Paraspeckle protein p54^{nrb} links Sox9-mediated transcription with RNA processing during chondrogenesis in mice

Kenji Hata,¹ Riko Nishimura,¹ Shuji Muramatsu,² Akio Matsuda,² Takuma Matsubara,¹ Katsuhiko Amano,¹ Fumiyo Ikeda,¹ Vincent R. Harley,³ and Toshiyuki Yoneda¹

¹Department of Molecular and Cellular Biochemistry, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan. ²Laboratory for Drug Discovery, Research Center, Asahi KASEI Pharma Corporation, Fuji, Shizuoka, Japan. ³Human Molecular Genetics Laboratory, Prince Henry's Institute of Medical Research, Monash Medical Centre, Melbourne, Victoria, Australia.

The Sox9 transcription factor plays an essential role in promoting chondrogenesis and regulating expression of chondrocyte extracellular-matrix genes. To identify genes that interact with Sox9 in promoting chondrocyte differentiation, we screened a cDNA library generated from the murine chondrogenic ATDC5 cell line to identify activators of the collagen, type II, α 1 (*Col2a1*) promoter. Here we have shown that paraspeckle regulatory protein 54-kDa nuclear RNA-binding protein (p54^{nrb}) is an essential link between Sox9-regulated transcription and maturation of Sox9-target gene mRNA. We found that p54^{nrb} physically interacted with Sox9 and enhanced Sox9-dependent transcriptional activation of the *Col2a1* promoter. In ATDC5 cells, p54^{nrb} colocalized with Sox9 protein in nuclear paraspeckle bodies, and knockdown of p54^{nrb} suppressed Sox9-dependent *Col2a1* expression and promoter activity. We generated a p54^{nrb} mutant construct lacking RNA recognition motifs, and overexpression of mutant p54^{nrb} in ATDC5 cells markedly altered the appearance of paraspeckle bodies and inhibited the maturation of *Col2a1* mRNA. The mutant p54^{nrb} inhibited chondrocyte differentiation of mesenchymal cells and mouse metatarsal explants. Furthermore, transgenic mice expressing the mutant p54^{nrb} in the chondrocyte lineage exhibited dwarfism associated with impairment of chondrogenesis. These data suggest that p54^{nrb} plays an important role in the regulation of Sox9 function and the formation of paraspeckle bodies during chondrogenesis.

Introduction

Chondrogenesis is an important biological event in endochondral bone development, skeletogenesis, and tissue patterning (1, 2). After condensation of chondrogenic mesenchymal cells, they begin to differentiate into chondrocytes (3). The transcription factor Sox9 contains a SRY-related high-mobility group box and promotes chondrocyte differentiation and the expression of cartilage-specific extracellular matrix genes, including collagens and proteoglycans (3). In humans, heterozygous *SOX9* mutations cause campomelic dysplasia characterized by severe chondrodysplasia (4, 5). Heterozygous *Sox9* mutant mice or mice lacking Sox9 function show impaired endochondral bone formation (6, 7). These findings indicate that Sox9 plays an essential role in chondrogenesis.

Sox9 is also implicated in the expression of Sox5 and Sox6, both of which form a transcriptional complex with Sox9 to control the expression of collagen, type II, α 1 (*Col2a1*) and aggrecan (*Acan*) during chondrocyte differentiation (7). In addition, PGC-1 α functions as a transcriptional coactivator for Sox9 (8). Moreover, the functional and physical interaction of Sox9 and β -catenin is important evidence for crosstalk between Sox9 and Wnt signaling (9). These findings have contributed to identification of transcriptional components for Sox9 that are implicated in chondrogenesis. However, other modes of the regulation of chondrogenic genes,

including chromatin remodeling, histone modification, and RNA processing, are not well understood.

Because the transcription factors form a large complex that sequentially and dynamically controls expression of target genes in collaboration with the partner molecules, identification of the components of the "transcriptional factory" formed by Sox9 would lead to a breakthrough in our understanding of the mechanisms of Sox9-regulated chondrogenesis. In this study, we identify 54-kDa nuclear RNA-binding protein (p54^{nrb}) as an interacting partner of Sox9 and show that p54^{nrb} controls transcription and mRNA processing of the *Col2a1* gene. Furthermore, we demonstrate that p54^{nrb} plays an important role in chondrogenesis in vitro and in vivo. Thus p54^{nrb} is an essential transcriptional regulator that links the Sox9-dependent transcription to target gene mRNA maturation during chondrogenesis.

Results

Physical and functional interaction of p54^{nrb} with Sox9. To identify factors involved in Sox9-mediated regulation of chondrocyte differentiation, we screened a cDNA library generated from the chondrogenic cell line, ATDC5 (10), by performing a luciferase reporter assay using the *Col2a1* gene promoter. Four cDNA clones stimulated the *Col2a1* gene promoter (Figure 1A). One of these encodes the full-length p54^{nrb} protein (11–13), which contains 2 RNA recognition motifs (RRMs) and is localized to nuclear paraspeckle bodies (14). We have confirmed that p54^{nrb} mRNA is expressed in ATDC5 cells and in primary mouse chondrocytes (data not shown). To examine the functional relationship between p54^{nrb} and Sox9, we determined the effect of p54^{nrb} on the transcriptional

Nonstandard abbreviations used: BMP, bone morphogenetic protein; *Col2a1*, collagen, type II, α 1; p54^{nrb}, 54-kDa nuclear RNA-binding protein; PSP1, paraspeckle protein 1; RRM, RNA recognition motif.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 118:3098–3108 (2008). doi:10.1172/JCI31373.

