Arid5a cooperates with Sox9 to stimulate chondrocyte-specific transcription

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ABSTRACT **SRY-box–containing gene 9 (Sox9) is an essential transcription factor in chondrocyte lineage determination and differentiation. Recent studies demonstrated that Sox9 controls the transcription of chondrocyte-specific genes in association with several other transcriptional regulators. To further understand the molecular mechanisms by which Sox9 influences transcriptional events during chondrocyte differentiation, we attempted to identify transcriptional partners of Sox9 and to examine their roles in chondrocyte differentiation. We isolated AT-rich interactive domain–containing protein 5a (Arid5a; also known as Mrf1) as an activator of the *Col2a1* gene promoter from an ATDC5 cDNA library. Arid5a was highly expressed in cartilage and induced during chondrocyte differentiation. Furthermore, Arid5a physically interacted with Sox9 in nuclei and up-regulated the chondrocyte-specific action of Sox9. Overexpression of Arid5a stimulated chondrocyte differentiation in vitro and in an organ culture system. In contrast, Arid5a knockdown inhibited *Col2a1* expression in chondrocytes. In addition, Arid5a binds directly to the promoter region of the *Col2a1* gene and stimulates acetylation of histone 3 in the region. Our results suggest that Arid5a may directly interact with Sox9 and thereby enhance its chondrocyte-specific action.**

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INTRODUCTION

Endochondral ossification is strictly regulated by several hormones, cytokines, and growth factors that activate downstream signaling and regulate transcription factors (de Crombrugghe *et al.*, 2000; Ornitz and Marie, 2002; Kronenberg, 2003; Nishimura *et al.*, 2008). One transcription factor, Sox9, plays indispensable roles in endochondral ossification in vertebrates. Mutations of the *SOX9* gene result in campomelic dysplasia, which is characterized by severe chondrodysplasia and autosomal sex reversal (Foster *et al.*, 1994; Wagner *et al.*, 1994; Sock *et al.*, 2003). *Sox9* conditional knockout mice completely lack cartilage development (Akiyama *et al.*, 2002). Several studies indicate that Sox9 is an important transcription factor that regulates the expression of chondrocyte-specific genes, including *Col2a1*, *Col11a2*, *Agc1*, *S100a1*, *S100b*, and *Pthrp*, which encode collagen type IIα1, collagen type XIα2, aggrecan, S100 calcium binding protein A1, S100 calcium binding protein B, and parathyroid hormone–related peptide, respectively (Bell *et al.*, 1997; Bridgewater *et al.*, 1998; Saito *et al.*, 2007; Han and Lefebvre, 2008; Amano *et al.*, 2009). In contrast, Sox9 appears to negatively regulate the late stage of chondrogenesis (Saito *et al.*, 2007; Amano *et al.*, 2009; Hattori *et al.*, 2010). Thus, Sox9 has complex roles in endochondral ossification.

Transcription factors can form large molecular complexes that mediate chromatin remodeling, histone modification, transcription, and splicing of primary transcripts (Cook, 1999; Xu and Cook, 2008). Several studies show that Sox9 forms a transcriptional complex with several other transcriptional regulators and temporally and spatially regulates chondrogenesis in the context of this complex. Peroxisome proliferator-activated receptor-γ coactivator-1α, Smad3, CREB binding protein/p300, and Tat interactive protein-60 have been shown to participate in Sox9-induced,

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Abbreviations used: Arid5a, AT-rich interactive domain–containing protein 5a (also known as Mrf1); BMP2, bone morphogenetic protein 2; BS, binding site; ChIP, chromatin IP; DN, dominant negative; FCS, fetal calf serum; H3 histone 3; H4, histone 4; H&E, hematoxylin and eosin; HAT, histone acetyl transferase; His, histidine; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; RT, reverse transcription; Sox5, SRY-box–containg gene 5; Sox6, SRY-box–containg gene 6; Sox9, SRY-box–containing gene 9; TK, thymidine kinase.

