promotes the early chondrogenesis through the activation of Sox9 (Furumatsu et al., 2005a). However, the cross-talk between TGF-β signal and Sox9 in the epigenetic regulation for initiating chondrogenesis is still unclear. Here, we further analyzed a crucial role of Smad3 in the Sox9-dependent chondrogenesis on chromatin. In this study, Smad3 enhanced the Sox9-mediated transcription in luciferase reporter assay systems (Fig. 1B and C). The increase of relative luciferase activity with Smad3 was higher in pGL3-585E, which contains a native set of Col2a1 promoter and enhancer, than in 12 × 48-pGL3-P systems. These findings might be caused by the binding affinity of Sox9 against each reporter plasmid. The activity of 12 × 48-pGL3-P containing high copies of Sox9-binding site might be already excited by the cotransfection of Sox9. A dose-dependent transactivation by Smad3 was observed in both systems,