Figure 1

Physical and functional interactions of p54^{nrb} with Sox9. **(A)** Stimulation of *Col2a1* promoter activity by cDNA clones isolated from ATDC5. ATDC5 cells were transfected with each cDNA together with the *Col2a1*-luciferase construct, and luciferase activity of cell lysates was measured. Clone 3 (cl 3) is p54^{nrb}. Cont, control. **(B)** Costimulatory effect of p54^{nrb} and Sox9 on *Col2a1* promoter activity. ATDC5 cells were transfected with p54^{nrb} together with Sox9, and luciferase activity of cell lysates was measured. **(C)** No upregulation of *Col2a1* promoter activity by p54^{nrb} in HeLa cells. HeLa cells were transfected with p54^{nrb} together with Sox9, and luciferase activity of cell lysates was measured. **(D)** Absence of effect of p54^{nrb} on transcriptional activity of Runx2. The osteocalcin gene promoter luciferase constructs were transfected into C3H10T1/2 cells with the plasmid as indicated. The luciferase activity of cell lysates was measured. **(E)** Physical association of p54^{nrb} with Sox9. Cell lysates expressing Myc-p54^{nrb}, HA-Sox9, or both were determined by immunoblotting with anti-HA antibody, followed by immunoprecipitation with anti-Myc antibody.

activity of Sox9. Overexpression of p54^{nrb} markedly enhanced Sox9 transactivation of the *Col2a1* gene promoter (Figure 1B). We did not observe the upregulation of the *Col2a1* promoter activity by p54^{nrb} in HeLa cells where Sox9 is not expressed (Figure 1C). In contrast, p54^{nrb} did not affect the transcriptional activity of Runx2, an essential transcription factor for osteoblast differentiation (15) (Figure 1D), suggesting that p54^{nrb} specifically stimulates the transcriptional activity of Sox9. To investigate the basis of the functional cooperation between p54^{nrb} and Sox9, we tested whether p54^{nrb} and Sox9 proteins interact. As shown in Figure 1E, coimmunoprecipitation experiments indicated a physical association between p54^{nrb} and Sox9. These results indicate that p54^{nrb} functions as a transcriptional partner for Sox9. Next we attempted to define whether the functional interaction between p54^{nrb} and Sox9 is required for regulation of the *Col2a1* gene promoter activity. Overexpression of a dominant-negative Sox9 mutant, which lacks binding activity to p54^{nrb}, markedly inhibited stimulation of *Col2a1* promoter activity by p54^{nrb} (Figure 2, A and B). A mutant of p54^{nrb}, ΔM, which lacks binding activity to Sox9 (Figure 2, C and D), failed to stimulate the transcriptional activity of Sox9 (Figure 2E). Furthermore, knockdown of p54^{nrb} (Figure 2, F and G) clearly inhibited transcriptional activity of Sox9 on the *Col2a1* gene promoter (Figure 2H). These results demonstrated that p54^{nrb} is a critical transcriptional partner of Sox9 and that this partnership upregulates *Col2a1* gene promoter activity.

Interaction of p54^{nrb} and Sox9 in paraspeckle nuclear bodies. It has been reported that p54^{nrb} colocalizes and interacts with paraspeckle protein 1 (PSP1) in the paraspeckle nuclear bodies (14, 16). Although nuclear paraspeckles are assumed to be involved in nuclear events,

including DNA replication, transcription, and mRNA maturation (17), their biological roles remain unclear. First, we determined the subcellular localization of p54^{nrb} in ATDC5 cells using Venus-tagged p54^{nrb}. p54^{nrb} was confined to the nucleus within nuclear speckles (Figure 3A) and colocalized with PSP1 (Figure 3A) but not with SC-35 (Figure 3B), a nuclear speckle body protein implicated in RNA splicing (18, 19). Consistent with results shown in Figure 1, p54^{nrb} is colocalized with Sox9 in transfected cells and primary mouse chondrocytes (Figure 3, C–F). Like p54^{nrb}, Sox9 colocalized with PSP1 but not with SC-35 (Figure 3, G and H). When Sox9 is co-overexpressed, the size of paraspeckle bodies became smaller (Figure 3, C and D). Because the size of nuclear speckle has been shown previously to be inversely correlated with the state of transcription of the cell (17), our data suggest that interaction between p54^{nrb} and Sox9 stimulates transcriptional activity.

Role of p54^{nrb} in mRNA processing of *Col2a1*. Because 2 RRM domains of p54^{nrb} are highly homologous to the RRM domain of poly-pyrimidine tract-binding protein-associated splicing factor (PSF), we speculated that p54^{nrb} is involved in splicing of the target genes necessary for chondrogenesis. We examined the processing of *Col2a1* mRNA by performing a minigene assay using a *Col2a1* gene cassette, containing the alternatively spliced exon 2 driven by the native promoter (20). Overexpression of Sox9 or p54^{nrb} produced a spliced transcript of the *Col2a1* (IIA) containing exon 2 (20, 21) (Figure 4, A and B). Furthermore, co-overexpression of Sox9 and p54^{nrb} produced more transcripts of *Col2a1* (IIA) than expression of Sox9 or p54^{nrb} alone (Figure 4, A and B). In addition, co-overexpression of Sox9 and p54^{nrb} also produced type II B isoform (Figure 4A), which is preferentially expressed in differentiated chondrocytes (21). However, p54^{nrb} is not involved in splicing of the fibronectin gene (Figure 4C). These results suggest that p54^{nrb} specifically controls the splicing and maturation of *Col2a1* mRNA. To further understand the role of p54^{nrb} in this process, we generated a mutant of p54^{nrb} (Δ224), lacking 2 RRM domains (Figure 2C). In ATDC5 cells in which the mutant was expressed, the shape of paraspeckle bodies was rounded (Figure 5A), without affecting the proliferation of ATDC5 cells (Figure 5B), and not colocalized with SC-35 (Figure 5C). Interestingly, however, the mutant p54^{nrb} still upregulated the transcriptional activity of Sox9 (Figure 5D) and retained the binding capacity to Sox9 (Figure 2D). In addition, where mutant p54^{nrb} colocalized with Sox9, the size of speckles was smaller (Figure 5E). Next, we evaluated the role of both the

