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Transcriptional induction of SOX9 by NF-κB family member RelA in chondrogenic cells

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Summary

Objective: Although SOX9 is a key molecule for chondrogenic differentiation, little is known about the upstream signal. The present study attempted to identify transcription factors to induce SOX9 expression and examined the mechanism.

Methods: Sequences of about 1 kb of 5′-end flanking regions were compared between human and mouse SOX9 genes. *In vivo* localization was examined by immunohistochemistry in the limb cartilage of fetal mice. Promoter activities of the SOX9, SOX6, and type II collagen (COL2A1) genes were determined in human non-chondrogenic HeLa cells and mouse chondrogenic ATDC5 cells transfected with a luciferase-reporter gene containing the promoter fragments. Protein–DNA binding was examined by electrophoretic mobility shift and chromatin immunoprecipitation assays. The chondrogenic differentiation was assessed by endogenous SOX9, SOX6, and COL2A1 mRNA levels, and by Alcian blue staining and alkaline phosphatase activity.

Results: Among transcription factors whose binding motifs were identified in the highly-conserved regions between human and mouse SOX9 promoters, a nuclear factor kappa B (NF-κB) member RelA strongly activated the promoter activity. RelA and SOX9 were co-localized in the limb cartilage. Deletion, mutagenesis, and tandem-repeat analyses identified the core region responsive to RelA at the NF-κB binding motif to be around −250 bp of the human SOX9 promoter, and this was confirmed to show specific binding to RelA. RelA induced the chondrogenic differentiation parameters in HeLa and ATDC5 cells.

Conclusion: We have identified RelA as a transcriptional factor for SOX9 induction and chondrogenic differentiation *via* binding to an NF-κB binding motif in the SOX9 promoter.

Key words: SOX9, RelA, Chondrocyte, Transcription.

Introduction

Skeletal development is initiated by the recruitment of undifferentiated mesenchymal cells into condensations, which differentiate into pre-chondrocytes and then chondrocytes that produce cartilage-specific extracellular matrix proteins like type II collagen (COL2A1)[1]. Sex-determining region Y-type high mobility group box 9 (SOX9) is expressed in the mesenchymal cells, pre-chondrocytes and chondrocytes[2,3], and functions as a master transcriptional activator of COL2A1 and other chondrocyte-specific matrix proteins, in cooperation with the co-factors SOX6 and L-SOX5[4–9]. Expressions of the SOX6 and L-SOX5 are also controlled by SOX9[4,10]. Studies in mice have shown that SOX9 is essential for multiple steps in the chondrogenic differentiation pathway: conditional ablation of the SOX9 gene in the limb buds before mesenchymal condensation resulted in a complete absence of chondrocytes, whereas the conditional ablation after mesenchymal condensation resulted in a severe generalized chondrodysplasia[10–12]. In humans as well, heterozygous mutations of the SOX9 gene cause a severe chondrodysplasia, known as campomelic dysplasia[11,13]. Furthermore, we previously reported that SOX9 in combination with SOX6 and L-SOX5 (the SOX trio) stimulated chondrogenesis even from non-chondrogenic cells of mouse and human origins, implicating a possible clinical application of this signal to cartilage regeneration[14]. Despite the substantial information about the expression profiles and the target genes of SOX9, little is known about the upstream signaling or the functional regulation of the SOX9 promoter. To identify transcription factors that induce SOX9 expression, the present study initially compared the genomic sequences of proximal promoter regions between human and mouse SOX9 genes, and identified several highly-conserved regions containing putative transcription

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factor-binding motifs. Among the candidate transcription factors, our further analyses found that nuclear factor kappa B (NF-κB) family member RelA (NF-κB p65) most strongly activated the human SOX9 promoter activity.

The NF-κB family of transcription factors plays a crucial role in a broad range of biological processes, including immune responses, inflammation, proliferation, differentiation and apoptosis[15–17]. The family includes RelA, RelB, Rel, p105/p50 and p100/p52, each of which contains a Rel homology domain that mediates DNA binding and dimerization. Numerous studies have established that IκB proteins are phosphorylated and degraded by a large protein complex IκB kinase (IKK) in response to several signals, thereby allowing free NF-κB complexes to translocate from the cytoplasm into the nucleus, leading to target gene transactivation[18,19]. The NF-κB family genes are expressed in the chick limb cartilage, and the blockage of the NF-κB activity caused the arrest of the limb outgrowth[20]. The IKKα-deficient mice also exhibited suppression of limb outgrowth[21,22]. Since these lines of evidence implicate the interaction between NF-κB and SOX9 signals during skeletal development, the present study investigated the mechanism underlying the transcriptional regulation of the SOX9 promoter by RelA.

Materials and methods

COMPARISON OF THE PROXIMAL PROMOTER SEQUENCES OF THE HUMAN, MOUSE AND CHICK SOX9 GENES

We compared the sequences of the 5′-end flanking regions relative to the transcription start site among 4 kb human, 4 kb mouse and 300 bp chick SOX9 gene, using BLASTN search[23]. The detected sequences were aligned by the Vector-NTI software (Invitrogen), and the transcription factor-binding motifs were predicted using the TFSEARCH web site (Computational Biology Research Center, AIST, Japan).

CELL CULTURES

The human epithelial cell line HeLa (RIKEN Cell Bank, Tsukuba, Japan) was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Mouse chondrogenic ATDC5 cells (RIKEN Cell Bank) were grown and maintained in DMEM/F12 (1:1) with 5% FBS. To induce chondrogenic differentiation, ATDC5 cells were cultured in the presence of insulin-transferring-sodium selenite media supplement (ITS) (Sigma) for 3 weeks and replaced by αMEM/5% FBS with 4 mM inorganic phosphate (Pi) for 2 d[24].

CONSTRUCTION OF EXPRESSION VECTORS

Full-length human cDNA sequences of the transcription factors were polymerase chain reaction (PCR)-amplified and cloned into pCMV-HA vector (Clontech, Palo Alto, CA, USA). The Gene Bank access numbers are as follows: NFAT1 NM_012340.3, NFAT2 NM_006162.3, NFAT3 NM_004554.4, NFAT4 NM_004555.2, NFAT5 NM_138714, Fos NM_005252.2, Jun NM_002228.3, Fra-1 NM_005438.3, RelA NM_021975.2, RelB NM_006509.2, Rel NM_002908.2, p105 NM_003998.2, p100 NM_001077493.1, ATF1 NM_005171.3, ATF2 NM_001880.2, ATF4 NM_001675.2, ATF6 NM_007348.2, ATF7 NM_001130059.1, CREB NM_004379.2, C/EBPα NM_004364.2, C/EBPβ NM_005194.2, C/EBPδ NM_005195.3, C/EBPε NM_001805.2, GATA-1 NM_ 002049.3, GATA-2 NM_032638.3, GATA-3 NM_001002295.1, GATA-4 NM_002052.3, GATA-5 NM_080473.4, GATA-6 NM_005257.3. The primer sequences are available upon request.