chondrocyte-specific actions (Kawakami *et al.*, 2005; Furumatsu *et al.*, 2005a, 2005b; Hattori *et al.*, 2008). Sox family members SRY-box–containing gene 5 (Sox5) and SRY-box–containing gene 6 (Sox6) are essential for chondrogenesis (Smits *et al.*, 2001, 2004) and appear to interact with Sox9 during chondrogenesis (Lefebvre *et al.*, 1996). $p54^{nrb}$ has been identified as a member of the transcriptional complex assembled by Sox9 and has been shown to be a coupling molecule that mediates Sox9-regulated transcription and splicing during chondrogenesis (Hata *et al.*, 2008). Sox9 plays important roles not only in cartilage but also in heart, testis, and immune cells (Akiyama *et al.*, 2004; Barrionuevo *et al.*, 2006; Horiuchi *et al.*, 2009); therefore how Sox9 specifically mediates chondrocyte linage determination and differentiation is of interest. Identification and characterization of the Sox9 transcriptional partners will contribute to further understanding of the molecular basis by which Sox9 regulates chondrocyte linage determination and differentiation.

In this study, we attempted to further understand the transcriptional regulation of the *Col2a1* gene, because *Col2a1* encodes a major cartilage matrix component (Bell *et al.*, 1997; de Crombrugghe *et al.*, 2000; Kronenberg, 2003) by Sox9. We isolated AT-rich interactive domain–containing protein 5a (Arid5a, also known as Mrf1) as a transcriptional partner of Sox9. Furthermore, we found that Arid5a stimulated chondrocyte differentiation in collaboration with Sox9. Collectively, our results suggest that Arid5a may be an important transcriptional partner of Sox9 during chondrocyte differentiation.

RESULTS

Expression of Arid5a in cartilage and during chondrocyte differentiation

To understand the molecular mechanisms by which Sox9 regulates chondrocyte linage determination and differentiation, we attempted to identify a transcriptional partner of Sox9. Therefore we performed mammalian expression cloning in ATDC5, a chondrogenic cell line, using an ATDC5 cDNA library and a *Col2a1* reporter construct (Muramatsu *et al.*, 2007; Hata *et al.*, 2008). We isolated *Arid5a*, which belongs to the Arid family of DNA binding proteins (Wilsker *et al.*, 2002), as a positive clone that increased the activity of a *Col2a1* reporter, from the ATDC5 cDNA library. Interestingly, *Arid5b* (also known as *Mrf2* and *Desert*)-deficient mice show skeletal abnormalities and dwarfism (Lahoud *et al.*, 2001; Whitson *et al.*, 2003; Schmahl *et al.*, 2007), suggesting that Arid5b might play a role in bone and/or cartilage development. Because Arid5a shares the highest identity with Arid5b among Arid family members, and because the role of Arid5a in vivo, particularly in cartilage development, is currently unknown, we decided to investigate the role of Arid5a in chondrocyte differentiation. To examine whether *Arid5a* is expressed in cartilage, we performed real-time reverse transcription (RT)-PCR analysis in several mouse tissue types. *Arid5a* was highly expressed in cartilage, heart, and testis, where *Sox9* function is also essential (Figure 1A) (Akiyama *et al.*, 2004; Nel-Themaat *et al.*, 2009). *Arid5a* was also highly expressed in bone tissues (Figure 1B). To confirm the results, we performed immunohistochemical analyses of the growth plate of the mouse tibia. We confirmed that an anti-Arid5a polyclonal antibody recognized Arid5a protein as determined by immunoblotting analysis (Figure 1C). Immunohistochemical analyses indicated that Arid5a and Sox9 have similar expression patterns in the cartilage of the mouse growth plate (Figure 1D). These results suggested that Arid5a might be involved in chondrocyte differentiation and associated with Sox9. To understand the involvement of Arid5a in chondrocyte differentiation, we determined whether Arid5a expression was associated with chondrocyte differentiation. To address this question, we overexpressed Sox9 in ATDC5 cells using an adenovirus expression system (Figure 1E) and examined the expression of endogenous *Arid5a* as determined by real-time RT-PCR analysis. As shown in Figure 1F, endogenous *Arid5a* was dramatically induced along with the up-regulation of endogenous *Col2a1* expression. Similarly, concomitant overexpression of Sox9, Sox5, and Sox6, which induce chondrocyte differentiation (Amano *et al.*, 2009), also up-regulated endogenous *Col2a1* and *Arid5a* expression in a mesenchymal cell line, C3H10T1/2, which can differentiate into chondrocytes (Figure 1, G and H). These results suggested that Arid5a was associated with chondrocyte differentiation.