Figure 2

Importance of association of p54^{nrB} with Sox9 in upregulation of *Col2a1* gene promoter activity. (A) Inhibition of p54^{nrB}-stimulated *Col2a1* promoter activity by dominant-negative Sox9 (DN). Luciferase activity of ATDC5 cell lysates transfected with *Col2a1* luciferase construct, together with expression vectors as indicated was measured. (B) No association of dominant-negative Sox9 with p54^{nrB}. The cell lysates expressing wild-type or the mutants of HA-Sox9 were precipitated (Ppt) with His-tag-p54^{nrB} protein, and then the precipitates were determined by immunoblotting with anti-HA antibody. ΔHMG, a mutant lacking the HMG domain. (C) Schematic diagram of the mutants of p54^{nrB}. ΔM, Δ224, and Δ313 are mutants of p54^{nrB}. (D) Analysis of binding domain of p54^{nrB} with Sox9. The cell lysates expressing wild-type or mutants of p54^{nrB} were precipitated with tandem affinity purification-tagged (TAP-tagged) Sox9 protein, and then the precipitates were determined by immunoblotting with anti-Myc antibody. (E) The mutant p54^{nrB} (ΔM) failed to transactivate the transcriptional activity of Sox9. ATDC5 cells were transfected with constructs as indicated, and luciferase activity of cell lysates was measured. (F) Knockdown of p54^{nrB} by shRNA. shRNA expression vector for GFP or p54^{nrB} (shGFP or shp54^{nrB}) was transfected into ATDC5 cells, and the total RNA of the cells was determined by RT-PCR analyses. (G) Knockdown of p54^{nrB} by shRNA. ATDC5 cells were transfected with shGFP or shp54^{nrB}, and the cell lysates were examined by immunoblotting with anti-p54^{nrB} and β-actin antibodies. (H) Inhibition of Sox9-dependent *Col2a1* promoter activity by knockdown of p54^{nrB}. Luciferase activity was measured in cell lysates transfected with expression vectors as indicated.

p54^{nrB} RRM and paraspeckle nuclear bodies in the processing of *Col2a1* mRNA. Overexpression of the mutant p54^{nrB} blocked the maturation of *Col2a1* mRNA (Figure 5F). We confirmed that this effect of the mutant p54^{nrB} is not due to inhibition of transcription of the minigene (Figure 4B). Collectively, the results indicate that p54^{nrB} regulates both the splicing of *Col2a1* mRNA and the formation of paraspeckle bodies via RRM.

Regulation of chondrocyte differentiation by p54^{nrB}. To investigate whether p54^{nrB} is involved in chondrogenesis, we examined its effect on chondrocyte differentiation. Overexpression of p54^{nrB} in ATDC5 cells induced the endogenous expression of *Col2a1* and

Acan (Figure 6, A–D). In contrast, knockdown of p54^{nrB} clearly inhibited Sox9-induced *Col2a1* expression (Figure 6E). Next, we examined the effect of the p54^{nrB} mutant that impaired *Col2a1* mRNA processing and paraspeckle body formation. Overexpression of the p54^{nrB} mutant dramatically inhibited Sox9-induced *Col2a1* expression (Figure 6, C and D). Also, the mutant p54^{nrB} abolished bone morphogenetic protein 2-induced (BMP2-induced) chondrocyte differentiation (Figure 6F). On the other hand, the mutant p54^{nrB} did not affect BMP2-induced osteoblast differentiation (Figure 6G). Furthermore, organ culture experiments using mouse fetal metatarsals indicated that while wild-type

Figure 3

Colocalization of p54^{nrb} with Sox9 in paraspeckle nuclear bodies. (A) Colocalization of p54^{nrb} with PSP1 in the nucleus of ATDC5 cells. ATDC5 cells transfected with Venus-tagged p54^{nrb} (Venus-p54^{nrb}) and DsRed-tagged PSP1 (DsRed-PSP1) were monitored under a fluorescence microscope. (B) p54^{nrb} is not localized with SC-35. ATDC5 cells transfected with Venus-tagged p54^{nrb} were immunostained with anti-SC-35 antibody. (C) Colocalization of p54^{nrb} and Sox9 in the nucleus. ATDC5 cells transfected with Venus-tagged p54^{nrb} and DsRed-tagged Sox9 (DsRed-Sox9) were monitored under a fluorescence microscope. (D and E) Colocalization of p54^{nrb} and Sox9 in the nucleus. C3H10T1/2 cells transfected with Venus-tagged p54^{nrb} and DsRed-tagged Sox9 were monitored under a confocal microscope. C3H10T1/2 cells expressing both Venus-p54^{nrb} and DsRed-Sox9 show smaller size of speckle than C3H10T1/2 cells expressing only Venus-p54^{nrb} (D). Nucleuses of cells were stained with DAPI. (F) Colocalization of p54^{nrb} and Sox9 in murine primary chondrocytes. Primary chondrocytes isolated from mouse ribs were immunostained with anti-p54^{nrb} and anti-Sox9 antibodies and photographed under a confocal microscope. (G) Colocalization of Sox9 with PSP1 in the nucleus of ATDC5 cells. ATDC5 cells transfected with DsRed-tagged Sox9 and Venus-tagged PSP1 (Venus-PSP1) were monitored under a fluorescence microscope. (H) Sox9 does not affect localization of SC-35. ATDC5 cells transfected with DsRed-tagged Sox9 were immunostained with anti-SC-35 antibody. Original magnification, ×400.

Figure 4

Processing of *Col2a1* mRNA by p54^{nrb}. **(A)** *Col2a1* mRNA processing by p54^{nrb}. A minigene construct of the *Col2a1* gene was transfected with expression plasmids as indicated into ATDC5 cells. Total RNA isolated from the cells was determined by RT-PCR analyses using the primers (see Methods) specific for the minigene products (upper panel) or β-actin (bottom panel) Ex1, exon 1. **(B)** Effect of wild-type and a mutant p54^{nrb} on processing of *Col2a1* mRNA. A minigene construct of the *Col2a1* gene was transfected with the expression vectors as indicated into ATDC5 cells. Expression of spliced minigene product (upper panel) and nonspliced minigene product (bottom panel) was determined by real-time PCR using the primers (see Methods) specific for the spliced and nonspliced minigene products. Expression levels were normalized with β-actin expression. **(C)** Effect of p54^{nrb} on processing of fibronectin mRNA. HEK293 cells were transfected with fibronectin minigene construct (7iBi) together with mock (Cont), SRP40 expression vector, or p54^{nrb} expression vector. Total RNA isolated from the cells was determined by RT-PCR analyses using the primers (see Methods) specific for the minigene products (upper panel) or GAPDH (bottom panel).

tent with the results shown in Figure 5B, proliferation of chondrocytes was not influenced in the transgenic mice. Collectively, these data suggest that inhibition of mRNA processing of *Col2a1* by p54^{nrb} leads to delay of ossification, which resulted in dwarfism seen in the transgenic mice.