LUCIFERASE ASSAY

The human SOX9 promoter region from −927 to +84 bp relative to the transcriptional start site (TSS) was obtained by PCR using human genomic DNA as a template and were cloned into the EcoRI and HindIII sites of the modified pGL3 vector containing additional cloning sites between the XhoI and HindIII sites of the original plasmid, the pGL3-basic vector (Promega, Madison, WI, USA). Deletion and mutation constructs were created by PCR technique. Tandem-repeat constructs were created by ligating the double strand oligonucleotides into EcoRI site of the modified pGL3 vector. Transfection of HeLa and ATDC5 cells was performed in quadruplicate in 48-well plates using FuGENE 6 transfection reagent (Roche, Mannheim, Germany): FuGENE 6 with a total amount of 150 ng of plasmid DNA, 100 ng of pGL3 reporter vector, 50 ng of effector vector, and 4 ng of pRL-TK vector (Promega) for internal control per well. Cells were harvested 48 h after the transfection. The luciferase assay was performed with a dual-luciferase-reporter assay system (Promega) using a GloMax 96 Microplate Luminometer (Promega). The results were shown as the ratio of the firefly activities to the renilla activities. For the SOX6 promoter assay, a luciferase-reporter construct containing the human SOX6 promoter and exon 1 region (−517 to IVS1 + 23) was generated and transfected in HeLa and ATDC5 cells as reported previously[25]. For the COL2A1 promoter assay, a luciferase-reporter construct containing the four repeats of the 49 bp SOX9 enhancer and the basal promoter (from −183 to +23) in the human COL2A1 gene was generated and transfected in the cells[26].

IMMUNOHISTOCHEMISTRY

Tissues from C57BL6 mouse embryos (E17.5) were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) overnight at 4°C, embedded in paraffin and cut into 5 μm sections. Sections were incubated overnight at 4°C with primary antibodies to RelA (C-20) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SOX9 (1:500; Santa Cruz Biotechnology), as well as the non-immune serum as the control. The localizations were detected with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

RelA protein was prepared by *in vitro* translation using the TNT T7 Coupled Reticulocyte Lysate System (Promega) and pCITE4 vector (Novagen, Milwaukee, WI, USA) into which RelA complementary DNA was cloned. The translation product was verified by Western blotting. Nuclear extracts were prepared from undifferentiated and differentiated ATDC5 cells before and after the culture with ITS for 3 weeks and Pi for 2 d, respectively. EMSA was carried out using a DIG gel shift kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Binding reactions were incubated for 30 min at room temperature. For competition analyses, 100-fold excess of unlabeled competitor probe was included in the binding reaction. For the supershift experiments, 1 μL of the antibody to RelA (C-20) above was added after 30 min of the binding reaction, and the reaction was incubated for an additional 30 min at room temperature. Samples were loaded onto Novex 6% TBE gels (Invitrogen), and electrophoresed at 100 V for 60 min.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

The ChIP assay was performed with a OneDay ChIP kit (Diagenode, Liege, Belgium) according to the manufacturer's instructions. *In vivo* cross-linking was performed 2 d after the transfection of HeLa cells with EV, RelA using FuGENE 6, and then the lysates were sonicated to shear genomic DNA. For immunoprecipitation, an antibody to RelA and the control normal rabbit immunoglobulin G (IgG) were used. Two primer sets, one spanning (−478/−239 bp) and the other not spanning (−3781/−3625 bp) the identified NF-κB motif, were employed. PCR was performed using Ex Taq (Takara Bio, Otsu, Japan) in the presence of 10% dimethyl sulfoxide.

GENE TRANSFER

For transient gene transfer, 2×10^5 HeLa cells were cultured in 6-well plates to subconfluency, and transfected with 1 μg of expression vector of RelA or the control empty vector (EV) using FuGENE 6. After 48 h, total mRNA of harvested cells was extracted and analyzed by real-time RT-PCR as described below.

Production of retroviral vectors was performed as described previously[27]. For retroviral gene transfer, 2×10^6 Plat-E cells were plated in 6-well plates, transfected with 2 μg pMx vector of RelA or the control green fluorescent protein (GFP) using FuGENE 6, and the conditioned medium was collected after 48 h. On the day before retroviral transfection, 3×10^5 of ATDC5 cells were plated onto a 60-mm culture dish. For the transfection, 4 mL of the conditioned medium containing the retrovirus was added to the cells with 32 μg of polybrene. Selection of the retrovirus-introduced cells was started 48 h after transfection in the medium containing 10 μg/mL of blasticidin.

REAL-TIME RT-PCR

Total RNA from cells was isolated with an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and an aliquot (1 μg) was reverse-transcribed with QuantiTect Reverse Transcription (Qiagen) to make single-stranded cDNA. Real-time RT-PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR Master Mix (Qiagen)

according to the manufacturer's instructions. Standard plasmids were synthesized with a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. All reactions were run in quadruplicate. Copy numbers of target gene messenger RNA (mRNA) in each total RNA were calculated by reference to standard curves and were adjusted to the human or mouse standard total RNA (ABI) with the human GAPDH or rodent GAPDH as an internal control. The primer sequences are available upon request.

CHONDROCYTE DIFFERENTIATION ASSAYS

For the Alcian blue staining, the cells were fixed with 10% (vol/vol) formaldehyde, and stained with 0.3% Alcian blue 8GS (Fluka, Buchs, Switzerland) in 0.1 N HCl. Alkaline phosphatase (ALP) staining was performed by a solution containing 0.01% naphtol AS-MX phosphate disodium salt, 1% *N,N*-dimethyl-formamide and 0.06% fast blue BB (Sigma). ALP activity was measured with a Lab Assay ALP kit (Wako, Osaka, Japan).

Results

IDENTIFICATION OF TRANSACTIVATORS OF THE SOX9 PROMOTER BY COMPARISON BETWEEN HUMAN AND MOUSE GENES

To identify transcription factors that activate the SOX9 promoter, we initially performed exhaustive comparison of the sequences of about 4 kb of the 5′-end flanking regions between human and mouse genes, and found that the 1.0 kb upstream of the TSS was about 80% conserved between the species. The sequence search identified the binding motifs of NFAT, AP-1, NF-κB, Sp1, CREB/ATF, CCAAT, and GATA in the highly-conserved regions (Fig. 1). The sequence of the motifs at the proximal region showed good conservation in the chick gene as well as in the human and mouse ones.