Interaction of Arid5a with Sox9 during chondrocyte differentiation

To address whether Arid5a was a transcriptional partner of Sox9, we performed coimmunoprecipitation experiments to examine the relationship between Arid5a and Sox9 in BOSC23 cells, which are readily transfected. When we overexpressed both Flag-tagged Arid5a (Flag-Arid5a) and HA-tagged-Sox9 (HA-Sox9) in BOSC23 cells, Flag-Arid5a coprecipitated with HA-Sox9 (Figure 2A). To further examine the relationship between Arid5a and Sox9, cellular localization of Arid5a and Sox9 was assessed in ATDC5 cells by overexpressing Venus-tagged Arid5a (Venus-Arid5a) and DsRed-tagged Sox9 (DsRed-Sox9). We found that Venus-Arid5a was localized in the nucleus, and it formed granular structures (Figure 2B). When we introduced both Venus-Arid5a and DsRed-Sox9 in ATDC5 cells, Venus-Arid5a was closely associated with DsRed-Sox9 in the nucleus (Figure 2B). We then investigated the functional interaction of Arid5a with Sox9 by performing reporter assays using a *Col2a1* reporter construct containing the human Col2a1 gene promoter (–89 to +16) and four 48-base pair tandem repeats of the Sox9 binding element in ATDC5 cells. As expected, overexpression of Arid5a significantly increased *Col2a1* reporter activity (Figure 2C). Importantly, overexpression of Arid5a markedly enhanced the transcriptional activity of Sox9 on the *Col2a1* reporter (Figure 2C). Conversely, a dominant-negative (DN) form of Sox9 that lacks the transcriptional activation domain (Amano *et al.*, 2009) suppressed *Col2a1* reporter activity induced by Arid5a (Figure 2D). These results suggest that Arid5a and Sox9 interact to cooperatively regulate *Col2a1* reporter activity.

To determine whether Arid5a regulated chondrocyte differentiation, by using an adenovirus expression system we overexpressed Arid5a with or without Sox9 in ATDC5 cells and then we examined expression of endogenous *Col2a1* by using real-time RT-PCR. Overexpression of Arid5a overexpression significantly increased endogenous *Col2a1* expression (Figure 3, A and B). Furthermore, Arid5a overexpression markedly stimulated chondrocyte-specific activity of Sox9 in ATDC5 cells as determined by endogenous *Col2a1* expression (Figure 3B). Together with coimmunoprecipitation experiments, these results confirm that Arid5a physically and functionally interacts with Sox9 to regulate chondrocyte differentiation.

To further understand the role of Arid5a in chondrocyte differentiation, we next performed knockdown experiments using Arid5a siRNA or synthetic Arid5a miRNA in ATDC5 cells. Knockdown of Arid5a mediated by siRNA suppressed endogenous *Col2a1* expression in ATDC5 cells (Figure 4, A and B). Infection of adenovirus carrying the synthetic Arid5a miRNA suppressed expression of endogenous *Arid5a* without affecting overexpression of *SOX9* or endogenous *Sox9* levels (Figure 4C). Consistent with the results shown in Figure 4B, knockdown of Arid5a using the synthetic Arid5a miRNA inhibited expression of endogenous *Col2a1* induced by