Discussion

The expression of the genes necessary for chondrogenesis is exquisitely and harmoniously regulated by several transcription factors (1). Several lines of evidence demonstrate the indispensable role of Sox9 for the commitment of mesenchymal cells into chondrocytes and the early stages of chondrocyte differentiation (3). In the present study, we have identified p54^{nrb} as an important transcriptional partner of Sox9 during chondrogenesis. We found that p54^{nrb} stimulates the transcriptional activity of Sox9 and chondrogenic action of Sox9. In contrast, knockdown of p54^{nrb} inhibited the activity of Sox9. Consistently, a dominant-negative mutant of Sox9 dramatically suppressed the stimulatory effect of p54^{nrb} on *Col2a1* promoter activity. The interaction between p54^{nrb} and Sox9 was confirmed by coimmunoprecipitation experiments and colocalization in the nucleus of ATDC5 cells and primary chondrocytes. Collectively, these results clearly indicate that p54^{nrb} forms a transcriptional factory with Sox9.

Because p54^{nrb} has been shown to play a role in regulation of splicing or termination of mRNA synthesis (22), we examined whether p54^{nrb} regulates the processing of *Col2a1* mRNA. Minigene assay showed that p54^{nrb} overexpression stimulates the processing of *Col2a1* mRNA. Moreover, the mutant of p54^{nrb} lacking RRMs inhibited *Col2a1* mRNA processing. Consistently, the mutant p54^{nrb} also blocked *Col2a1* expression induced by Sox9. However, interestingly, the mutant p54^{nrb} retains the ability to upregulate the transcriptional activity of Sox9 on the *Col2a1* gene promoter. These results indicate that p54^{nrb} regulates both transcription and splicing of *Col2a1* mRNA through 2 different functional domains during chondrocyte differentiation and that p54^{nrb} is a critical Sox9 transcriptional factory component that couples the transcription and processing of *Col2a1* mRNA.

Several studies demonstrate that nuclear speckles are implicated in the regulation of transcription and splicing of target genes and, consequently, controlling of cell growth and differ-

p54^{nrb} stimulated chondrogenesis, the p54^{nrb} mutant suppressed it (Figure 6, H–J). These data indicate that p54^{nrb} plays a critical role in chondrocyte differentiation via its RRM domains.

Requirement of p54^{nrb} in chondrogenesis in vivo. To demonstrate the importance of p54^{nrb} in chondrogenesis in vivo, we generated transgenic mice, in which the mutant p54^{nrb} is specifically expressed in the chondrocyte lineage, using the *Col2a1* gene promoter (Figure 7, A–C). Transgenic mice showed dwarfism between the neonatal period and 3 weeks after birth (Figure 7, D–H). Histological analyses showed that the layer of proliferating chondrocytes was markedly thinner in the transgenic mice compared with wild-type littermates (Figure 7I). Consistent with the reduction in *Col2a1* expression by overexpression of the mutant p54^{nrb} (Figure 6, C and D), *Col2* expression was suppressed in the transgenic mice (Figure 7, J and K). These results indicate that p54^{nrb} plays a critical role in chondrocyte differentiation. To further understand the role of p54^{nrb} in chondrogenesis, we examined the transgenic mice at younger stage. As shown in Figure 8A, enchondral ossification at the birth was markedly impaired in the transgenic mice. In situ hybridization experiments also indicated the delayed chondrogenesis in the transgenic mice at E15 (Figure 8B). In contrast, consis-

Figure 5

Impairment of paraspeckle formation and *Col2a1* mRNA processing by a mutant p54^{nrp} (Δ244). (A) Impairment of paraspeckle body formation by a p54^{nrp} mutant. ATDC5 cells transfected with Venus-tagged wild-type or mutant p54^{nrp} and DsRed-PSP1 were visualized under a fluorescence microscope. Nucleuses of cells were stained with DAPI. (B) Effect of the mutant p54^{nrp} on proliferation of ATDC5 cells. Control, wild-type p54^{nrp}, or the mutant p54^{nrp} were transfected into ATDC5 cells, and the growth of the cells was determined by cell proliferation assay. (C) Wild-type and a mutant p54^{nrp} does not affect localization of SC-35. ATDC5 cells transfected with Venus-tagged wild-type or mutant p54^{nrp} were immunostained with anti-SC-35 antibody. (D) Upregulation of transcriptional activity of Sox9 by a p54^{nrp} mutant. ATDC5 cells were transfected with wild-type and a mutant p54^{nrp} together with Sox9, and luciferase activity of cell lysates was measured. (E) Colocalization of a mutant p54^{nrp} with Sox9. ATDC5 cells transfected with DsRed-tagged Sox9 and Venus-tagged p54^{nrp} mutant were monitored under a fluorescence microscope. (F) Impairment of *Col2a1* mRNA processing by a mutant p54^{nrp}. A minigene construct of the *Col2a1* gene was transfected with expression plasmids as indicated into ATDC5 cells. Total RNA isolated from the cells was determined by RT-PCR analyses using the primers (see Methods) specific for the minigene products (upper panel) or β-actin (bottom panel). Original magnification, ×400 (A, C, and E).

entiation (14, 23, 24). p54^{nrp} is also implicated in formation of paraspeckle bodies with PSP1 (14). However, the biological role of p54^{nrp} and paraspeckle body is unknown. In this study, we demonstrate that p54^{nrp} is critical for chondrogenesis. Overexpression of p54^{nrp} upregulated the expression of chondrogenic genes and stimulated chondrocyte differentiation in C3H10T1/2 cells. In the cells, knockdown experiments supported the importance of p54^{nrp} in chondrogenesis. In organ culture systems, p54^{nrp} stimu-

lated chondrogenesis. Furthermore, overexpression of the p54^{nrp} mutant lacking RRM1 suppressed chondrogenesis in vitro and in vivo. This mutation caused enlargement of paraspeckle size, without affecting the size of the authentic nuclear speckle formed by SC-35, which is also implicated in regulation of splicing of mRNA (18). Interestingly, Sox9 is localized in the paraspeckle body and decreased its size. Collectively, our results raise a possibility that the RRM domain interaction of p54^{nrp} with Sox9 dynamically reg-