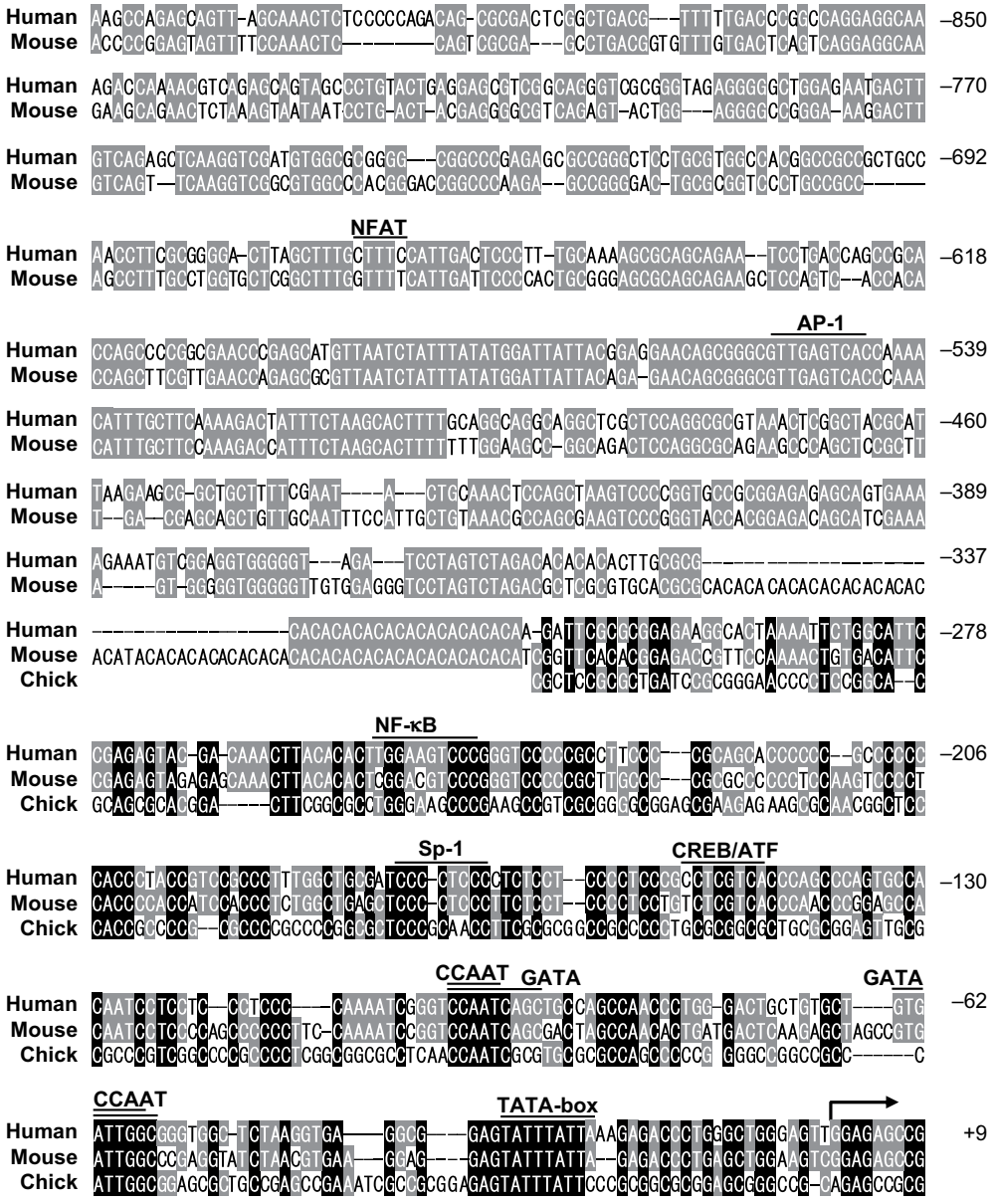


Fig. 1. Comparison of the proximal promoter sequences of the human, mouse and chick SOX9 genes. Conserved nucleotides in the two or three species are denoted by white letters shaded in gray or black, respectively. An arrow shows the transcription start sites of the human and mouse genes. Putative binding motifs of transcription factors identified in highly-conserved regions are indicated.

We therefore created expression vectors of the transcription factors whose binding motifs were identified: NFAT1, NFAT2, NFAT3, NFAT4, NFAT5, Fos, Jun, Fra-1, RelA, RelB, Rel, p105, p50, p100, p52, ATF1, ATF2, ATF4, ATF6, ATF7, CREB, C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, GATA-1, GATA-2, GATA-3, GATA-4, GATA-5, and GATA-6; and transfected them in human non-chondrogenic HeLa cells and mouse chondrogenic ATDC5 cells with a luciferase-reporter construct containing the 5′-end flanking region (−927/+84) of the human SOX9 gene (Fig. 2). Among the transcription factors, the luciferase-reporter assay revealed that an NF-κB family member RelA strongly activated the SOX9 promoter activity in both HeLa and ATDC5 cells, causing us to speculate that RelA is a potent transcriptional factor for SOX9 induction.

IN VIVO LOCALIZATION OF RelA AND SOX9 IN THE LIMB CARTILAGE

To know the possible interaction between RelA and SOX9 *in vivo*, we then examined the expression patterns of RelA and SOX9 in the limb cartilage of fetal mice (Fig. 3). Both RelA and SOX9 were well co-localized in resting chondrocytes, as well as in pre-hypertrophic and hypertrophic chondrocytes, suggesting the molecular interaction between RelA and SOX9 during the chondrocyte differentiation.

IDENTIFICATION OF THE CORE REGION RESPONSIVE TO RelA IN THE SOX9 PROXIMAL PROMOTER

To identify the region responsive to RelA in the human SOX9 proximal promoter, we performed the deletion analysis of the luciferase assay in HeLa and ATDC5 cells

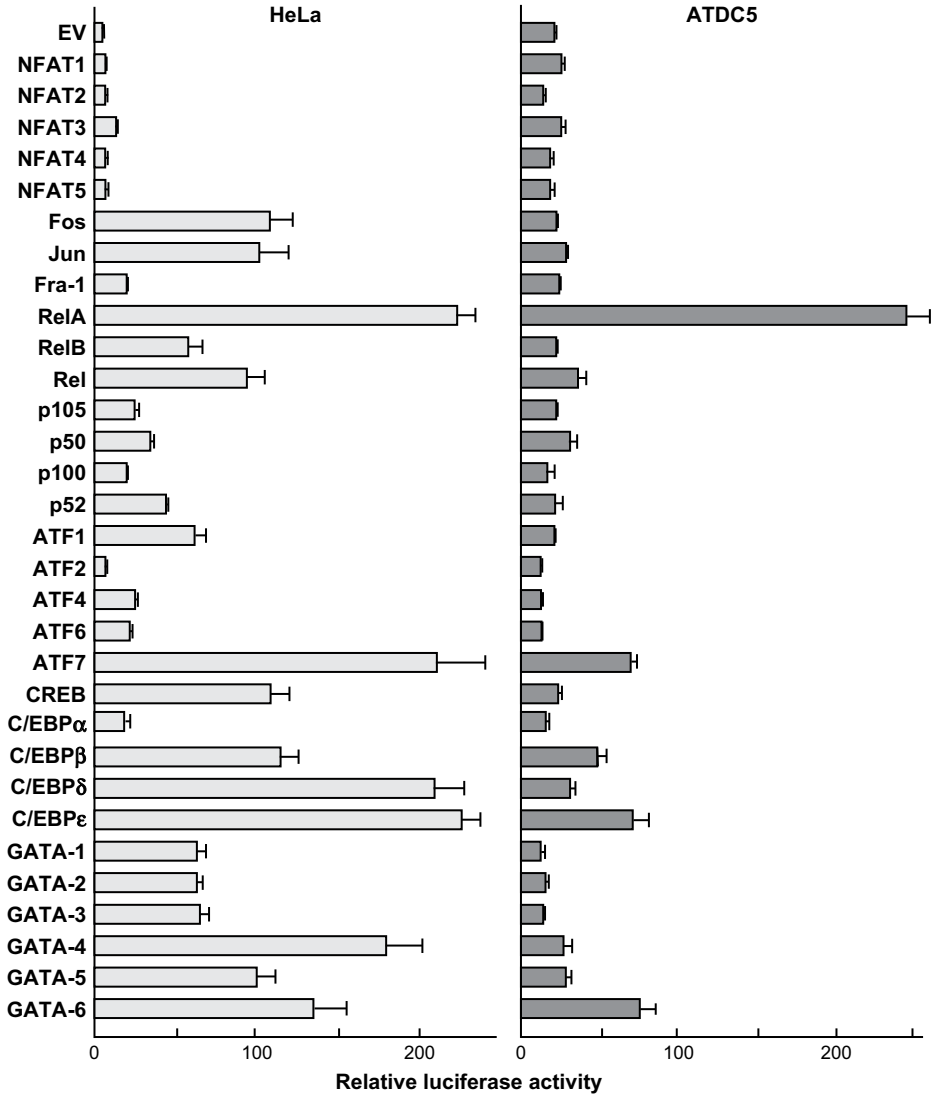


Fig. 2. Luciferase assay for the human SOX9 promoter activity by the transcription factors whose binding motifs were identified in Fig. 1. Human non-chondrogenic HeLa cells and mouse chondrogenic ATDC5 cells co-transfected with luciferase-reporter constructs containing the proximal 5′-end flanking region (from −927 to +84 bp relative to the TSS) of the human SOX9 gene, and the effecter vectors or the control EV. Data are shown as means (bars) ± S.E.M. (error bars) of relative luciferase activity (the ratio of the firefly activities to the renilla activities) for 4 wells/group.