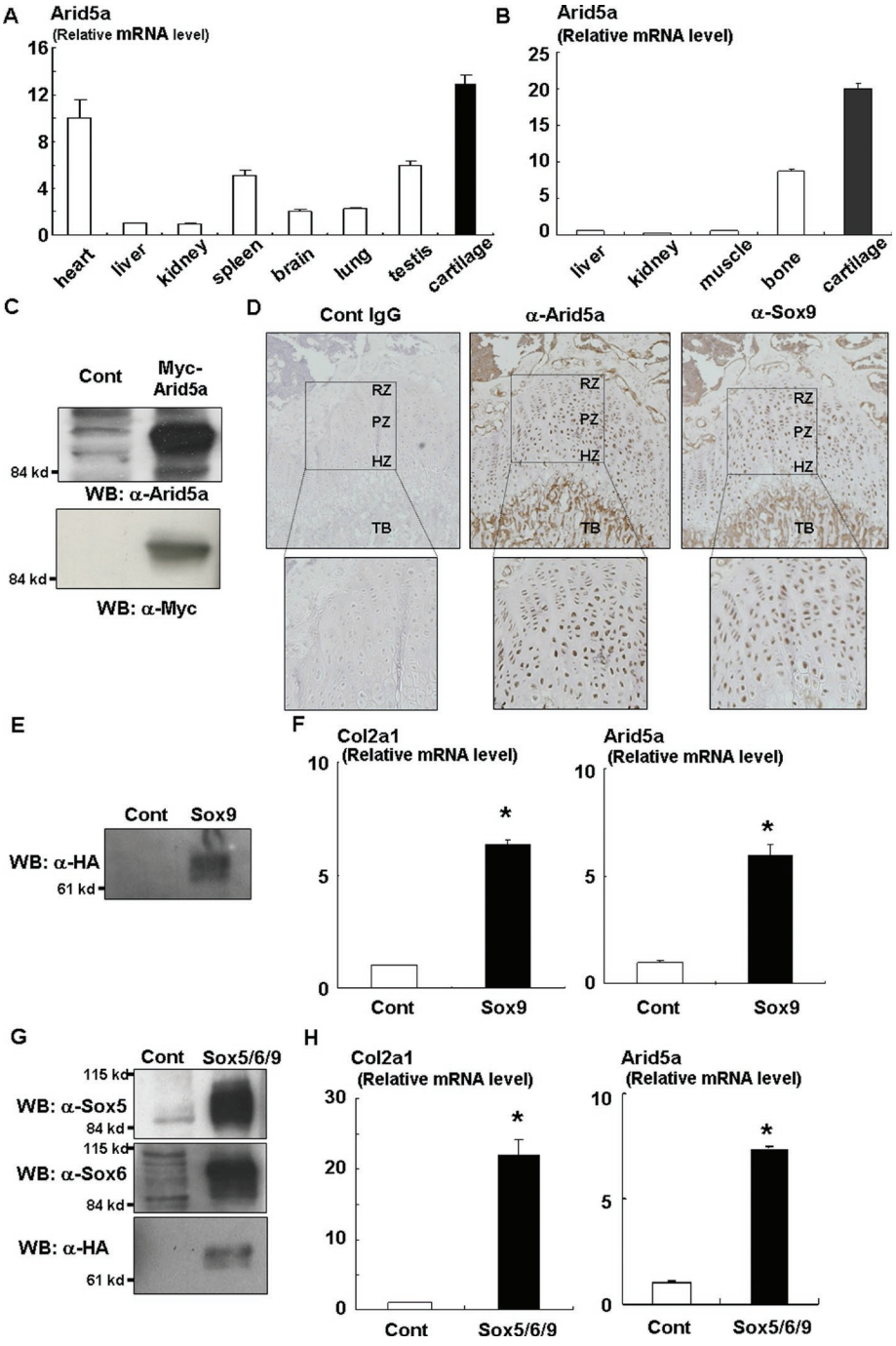

FIGURE 1: Expression of Arid5a in cartilage and during chondrocyte differentiation. (A and B) Total RNA was isolated from several tissues of 4-wk-old DDY mice, as indicated. Cartilage and bone were isolated from the rib and calvaria of the mice, respectively. These samples of total RNA were subjected to real-time RT-PCR analyses for Arid5a expression. Relative mRNA levels were normalized with β-actin expression. (C) BOSC23 cells were transfected with pcDNA3 (Cont) or 6xMyc-Arid5a expression vector. Two days after transfection, the cells were lysed and the lysates were subjected to immunoblotting with anti-Arid5a (top panel) or anti-Myc (bottom panel) antibody. (D) Paraffin sections of tibia excised from 1-d-old mice were immunostained with control IgG (left panel), anti-Arid5a antibody (middle panel), and anti-Sox9 antibody (right panel). The bottom panels show the magnified sections. RZ: resting chondrocyte zone, PZ: proliferating chondrocyte zone, HZ: hypertrophic chondrocyte zone, TB: trabecular bone. (E) ATDC5 cells infected with control (Cont) or HA-Sox9 adenoviruses were cultured for 2 d. Expression of HA-Sox9 was examined by immunoblotting with anti-HA antibody. (F) ATDC5 cells infected with control (Cont) or HA-Sox9 adenoviruses were cultured for 3 d. Total RNA isolated from the cells was determined by real-time RT-PCR analyses for *Col2a1* (left panel) and *Arid5a* (right panel). Relative mRNA levels were normalized with β-actin expression. *$p < 0.01$ (vs. control) as determined by Student's *t* test. (G) C3H10T1/2 cells were infected with adenoviruses carrying Sox5, Sox6, and HA-Sox9, and were cultured for 2 d. Expression of Sox5, Sox6, and HA-Sox9 was examined by immunoblotting with anti-Sox5 (top panel), anti-Sox6 (middle panel), or anti-HA antibodies (bottom panel). (H) C3H10T1/2 cells infected with control (Cont) or Sox5/6/9 adenoviruses were cultured for 3 d. Total RNA isolated from the cells was subjected to real-time RT-PCR analyses for *Col2a1* (left panel) and *Arid5a* (right panel). Relative mRNA levels were normalized with β-actin expression. *$p < 0.01$ (vs. control) as determined by Student's *t* test.