Figure 6

Role of $p54^{nrb}$ in the regulation of chondrocyte differentiation. (A) The $p54^{nrb}$ -induced *Col2a1* and *Acan* in ATDC5 cells. ATDC5 cells were transfected as indicated, and then subjected to RT-PCR analyses. (B) The $p54^{nrb}$ -induced type IIA and IIB forms of *Col2a1* in ATDC5 cells. ATDC5 cells were transfected as indicated, and then subjected to RT-PCR analyses using the specific primers (see Methods) for *Col2a1* (upper panel; the primer set can distinguish 2 isoforms of *Col2a1*), *Col2a1* (middle panel; the primer set cannot distinguish 2 isoforms of *Col2a1*), or β -actin. (C and D) ATDC5 cells were infected with Sox9 and/or Myc-tagged wild-type or a Myc-tagged mutant $p54^{nrb}$ (Δ 224) adenovirus and then subjected to northern (C) or western (D) blotting, respectively. (E) Knockdown of $p54^{nrb}$ suppressed Sox9-induced *Col2a1* expression. ATDC5 cells were transfected with expression vectors as indicated, and then total RNA of the cells was determined by RT-PCR analyses. (F) The $p54^{nrb}$ mutant blocked chondrocyte differentiation. C3H10T1/2 cells were infected with Myc-tagged wild-type or mutant $p54^{nrb}$ (Δ 224) adenovirus in the presence or absence of BMP2. The cells were examined by alcian blue staining. (G) No effects of $p54^{nrb}$ on osteoblastogenesis. C2C12 cells infected with Myc-tagged wild-type or mutant $p54^{nrb}$ (Δ 224) adenovirus in the presence or absence of BMP2. The cells were examined by alkaline phosphatase staining. (H and I) Requirement of $p54^{nrb}$ for chondrogenesis. Metatarsals isolated from mouse embryo were infected with control, Myc-tagged wild-type $p54^{nrb}$, or a Flag-tagged mutant $p54^{nrb}$ (Δ 224) adenovirus, incubated for 6 days, and subjected to immunostaining (H), histological analysis (I), and statistically analyses of the samples (J). PZ, proliferating zone; HZ, hypertrophic zone. All data were analyzed by ANOVA, followed by Student's *t* test. Values shown are mean $\pm$ SD. (**P* < 0.05, ***P* < 0.01 versus control). Original magnification, $\times 50$ (G); $\times 40$ (I).

Figure 7

Impaired chondrogenesis in the transgenic mice expressing the p54^{nrB} mutant. (A) The expression of the transgene in the chondrocytes isolated from the transgenic mice. Total RNA of chondrocytes isolated from wild-type or the transgenic mice was determined by RT-PCR analysis using specific primer (see Methods) for the transgene ($\Delta 224$) (upper panel) or β -actin (bottom panel). (B) Specific expression of p54^{nrB} mutant in chondrocytes of the transgenic mice. Total RNA isolated from brain (lane 1), kidney (lane 2), heart (lane 3), liver (lane 4), spleen (lane 5), chondrocytes (lane 6), or osteoblasts (lane 7) of the transgenic mice was determined by RT-PCR using specific primer (see Methods) for transgene ($\Delta 224$) (upper panel) or β -actin (bottom panel). (C) Overexpression of the mutant p54^{nrB} in the transgenic mice. Total RNA of chondrocytes isolated from wild-type or the transgenic mice was determined by real-time PCR analysis using a Taqman probe, which recognizes both intact and the mutant p54^{nrB}. (D and E) Dwarfism of the transgenic mice at birth. (F and G) Dwarfism of the transgenic mice at 3 weeks. D and F are macroscopic photographs and E and G are x-rays of the mice. (H) Body size of the transgenic mice. (I) H&E-stained section of the tibias of wild-type and transgenic mice at 2 weeks. (J) Col2 expression in the tibias of wild-type and transgenic mice. (K) Reduction in Col2 α 1 expression in the transgenic mice. Total RNA of chondrocytes isolated from wild-type or the transgenic mice was determined by real-time PCR analysis using a Taqman probe for Col2a1. Original magnification, $\times 40$ (I and J).

ulates the paraspeckle formation and mRNA processing necessary for chondrocyte differentiation.

In conclusion, our findings provide what we believe to be the novel paradigm whereby linkage between transcription and mRNA processing by a paraspeckle regulatory protein is critical for chondrocyte development. Thus, this finding provides a novel insight to our knowledge into the molecular basis of Sox9 regulation and chondrogenesis.

Methods

Cell and reagents. C3H10T1/2, ATDC5, HeLa, C2C12, and HEK293 cells were purchased from RIKEN and were cultured in α -MEM containing 10% fetal bovine serum. BOSC23 cells were kindly provided by Sakae Tanaka (Tokyo University Medical School, Tokyo, Japan). Recombinant BMP2 was

generated as described previously (25). Anti-HA, anti-Myc, anti-SC-35, anti-Sox9, anti-p54^{nrB}, and anti-type II collagen antibodies were purchased from Sigma-Aldrich, Santa Cruz Biotechnology Inc., Upstate, and Novotec.

Molecular cloning of p54^{nrB}. The full-length cDNA library of ATDC5 cells was generated in a pME18S-FL3 expression vector as previously described (26, 27). The 120,000 cDNA clones were screened using a luciferase reporter construct containing an 104-bp minimal promoter region and 4 copies of 48-bp Sox9-binding element but not any introduction of the Col2a1 gene (27). One positive clone encodes a full-length of p54^{nrB}.