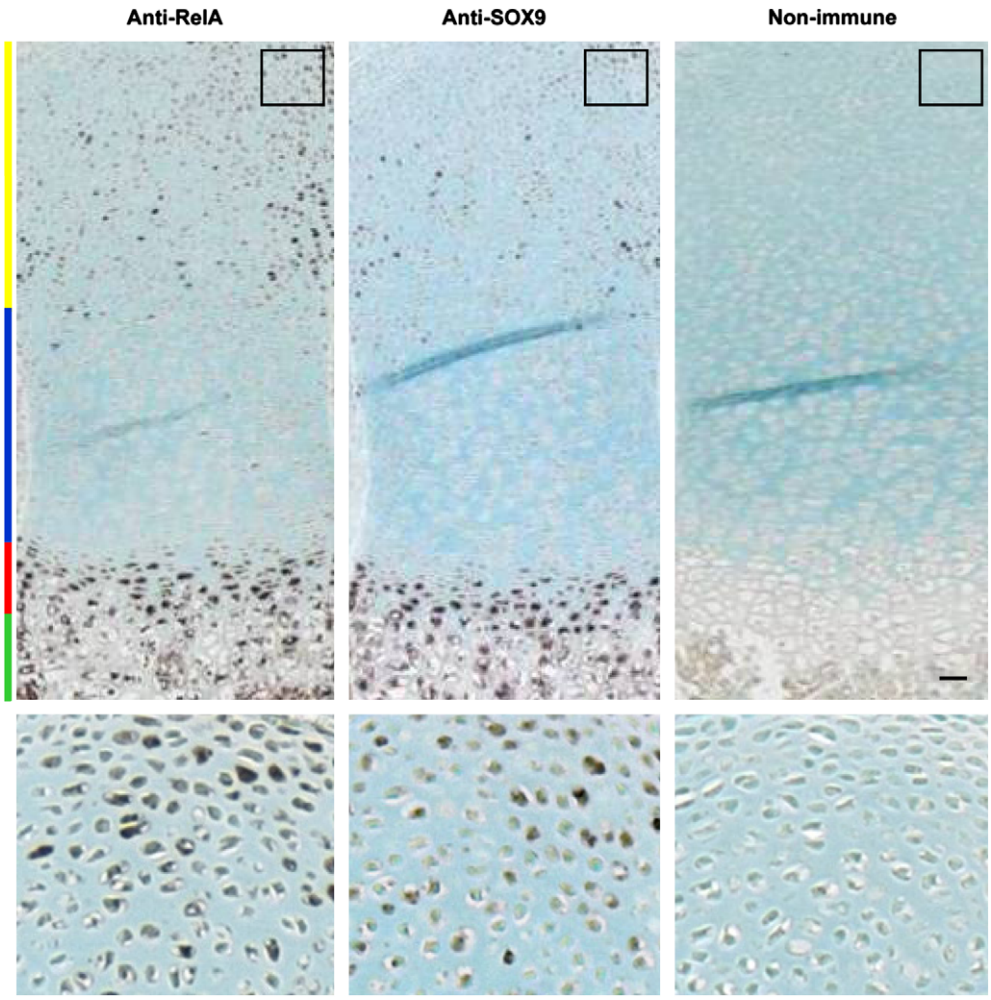


Fig. 3. Localizations of RelA and SOX9 by immunohistochemistry using the antibodies and the control non-immune serum in the proximal tibial limb cartilage of fetal mice (E17.5). Inset boxes in the top panels indicate the regions of the respective lower figures. Yellow, blue, red and green bars to the left of the panels indicate layers of resting, proliferative, pre-hypertrophic and hypertrophic zones, respectively. Scale bar, 100 μm.

co-transfected with RelA or the control EV. A series of 5′-deletions of the −927/+84 fragment identified the decreases of the transcriptional activity between −289 and −202 bp, and between −202 and −127 bp in both cells [Fig. 4(A)]. When we further compared the luciferase activity of the tandem repeats of the distal (−289/−203 bp) and proximal (−202/−128 bp) elements, only the distal responded to RelA in the repeat number-dependent manner, indicating that the region between −289 and −203 bp contains a core region responsive to RelA [Fig. 4(B)]. In fact, the identified −289/−203 bp element contained a consensus sequence of the NF-κB binding motif (HGGARNYYCC) at −252/−243 bp (TGGAAGTCCC), which was the only fully-matched sequence in the 1 kb SOX9 promoter.

We therefore prepared the 39 bp (−266/−228 bp) element containing the NF-κB motif for further analyses [Fig. 5(A)]. To examine the core responsive region in the element, we created two base mutations within the NF-κB binding motif, and further performed luciferase assay of tandem repeats of the wild-type and mutated elements in HeLa and ATDC5 cells. The repeat number-dependent transactivation of the wild-type element by RelA was suppressed by the mutagenesis in both cell types, confirming that the NF-κB binding motif is the core region responsive to RelA [Fig. 5(A)].

EMSA revealed the complex formation of the *in vitro*-translated RelA protein with the 39 bp element above [Fig. 5(B), lane 2]. Mutagenesis analyses in the element showed that the complex formation was abolished by mutations inside the NF-κB motif (m1 and m2), but not by outside mutations (m3) [Fig. 5(B), lanes 3–5]. Cold competition with excess amount of the unlabeled wild-type and outside mutation probes suppressed the complex formation, while that with the unlabeled inside mutation probes did not affect it [Fig. 5(B), lanes 6–9]. The binding between RelA and the NF-κB motif was confirmed by the complex formation of the 39 bp probe with nuclear extracts from ATDC5 cells. Interestingly, the complex formation was stronger by nuclear extracts from differentiated ATDC5 cells after the culture with differentiation medium than those of undifferentiated cells before the culture [Fig. 5(B), lanes 14 and 15]. Both complexes with the *in vitro*-translated RelA protein [Fig. 5(B), lane 12] and the ATDC5 nuclear extracts [Fig. 5(B), lane 18] underwent supershifts by addition of an antibody to RelA. These results demonstrate the specific binding between RelA and the NF-κB motif in the SOX9 promoter.

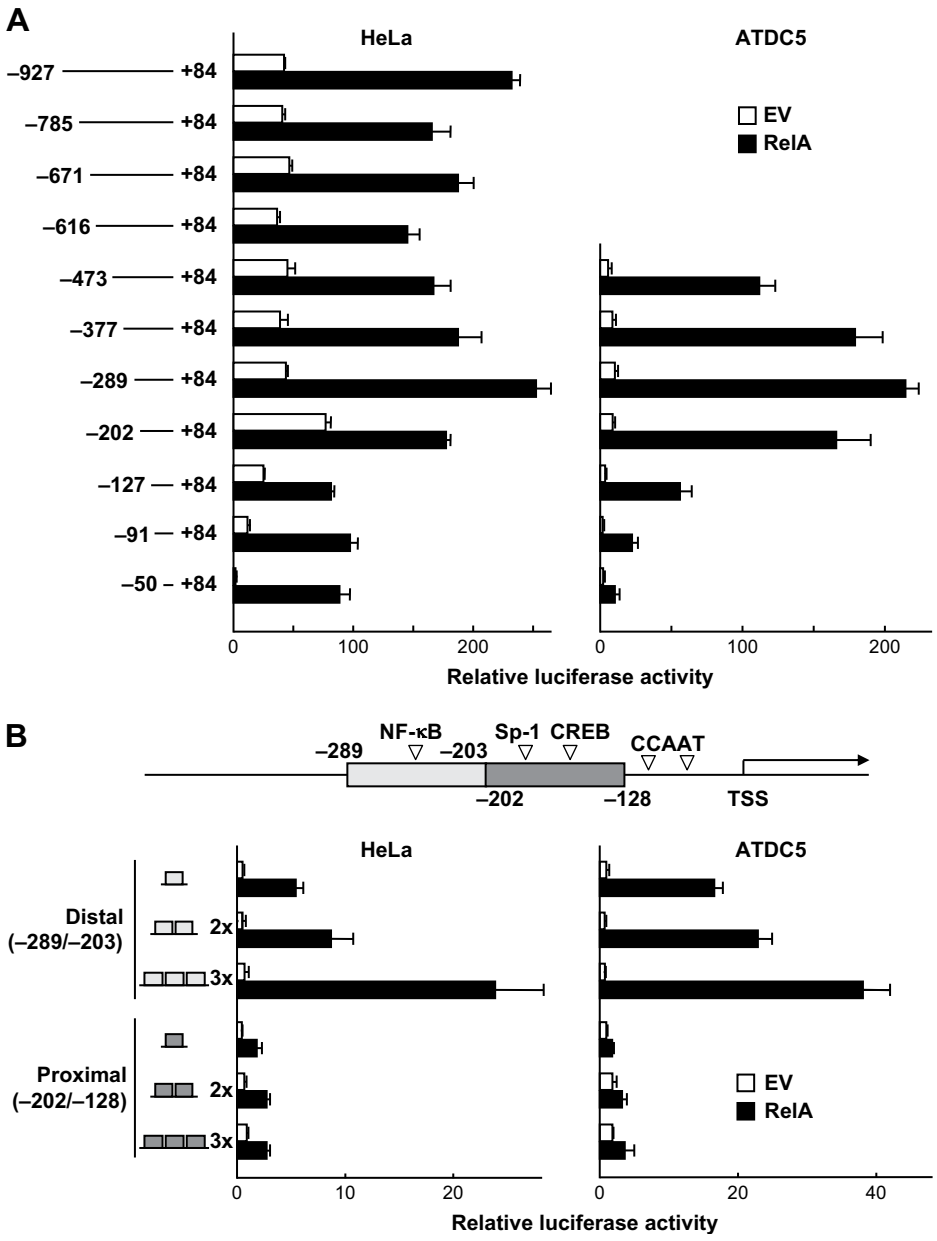


Fig. 4. (A) Identification of the region responsive to RelA by the deletion analysis of the luciferase assay in HeLa and ATDC5 cells. The cells with luciferase-reporter constructs containing the abovementioned −927/+84 region of the human SOX9 gene and the series of deletion fragments were co-transfected with RelA or the control EV. (B) Luciferase assays of the tandem repeats of the distal (−289/−203 bp relative to the TSS) and proximal (−202/−128 bp) elements that were identified in the deletion analysis above in HeLa and ATDC5 cells co-transfected with RelA or the control EV. The loci of consensus binding motifs that were identified by the present and previous studies (NF-κB, Sp1, CREB, and CCAAT) are indicated by open arrowheads. Data are shown as means (bars) ± S.E.M. (error bars) of relative luciferase activity for 4 wells/group.

ChIP assay showed the *in vivo* binding of RelA with the SOX9 promoter including the NF-κB motif [Fig. 5(C), left]. The specificity of the binding was confirmed because it was not immunoprecipitated by the non-immune IgG [Fig. 5(C), left], and no amplification was seen with a primer set that does not span the NF-κB motif [Fig. 5(C), right].

FUNCTIONAL ROLE OF RelA IN CHONDROGENIC DIFFERENTIATION

Finally, we investigated the involvement of RelA in chondrogenic differentiation. Among the NF-κB family members transfected in HeLa cells and ATDC5 cells, luciferase assays revealed that RelA transfection strongly stimulated the promoter activities of SOX6 and COL2A1, the representative chondrogenic markers besides SOX9 [Fig. 6(A)].

When RelA or the control EV was transiently transfected into HeLa cells, the RelA overexpression stimulated the mRNA levels of endogenous SOX9, SOX6, and COL2A1 [Fig. 6(B)]. To further investigate the function of RelA in chondrogenic differentiation, we established stable lines of ATDC5 cells with retroviral overexpression of RelA or the control GFP vector, and cultured them in the chondrogenic

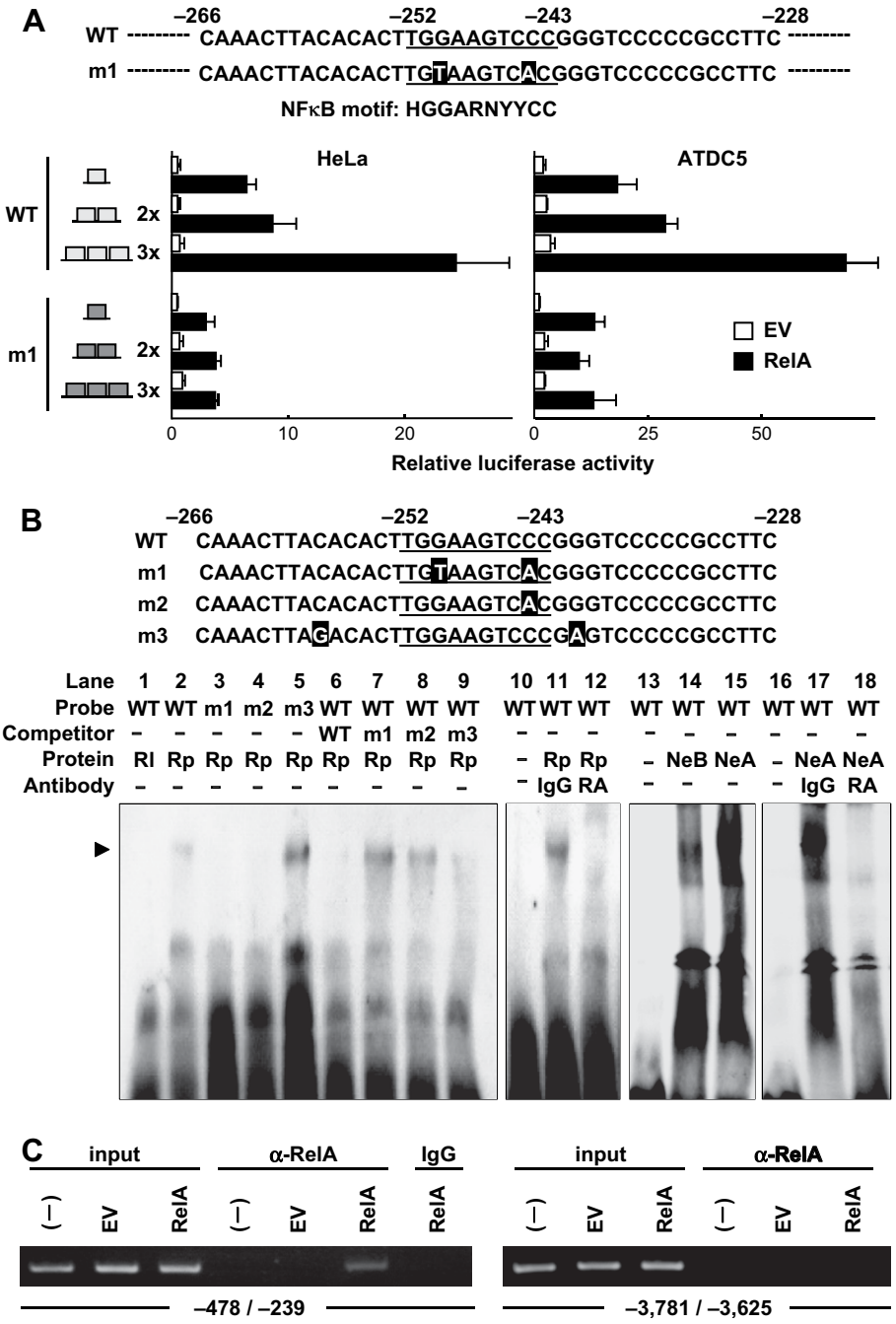
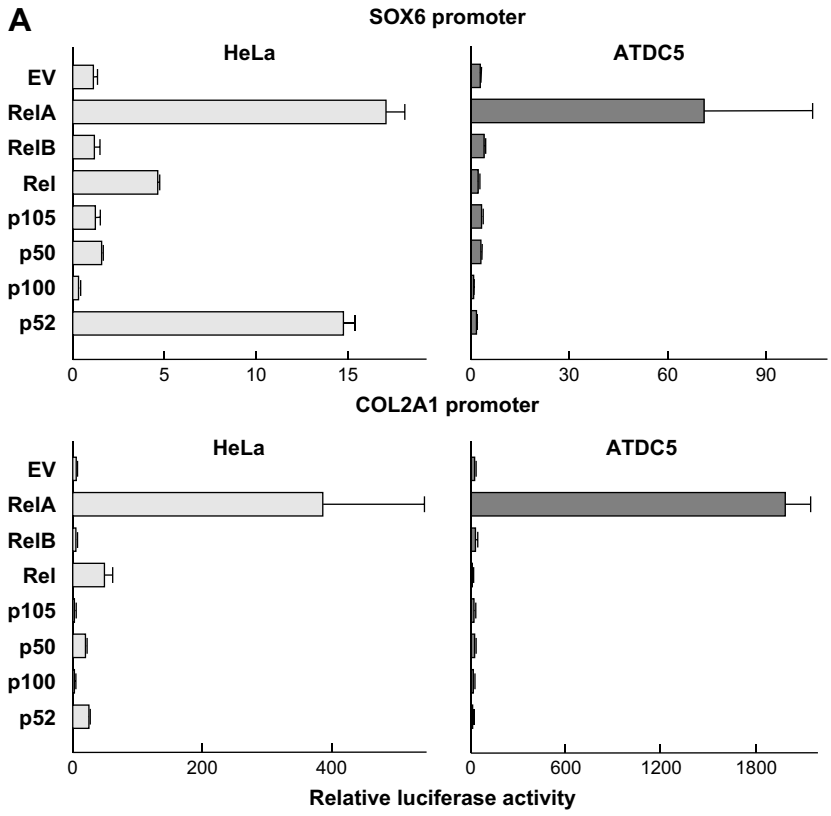