Constructs. HA-tagged wild-type and a dominant-negative mutant (aa 21–234) Sox9 have been described previously (28). Δ HMG mutant (a mutant lacking the HMG domain) Sox9 (aa 181–510) was generated by subcloning of the corresponding PCR product into HA-tag pcDNA3 vector. DsRed-Nuc construct was purchased from Clontech. PSP1 cDNA was

Figure 8

Delayed enchondral ossification in the transgenic mice. (A) The sections of wild-type and the transgenic mice at neonatal period were subjected to H&E, alcian blue, or von Kossa staining. (B) The sections of wild-type and the transgenic mice at E15 were determined by in situ hybridization (Col2a1, Col10a1, PTHR1, Ihh), H&E staining, and von Kossa staining. (C) Normal proliferation of chondrocytes in wild-type and the transgenic mice at E15. The sections of wild-type and the transgenic mice at E15 were determined by immunostaining with anti-PCNA antibody. Original magnification, $\times 25$ (A); $\times 40$ (B); $\times 20$ (C, left panel of WT and Tg); $\times 40$ (C, right panel of WT and Tg).

Immunoprecipitation and immunoblotting analysis. The cells were washed 3 times with ice-cold PBS and solubilized in lysis buffer (29). The lysates were centrifuged at 16,000 g for 15 minutes at 4°C and incubated with antibodies for 4 hours at 4°C, followed by immunoprecipitation with protein A-sepharose (Zymed) or protein G-agarose (Roche). Immunoprecipitates were washed 5 times with lysis buffer and boiled in SDS sample buffer, and supernatants were recovered as immunoprecipitate samples.

Cell lysates, nuclear extracts, cell matrix extracts, or immunoprecipitated samples were isolated were determined by immunoblotting using anti-Myc, anti-HA, or anti-type II collagen antibodies (25).

Production of recombinant His-tagged p54^{nrb} protein. PCR product containing the coding region of p54^{nrb} was subcloned into pCold vector (Takara). The construct was transformed into BL21 *E. coli* strain, and then His-tagged p54^{nrb} protein was generated and purified with Ni-NTA agarose beads (QIAGEN) according to the manufacturer's protocol. The purified His-tagged p54^{nrb} protein immobilized with the beads was used for binding assay with Sox9.

In vitro binding assay. Cell lysates were incubated with tandem affinity purification-tagged (TAP-tagged) Sox9 protein immobilized on IgG-Sepharose beads at 4°C for 2 hours. The beads were washed 5 times with lysis buffer, and then the proteins associated with TAP-tagged Sox9 were determined by immunoblotting using anti-Myc antibody. The association of p54^{nrb} with mutants of HA-tagged Sox9 was determined by immunoblotting with anti-HA antibody, following precipitation of cell lysates with recombinant His-tagged p54^{nrb} protein.

Immunocytochemical and immunohistochemical analyses. Cultured cells were washed 3 times with ice-cold PBS and fixed with 3.7% paraformaldehyde-PBS for 20 minutes. After 20-minute incubation with 0.1% Triton X-100-PBS, the cells were blocked with 1% BSA-containing PBS for 2 hours, incubated with anti-SC-35 antibody in 1% BSA-containing PBS, and then washed 6 times with PBS and incubated with FITC-conjugated affinity-purified anti-mouse IgG antibody (The Jackson Laboratory). The cells were washed extensively with PBS and visualized by fluorescence microscope (Zeiss) or confocal microscope (Zeiss). ATDC5 cells transfected with Venus-p54^{nrb}, Venus-p54^{nrb} ($\Delta 224$), DsRed-PSP1, or DsRed-Nuc (Clontech) were visualized by fluorescence microscope. Bones of mice were fixed in 3.8% formalin-PBS, decalcified in 4% EDTA, and then histologically analyzed by H&E staining and immunohistochemically analyzed using anti-type II collagen antibody. Undecalcified bone sections prepared from embryo or

a kind gift from Angus Lamond (University of Dundee, Dundee, United Kingdom) (14). To generate a mutant form of p54^{nrb} ($\Delta 224$), PCR products which encoded a portion of p54^{nrb} (aa 225–473) were subcloned into pcDNA3 (Invitrogen) tagged with a Flag epitope, Myc epitope, or Venus (an improved GFP) tag in N-terminus. The sequences of these constructs were confirmed by DNA sequence analysis. SRP40 cDNA was purchased from Open Biosystems Inc. Transfection of ATDC5 cells was carried out using Eugene6 (Roche) according to the manufacturer's protocol.

Reporter assay. The Col2a1 gene promoter fused to luciferase reporter construct was transfected to ATDC5 cells. Two days after transfection, cells were lysed, and luciferase activity was determined using specific substrates in a luminometer (Promega) according to manufacturer's protocol. Transfection efficiency was normalized by determining the activity of renilla luciferase.

neonatal mice were analyzed by H&E staining, von Kossa staining, alcian blue staining and immunohistochemical analysis using anti-PCNA antibody (DAKO Cytomation).

RT-PCR. Total RNA was isolated from cells using RNeasy mini kit (QIAGEN). After denaturation of total RNA at 70°C for 10 minutes, cDNA was synthesized with oligo-dT primer and RT (Clontech). PCR amplification was performed by using the specific primers for aggrecan (forward primer, 5'-TCCTCTCCGGTGGCAAAGAAGTTG-3'; reverse primer, 5'-CCAAGTTCAGGGTCACTGTTACCG-3'), β -actin (forward primer, 5'-TTCGAGCAGGAGATGGCCAC-3'; reverse primer, 5'-TCTGCATCCTGTCAGCAAT-3'), or GAPDH (forward primer, 5'-ACATCAAGAAGGTGTGAAGCAGG-3'; reverse primer, 5'-CTCTTGCTCTCAGATCCTTGCTGG-3'). To determine *Col2a1* mRNA expression, we used 2 sets of primers. One set of primers (forward primer, 5'-CAGGCCTCGCGTGAGCCATGAT-3'; reverse primer, 5'-GTTCTCCATCTCTGCCACG-3') distinguished 2 different isoforms of *Col2a1* (IIA and IIB). The other set of primers (forward primer, 5'-GGTTTGAGAGACCATGAAC-3'; reverse primer, 5'-TGGGTTCGCAATGGATTGTG-3') recognizes *Col2a1* (IIA and IIB). PCR products were loaded agarose gel and stained with ethidium bromide. To determine the expression level of transgene in chondrocytes of the transgenic mice, real-time PCR analysis was performed using Taqman probe that recognizes both endogenous and the mutant p54^{nrB}.