Fig. 5. (A) The 39 bp element (−265/−227 bp) containing the putative NF-κB motif (−251/−242 bp, underlined) in the identified region (wild-type; WT) and that with two base mutations at −243 and −249 bp (black boxes) within the NF-κB motif (m1). Luciferase assays of the tandem repeats of the WT and m1 elements in HeLa and ATDC5 cells co-transfected with RelA or the control EV. Data are shown as means (bars) ± S.E.M. (error bars) of relative luciferase activity for 4 wells/group. (B) EMSA for binding of the *in vitro*-translated RelA protein (Rp; lanes 1−12) or nuclear extract (Ne; lanes 13−18) with the WT or the mutated probes of the 39 bp element (−265/−227 bp) above. RI denotes reticulocyte lysate without the transcriptional/translational template. Mutations were created inside (m1 for double mutations and m2 for a single mutation) and outside (m3) the NF-κB motif. Cold competition with 100-fold excess of unlabeled WT or the mutated probes is also presented. Binding to the probe was compared between nuclear extracts of ATDC5 cells before (NeB) and after (NeA) the chondrogenic differentiation by ITS and Pi. The arrowhead indicates the shifted bands of the RelA−DNA probe complex. RA and IgG denote an antibody to RelA and non-immune IgG as the control, respectively. (C) ChIP assay for specific binding of RelA to the NF-κB motif. Cell lysates of HeLa cells with no transfection (−), transfected with EV, or RelA were amplified by a primer set spanning the NF-κB motif (−478/−239 bp, left panel) or not spanning the motif (−3781/−3625 bp, right panel) before (input) and after immunoprecipitation with an antibody to RelA (α-RelA) or the control non-immune IgG (α-IgG).


A
SOX6 promoter
HeLa
ATDC5
EV
RelA
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Rel
p105
p50
p100
p52
0
5
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15
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30
60
90
COL2A1 promoter
HeLa
ATDC5
EV
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p50
p100
p52
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200
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1200
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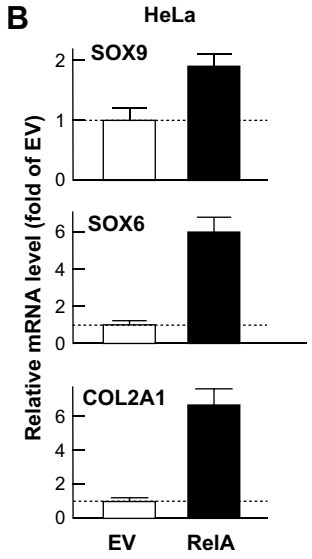
Relative luciferase activity


B
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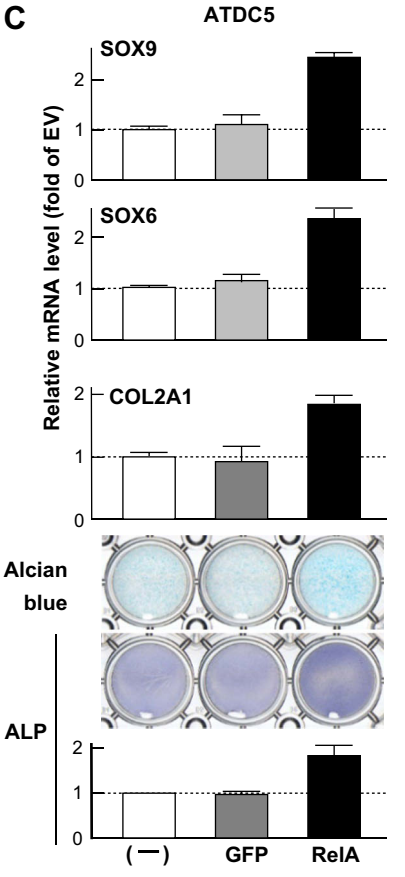
C
ATDC5
SOX9
SOX6
COL2A1
Relative mRNA level (fold of EV)
Alcian blue
ALP
(−)
GFP
RelA

differentiation medium [Fig. 6(C)]. In addition to the mRNA levels of SOX9, SOX6, COL2A1, the chondrogenic differentiation markers Alcian blue and ALP activity were stimulated by the RelA overexpression. The raw values of SOX9, SOX6, and COL2A1 mRNA levels normalized by G3PDH in the control HeLa cells were 1999 ± 29, 486 ± 90, and 21 ± 2 copies/μg of total RNA [Fig. 6(B)], while those in the control ATDC5 cells were 118 ± 8, 356 ± 11, and 6983 ± 214 copies/μg of total RNA, respectively [Fig. 6(C)], indicating that HeLa cells express negligible level of COL2A1 despite higher levels of SOX9 and SOX6 than ATDC5 cells. These results indicate that RelA functions as an inducer of chondrogenic differentiation, probably *via* the SOX9 transactivation.

Discussion

The present comparison of the promoters between human and mouse SOX9 genes found an NF-κB family member RelA to be a transcription factor of SOX9. We further identified an NF-κB binding motif around −250 bp as the core region responsive to RelA in the human SOX9 proximal promoter. In several previous studies, the sequences of the mouse and human SOX9 genes were compared and functional analyses of the SOX9 promoter were made. Morishita *et al.* identified a 30-bp region in the first intron as an ATDC5-specific enhancer, although the related transcriptional regulation remains unclear[28]. Kanai and Koopman showed that the region between −193 and −73 bp is essential for the sex- and tissue-specific expression of the mouse SOX9 gene[29]. These findings were consistent with those of a later study by Colter *et al.* on the human SOX9 promoter in which activity decreased when a deletion was made past position −172 bp[30]. In the region, they identified two CCAAT motifs that are important for the SOX9 promoter activity in chondrogenic cells. In the present deletion analysis of the luciferase assay, these motifs are located between −127 and −91 bp and between −91 and −50 bp [the top schema in Fig. 4(B)]. Although the transactivity induced by RelA did not differ between the regions, the baseline activity without the RelA stimulation was actually decreased between them [Fig. 4(A)]. This confirms the regulation of SOX9 transactivity by C/EBP proteins through its interaction with the two CCAAT motifs located more proximal to the present NF-κB motif in the SOX9 promoter. In fact, C/EBP proteins showed potent transactivation of SOX9 in both HeLa and ATDC5 cells (Fig. 2). More recently, the same group reported that the human SOX9 proximal promoter is also regulated by the cyclic-AMP response element binding (CREB) protein and Sp1[31]. In the present deletion analysis, the binding motifs are located between −202 and −128 bp, which we identified as the proximal element [the top schema in Fig. 4(B)]. Here again, the baseline transactivity was decreased as well as the RelA-induced activity. In the tandem-repeat experiments, the baseline transactivity was increased dependent on the repeat number of the proximal element, though not as strongly as that of the distal element (−202/−128) under the RelA stimulation [Fig. 4(B)]. Interestingly, the decrease in the deletion analysis and the increase in the tandem-repeat analysis of the proximal element were equivalently seen between the presence and absence of the RelA stimulation, while those of the distal element were apparent only under the RelA stimulation (Fig. 4). These indicate that the identified NF-κB motif in the distal element is specific to the RelA stimulation, while the proximal element including the CREB and Sp1 motifs functions as a basal regulatory region in the SOX9 proximal promoter.

The RelA overexpression enhanced the promoter activities and the endogenous mRNA levels of SOX6 and COL2A1 in HeLa and ATDC5 cells (Fig. 6). These may be at least partly mediated by the RelA effect on the SOX9 transactivation, since SOX9 is a crucial transcriptional activator of SOX6 and COL2A1[4–6,10]. Although the RelA overexpression enhanced chondrogenic differentiation shown by Alcian blue staining and ALP activity in differentiated ATDC5 cells after the stimulation by ITS and Pi [Fig. 6(C)], this was not reproducible in undifferentiated ATDC5 cells without the stimulation (data not shown). Considering that the promoter assays were performed in ATDC5 cells without the differentiation stimulation, there is a discrepancy between endogenous mRNA levels and exogenous promoter activities of SOX9, SOX6 and COL2A1 in the RelA actions on undifferentiated ATDC5 cells. This might be due to post-transcriptional negative regulation that was specific to endogenous mRNAs or the chromatin regulation occurring only in a genomic context, which are specific to undifferentiated ATDC5 cells. In fact, EMSA using nuclear extracts from ATDC5 cells revealed that complex formation with the NF-κB probe was much stronger in extracts from differentiated cells than in those from undifferentiated cells [Fig. 5(B), lanes 14 and 15]. Contrarily, our previous study has shown that the overexpression of SOX trio or the SOX9 alone potently stimulated chondrogenic differentiation even from non-chondrogenic cells[14], indicating that the RelA may not induce sufficiently high SOX9 levels to force chondrogenic differentiation in the absence of additional stimulation.

Although the present study focused on a region within 1 kb of the 5′-end flanking region of the SOX9 gene and identified RelA as the potent transactivator of the limited region, there are surely more distant regions that are critical for the SOX9 expression. The fact that translocation breakpoints in campomelic dysplasia patients have been mapped 50 kb- or more distant from SOX9[13,32] indicates the large genomic environment regulating SOX9 expression *in vivo*. In mice as well, suppression of limb outgrowth by the blockage of the NF-κB pathway was shown to be due to defects in fibroblast growth factor (FGF) signal which caused a failure in mesenchymal−epithelial communication, rather than to a defect in chondrogenesis[20,33]. In addition to the abovementioned signals that directly

Fig. 6. (A) Promoter activities of SOX6 and COL2A1 by the NF-κB family members in HeLa and ATDC5 cells. The cells were co-transfected with the luciferase-reporter construct containing the SOX6 promoter fragment (−517 to IVS1 + 23 in the human SOX6 gene) or the COL2A1 promoter fragment (four repeats of the 49 bp SOX9 enhancer and the basal promoter from −183 to +23 bp in the human COL2A1 gene), and the NF-κB family factors or the control EV. Data are shown as means (bars) ± S.E.M. (error bars) of relative luciferase activity (the ratio of the firefly activities to the renilla activities) for 4 wells/group. (B) mRNA levels of endogenous SOX9, SOX6, and COL2A1 determined by real-time RT-PCR in HeLa cells that were transiently transfected with RelA or the control EV. Data are shown as means (bars) ± S.E.M. (error bars) of relative mRNA level as compared to the EV-transfected cells for 3 wells/group. (C) mRNA levels of endogenous SOX9, SOX6, and COL2A1, Alcian blue staining, ALP staining and activity (relative to control) in stable lines of ATDC5 cells retrovirally transfected with RelA or the control green fluorescence protein (GFP) and in non-transfected parental cells (−) after culture for 3 weeks with ITS and 2 d with Pi. The relative mRNA data are shown as means (bars) ± S.E.M. (error bars) as compared to the non-transfected parental cells (−) for 3 wells/group.

activate the putative motifs such as CCAAT, CREB, and Sp1 within the 1 kb promoter, there are several pathways known to induce the SOX9 expression. FGFs have been shown to up-regulate SOX9 mRNA expression in chondrocytes through a MAP kinase pathway[34]. Bone morphogenetic proteins and hedgehog family members enhance SOX9 expression under certain conditions, while retinoic acid exhibits mixed results[8,35–37]. Hence, we surmise that RelA is not the principal transactivator of SOX9, but is a member of complicated molecular network for the transactivation. Addition of other signals to RelA will be needed to achieve strong SOX9 induction and efficient chondrogenic differentiation.

Proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α are known to be representative ligands for the NF-κB signal[38–41]. A previous report showed that IL-1 and TNF-α caused a suppression of SOX9 expression in chondrogenic cells, which may explain the deleterious role of the cytokines in cartilage degenerative disorders such as rheumatoid arthritis and osteoarthritis[42]. Interestingly, the report indicated that the SOX9 suppression is at least partly mediated by the NF-κB signal induced by the cytokines. Furthermore, a recent study showed that silencing of IKKβ enhanced the accumulation of glycosaminoglycan in conjunction with increased SOX9 expression in human osteoarthritis chondrocytes[43]. These indicate the down-regulation of SOX9 by the NF-κB signal, which seems contradictory to the present results showing the positive relationship between them. A previous study, however, has shown that the SOX9 suppression by NF-κB occurs not at the transcriptional level, but at the post-transcriptional level through the RNA sequence-dependent mechanism[38]. At the transcriptional level as well, there may be pathways other than NF-κB in the SOX9 suppression by the proinflammatory cytokines, since the human promoter study above has shown that IL-1 down-regulated SOX9 promoter activity through a reduction of Sp1 binding to the proximal promoter in chondrocytes[31]. Hence, the NF-κB and SOX9 signals may regulate chondrogenic differentiation and skeletal development *via* complicated mechanisms by various kinds of interactions with each other.