Minigene assay. A minigene containing exon 1–4 of the mouse *Col2a1* gene fused to the native promoter (20) was transfected to ATDC5 cells. Total RNA was isolated from the cells and determined by RT-PCR analysis as previously described (20). Quantification of spliced-minigene and nonspliced products was determined by real-time PCR analysis using Taqman probes specific for minigene products. Minigene assay for fibronectin was performed using 7iBi minigene construct as previously described (30).

Northern blotting. Total RNA was isolated from cells using RNeasy mini kit (QIAGEN). Total RNA was subjected to 1% formaldehyde-agarose gels in 2.2 M formaldehyde, 20 mM MOPS buffer, and 1 mM EDTA and transferred to nylon membrane (QIAGEN). After UV cross-linking and prehybridization, the membrane was hybridized with *Col2a1*-specific probe labeled with digoxigenin. The signals were detected using DIG detection kit (Roche).

Chondrocyte differentiation. C3H10T1/2 cells were cultured in α -MEM containing ascorbic acid and 10% FCS with or without BMP2 (300 ng/ml), in the presence of 5% CO₂ and 5% O₂ for a week (31). The cells were analyzed by staining with Alcian blue (31).

Knockdown of p54^{nrB}. shRNA constructs were generated in pFIV-H1cop-GFP (B-Bridge). The target sequence is mouse p54^{nrB}, 5'-GACCTTTACACAGCGTAGC-3'. The shRNA expression construct was transfected into ATDC5 using Eugene6 (Roche).

Generation of adenovirus. The recombinant adenovirus carrying HA-Sox9, Myc-tagged wild-type p54^{nrB}, Myc-tagged p54^{nrB} (Δ 224), or Flag-tagged p54^{nrB} (Δ 224) cDNA was constructed by homologous recombination between the expression cosmid cassette and the parental virus genome in HEK293 cells as previously described (29). The viruses were confirmed to retain no proliferative activity in the cells other than HEK293 cells, because they lack both E1A and E1B domain of adenovirus (29). Titers of the viruses were analyzed by modified point assay (29).

Generation and analyses of transgenic mice. Flag-p54^{nrB} (Δ 224) cDNA was fused to the *Col2a1* gene promoter (32). The transgenic mice were generated by pronuclear injection methods into BDF1 strain and backcrossed to a C57BL/6 background (29). Genomic DNA isolated from the tail was analyzed by PCR and dot-blotting analysis using specific primers or the

probes for transgene (forward primer, [for Flag epitope] 5'-ACAAGGACGATGATGACAAGG-3'; reverse primer, 5'-GTTCTCTTCTGCTGCTGAGTT-3'), respectively. Bones were fixed in 10% buffered formalin, decalcified, and subjected to staining with H&E or immunohistological analysis using anti-Col2 antibody. All experiments were performed with littermates mice under protocols approved by Osaka University Graduate School of Dentistry animal committee.

Cell proliferation assay. Cell proliferation assay was performed using cell proliferation reagent WST-1 (Roche) according to manufacture's protocol. Briefly, ATDC5 cells infected with p54^{nrB} or control adenovirus for 2 days were plated into 96-well plates (500/well) and incubated at 37°C in a 5% CO₂ atmosphere. On day 1, 2, 3, and 4, 10 μ l of cell proliferation reagent WST-1 was added to each well and incubated for 1 hour. The cell number was determined by analysis of absorption at 450 nm using a microplate reader (Bio-Rad).

Organ culture of mouse metatarsals. The 3 central metatarsal rudiments were isolated from each hind limb of E15.5 mice. The metatarsals were incubated in α -MEM supplemented with 0.05 mg/ml ascorbic acid, 0.3 mg/ml L-glutamine, 1 mM β -glycerophosphate, 0.2% BSA, and 300 ng/ml BMP2 in a humidified 5% CO₂ incubator at 37°C. Six days after incubation, metatarsal explants were subjected to histological analysis. The samples were statistically analyzed using ImagePro program (Media Cybernetics Inc.).

In situ hybridization. Tibias from C57BL/6 mouse embryos (at E15) were fixed in 4% paraformaldehyde-PBS overnight at 4°C, embedded in paraffin, and cut into 5- μ m sections. Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared using a DIG RNA labeling kit (Roche Biochemica) according to the manufacturer's instructions. We used a 0.4-kb fragment of mouse *Col2a1* cDNA, a 0.65-kb fragment of mouse *Col10a1* cDNA, a 0.57-kb fragment of mouse *Ihh* cDNA, and a 0.8-kb fragment of mouse *Pth1r* cDNA to generate antisense and sense probes. The signals were detected with ALP-conjugated anti-DIG antibody (Roche). All probes were kindly provided by Toshihisa Komori (Nagasaki University Dental School, Nagasaki, Japan).

Statistics. The data were statistically analyzed by 1-tailed Student's *t* test. Data represent mean $\pm$ SD. *P* values of less than 0.05 were considered significant.

Acknowledgments

We thank Benoit de Crombrughe for the *Col2a1* gene promoter, Angus Lamond for PSP1 cDNA, Gobinda Sarkar for *Col2a1* minigene cassette DNA, and Atsushi Miyawaki for Venus construct. We thank Masahiro Saito and Eisaku Nishida for technical help for in situ hybridization. This work was supported in part by the Ministry of Education, Science, Sports and Culture Grant-in-Aid (to R. Nishimura and T. Yoneda), Grant-in-Aid for Scientific Research on Priority Areas B (to T. Yoneda and R. Nishimura), The 21st Century COE Program (to T. Yoneda and R. Nishimura), Kato Memorial Bioscience Foundation (to K. Hata), and the NHMRC Australia (to V.R. Harley).

Received for publication December 29, 2006, and accepted in revised form June 11, 2008.

Address correspondence to: Riko Nishimura, Department of Molecular and Cellular Biochemistry, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka, 565-0871, Japan. Phone: 81-6-6879-2887; Fax: 81-6-6879-2890; E-mail: rikonisi@dent.osaka-u.ac.jp.