Regarding RelA, to date a description of the *in vivo* function has been limited to the embryonic lethality of the homo-knockout mice. Since a recent report demonstrated that Nkx3.2 supports chondrocyte survival by activating RelA *via* a ligand-independent mechanism[44], RelA might possibly function to maintain the chondrogenic phenotype through constitutive activation of SOX9. Further understanding of the molecular network related to the RelA/SOX9 axis will lead to elucidation of the mechanism underlying chondrogenic differentiation and cartilage formation under physiological and pathological conditions.

Conflict of interest

The authors declare that they have no conflicts of interest.

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References

1. de Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol 2000;19:389–94.
2. Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, *et al.* The Sry-related gene SOX9 is expressed during chondrogenesis in mouse embryos. Nat Genet 1995;9:15–20.
3. Healy C, Uwanogho D, Sharpe PT. Expression of the chicken SOX9 gene marks the onset of cartilage differentiation. Ann N Y Acad Sci 1996;785:261–2.
4. Lefebvre V, Li P, de Crombrugghe B. A new long form of SOX5 (LSOX5), SOX6 and SOX9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. EMBO J 1998;17:5718–33.
5. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol 1997;17:2336–46.
6. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, *et al.* SOX9 directly regulates the type-II collagen gene. Nat Genet 1997;16:174–8.
7. Bridgewater LC, Lefebvre V, de Crombrugghe B. Chondrocyte-specific enhancer elements in the Col11a2 gene resemble the Col2a1 tissue-specific enhancer. J Biol Chem 1998;273:14998–5006.
8. Sekiya I, Tsuji K, Koopman P, Watanabe H, Yamada Y, Shinomiya K, *et al.* SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. J Biol Chem 2000;275:10738–44.
9. Zhang P, Jimenez SA, Stokes DG. Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. J Biol Chem 2003;278:117–23.
10. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor SOX9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of SOX5 and SOX6. Genes Dev 2002;16:2813–28.
11. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. SOX9 is required for cartilage formation. Nat Genet 1999;22:85–9.
12. Bi W, Huang W, Whitworth DJ, Deng JM, Zhang Z, Behringer RR, *et al.* Haploinsufficiency of SOX9 results in defective cartilage primordia and premature skeletal mineralization. Proc Natl Acad Sci U S A 2001;98:6698–703.
13. Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, *et al.* Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 1994;79:1111–20.
14. Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, Takato T, *et al.* The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum 2004;50:3561–73.
15. Meffert MK, Baltimore D. Physiological functions for brain NF-κB. Trends Neurosci 2005;28:37–43.
16. Li Q, Withoff S, Verma IM. Inflammation-associated cancer: NF-κB is the lynchpin. Trends Immunol 2005;26:318–25.
17. Bonizzi G, Karin M. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 2004;25:280–8.
18. Hayden MS, Ghosh S. Signaling to NF-κB. Genes Dev 2004;18:2195–224.
19. Chen LF, Greene WC. Shaping the nuclear action of NF-κB. Nat Rev Mol Cell Biol 2004;5:392–401.
20. Kanegae Y, Tavares AT, Izpisua Belmonte JC, Verma IM. Role of Rel/NF-κB transcription factors during the outgrowth of the vertebrate limb. Nature 1998;392:611–4.
21. Takeda K, Takeuchi O, Tsujimura T, Itami S, Adachi O, Kawai T. Limb and skin abnormalities in mice lacking IKKα. Science 1999;284:313–6.
22. Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, *et al.* IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev 1999;13:1322–8.
23. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
24. Magne D, Bluteau G, Faucheux C, Palmar G, Viqnes-Colombeix C, Pilet P, *et al.* Phosphate is a specific signal for ATDC5 chondrocyte maturation and apoptosis-associated mineralization: possible implication of apoptosis in the regulation of endochondral ossification. J Bone Miner Res 2003;18:1430–42.
25. Ikeda T, Saito T, Ushita M, Yano F, Kan A, Itaka K, *et al.* Identification and characterization of the human SOX6 promoter. Biochem Biophys Res Commun 2007;357:383–90.

26. Zhou G, Lefebvre V, Zhang Z, Eberspaecher H, de Crombrugghe B. Three high mobility group-like sequences within a 48-base pair enhancer of the Col2a1 gene are required for cartilage-specific expression *in vivo*. J Biol Chem 1998;273:14989–97.
27. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 2000;7: 1063–6.
28. Morishita M, Kishino T, Furukawa K, Yonekura A, Miyazaki Y, Kanematsu T, *et al*. A 30-base-pair element in the first intron of SOX9 acts as an enhancer in ATDC5. Biochem Biophys Res Commun 2001;288:347–55.
29. Kanai Y, Koopman P. Structural and functional characterization of the mouse SOX9 promoter: implications for campomelic dysplasia. Hum Mol Genet 1999;8:691–6.
30. Colter DC, Piera-Velazquez S, Hawkins DF, Whitecavage MK, Jimenez SA, Stokes DG. Regulation of the human SOX9 promoter by the CCAAT-binding factor. Matrix Biol 2005;24:185–97.
31. Piera-Velazquez S, Hawkins DF, Whitecavage MK, Colter DC, Stokes DG, Jimenez SA. Regulation of the human SOX9 promotor by Sp1 and CREB. Exp Cell Res 2007;313:1069–79.
32. Wirth J, Wagner T, Meyer J, Pfeiffer RA, Tietze HU, Schempp W, *et al*. Translocation breakpoints in three patients with campomelic dysplasia and autosomal sex reversal map more than 130 kb from SOX9. Hum Genet 1996;97:186–93.
33. Sil AK, Maeda S, Sano Y, Roop DR, Karin M. IκB kinase-α acts in the epidermis to control skeletal and craniofacial morphogenesis. Nature 2004;428:660–4.
34. Murakami S, Kan M, McKeehan WL, de Crombrugghe B. Up-regulation of the chondrogenic SOX9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. Proc Natl Acad Sci U S A 2000;97:1113–8.
35. Zehentner BK, Dony C, Burtscher H. The transcription factor SOX9 is involved in BMP-2 signaling. J Bone Miner Res 1999;14:1734–41.
36. Semba I, Nonaka K, Takahashi I, Takahashi K, Dashner R, Shum L. Positionally-dependent chondrogenesis induced by BMP4 is co-regulated by SOX9 and Msx2. Dev Dyn 2000;217:401–14.
37. Sekiya I, Koopman P, Tsuji K, Mertin S, Harley V, Yamada Y, *et al*. Transcriptional suppression of SOX9 expression in chondrocytes by retinoic acid. J Cell Biochem 2001;81:71–8.
38. Sitcheran R, Cogswell PC, Boldwin Jr AS. NF-κB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism. Genes Dev 2003;17:2368–73.
39. Silverman N, Maniatis T. NF-κB signaling pathways in mammalian and insect innate immunity. Genes Dev 2001;15:2321–42.
40. Ghosh S, Karin M. Missing pieces in the NF-κB puzzle. Cell 2002; 109(Suppl):S81–96.
41. Li Q, Verna IM. NF-κB regulation in the immune system. Nat Rev Immunol 2002;10:725–34.
42. Murakami S, Lefebvre V, Crombrugghe B. Potent inhibition of the master chondrogenic factor SOX9 gene by interleukin-1 and tumor necrosis factor-α. J Biol Chem 2000;275:3687–92.
43. Olivotto E, Borzi RM, Vitellotti R, Pagani S, Facchini A, Battistelli M, *et al*. Differential requirements for IKKα and IKKβ in the differentiation of primary human osteoarthritic chondrocytes. Arthritis Rheum 2008;58: 227–39.
44. Park M, Yong Y, Choi SW, Kim JH, Lee JE, Kim EW. Constitutive RelA activation mediated by Nkx3.2 controls chondrocyte viability. Nat Cell Biol 2007;9:287–98.