1. Kronenberg, H.M. 2003. Developmental regulation of the growth plate. *Nature*. **423**:332–336.
2. de Crombrughe, B., et al. 2000. Transcriptional mechanisms of chondrocyte differentiation. *Matrix*

Biol. **19**:389–394.

3. de Crombrughe, B., Lefebvre, V., and Nakashima, K. 2001. Regulatory mechanisms in the pathways of cartilage and bone formation. *Curr. Opin. Cell*

Biol. **13**:721–727.

4. Morais da Silva, S., et al. 1996. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals

- and birds. *Nat. Genet.* **14**:62–68.
5. McDowall, S., et al. 1999. Functional and structural studies of wild type SOX9 and mutations causing campomelic dysplasia. *J. Biol. Chem.* **274**:24023–24030.
6. Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R., and de Crombrughe, B. 1999. Sox9 is required for cartilage formation. *Nat. Genet.* **22**:85–89.
7. Akiyama, H., et al. 2002. 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.* **16**:2813–2828.
8. Kawakami, Y., et al. 2005. Transcriptional coactivator PGC-1 α regulates chondrogenesis via association with Sox9. *Proc. Natl. Acad. Sci. U. S. A.* **102**:2414–2419.
9. Akiyama, H., et al. 2004. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.* **18**:1072–1087.
10. Shukunami, C., et al. 1996. Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. *J. Cell Biol.* **133**:457–468.
11. Hallier, M., Tavitian, A., and Moreau-Gachelin, F. 1996. The transcription factor Spi-1/PU.1 binds RNA and interferes with the RNA-binding protein p54nrb. *J. Biol. Chem.* **271**:11177–11181.
12. Basu, A., Dong, B., Krainer, A.R., and Howe, C.C. 1997. The intracisternal A-particle proximal enhancer-binding protein activates transcription and is identical to the RNA- and DNA-binding protein p54nrb/NonO. *Mol. Cell. Biol.* **17**:677–686.
13. Peters, U., et al. 1997. AFX1 and p54nrb: fine mapping, genomic structure, and exclusion as candidate genes of X-linked dystonia parkinsonism. *Hum. Genet.* **100**:569–572.
14. Fox, A.H., et al. 2002. Paraspeckles: a novel nuclear domain. *Curr. Biol.* **12**:13–25.
15. Komori, T., et al. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell.* **89**:755–764.
16. Fox, A.H., Bond, C.S., and Lamond, A.I. 2005. P54nrb forms a heterodimer with PSP1 that localizes to paraspeckles in an RNA-dependent manner. *Mol. Biol. Cell.* **16**:5304–5315.
17. Lamond, A.I., and Spector, D.L. 2003. Nuclear speckles: a model for nuclear organelles. *Nat. Rev. Mol. Cell Biol.* **4**:605–612.
18. Sahlas, D.J., Milankov, K., Park, P.C., and De Boni, U. 1993. Distribution of snRNPs, splicing factor SC-35 and actin in interphase nuclei: immunocytochemical evidence for differential distribution during changes in functional states. *J. Cell Sci.* **105**:347–357.
19. Tabellini, G., et al. 2003. Diacylglycerol kinase-theta is localized in the speckle domains of the nucleus. *Exp. Cell Res.* **287**:143–154.
20. Nishiyama, T., Hatano, H., Kurosaka, M., Bolander, M.E., and Sarkar, G. 2003. Cis-acting intronic elements that regulate cartilage-specific alternative splicing of the type II collagen (Col2) pre-mRNA lie at or near splice site junction sequences flanking exon 2 of the gene. *J. Bone Miner. Res.* **18**:1716–1722.
21. Zhu, Y., Oganessian, A., Keene, D.R., and Sandell, L.J. 1999. Type IIA procollagen containing the cysteine-rich amino propeptide is deposited in the extracellular matrix of prechondrogenic tissue and binds to TGF-beta1 and BMP-2. *J. Cell Biol.* **144**:1069–1080.
22. Kameoka, S., Duque, P., and Konarska, M.M. 2004. p54(nrb) associates with the 5' splice site within large transcription/splicing complexes. *EMBO J.* **23**:1782–1791.
23. Dye, B.T., and Patton, J.G. 2001. An RNA recognition motif (RRM) is required for the localization of PTB-associated splicing factor (PSF) to subnuclear speckles. *Exp. Cell Res.* **263**:131–144.
24. Ishitani, K., et al. 2003. p54nrb acts as a transcriptional coactivator for activation function 1 of the human androgen receptor. *Biochem. Biophys. Res. Commun.* **306**:660–665.
25. Hata, K., et al. 2005. A CCAAT/enhancer binding protein beta isoform, liver-enriched inhibitory protein, regulates commitment of osteoblasts and adipocytes. *Mol. Cell. Biol.* **25**:1971–1979.
26. Matsuda, A., et al. 2003. Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. *Oncogene.* **22**:3307–3318.
27. Muramatsu, S., et al. 2007. Functional gene screening system identified TRPV4 as a regulator of chondrogenic differentiation. *J. Biol. Chem.* **282**:32158–32167.
28. Preiss, S., et al. 2001. Compound effects of point mutations causing campomelic dysplasia/autosomal sex reversal upon SOX9 structure, nuclear transport, DNA binding, and transcriptional activation. *J. Biol. Chem.* **276**:27864–27872.
29. Ikeda, F., et al. 2004. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J. Clin. Invest.* **114**:475–484.
30. Du, K., Peng, Y., Greenbaum, L.E., Haber, B.A., and Taub, R. 1997. HRS/SRp40-mediated inclusion of the fibronectin EIIIB exon, a possible cause of increased EIIIB expression in proliferating liver. *Mol. Cell. Biol.* **17**:4096–4104.
31. Fischer, L., Boland, G., and Tuan, R.S. 2002. Wnt-3A enhances bone morphogenetic protein-2-mediated chondrogenesis of murine C3H10T1/2 mesenchymal cells. *J. Biol. Chem.* **277**:30870–30878.
32. Metsaranta, M., Toman, D., de Crombrughe, B., and Vuorio, E. 1991. Mouse type II collagen gene. Complete nucleotide sequence, exon structure, and alternative splicing. *J. Biol. Chem.* **266**:16862–16869.