Sox9 (Figure 4C). In addition, knockdown of Arid5a using the synthetic Arid5a miRNA suppressed induction of endogenous Col2 expression by Sox9, Sox5, and Sox6 as determined by immunoblotting analysis (Figure 4D). Together, these results suggest that collaboration between Arid5a and Sox family members is necessary for chondrocyte differentiation.

Stimulation of early-stage chondrocyte differentiation by Arid5a

To further investigate the role of Arid5a in chondrocyte differentiation, we used organ culture experiments (Yasoda *et al.*, 1998) using metatarsals dissected from 15.5-d-old mouse embryos. Overexpression of Arid5a by adenovirus infection increased the size of the epiphysis of the metatarsals (Figure 5A). We quantitatively analyzed the metatarsals using classification of chondrocyte zone (Yasoda *et al.*, 1998). Overexpression of Arid5a enlarged the noncalcified chondrocyte zone in the epiphysis (Figure 5B), suggesting that Arid5a stimulated chondrocyte differentiation. Histological analyses indicated that the overexpression of Arid5a increased the number of small and round proliferative chondrocytes (Figure 5C). Overexpression of Arid5a enlarged the Col2-positive and Col10-negative area in the metatarsals (Figure 5, D and E). To confirm proliferative action of Arid5a during chondrocyte differentiation, we examined the effect of Arid5a overexpression on proliferation of C3H10T1/2 cells in the presence of bone morphogenetic protein 2 (BMP2), which induces chondrocytic differentiation in these cells (Hata *et al.*, 2008), by performing a WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benezene disulfonate) cell proliferation assay. We found that overexpression of Arid5a increased the proliferation of C3H10T1/2 cells (Figure 5F). Interestingly, overexpression of Arid5a decreased the calcified zone in the metatarsals (Figure 5G). To further examine the effect of Arid5a on calcification of

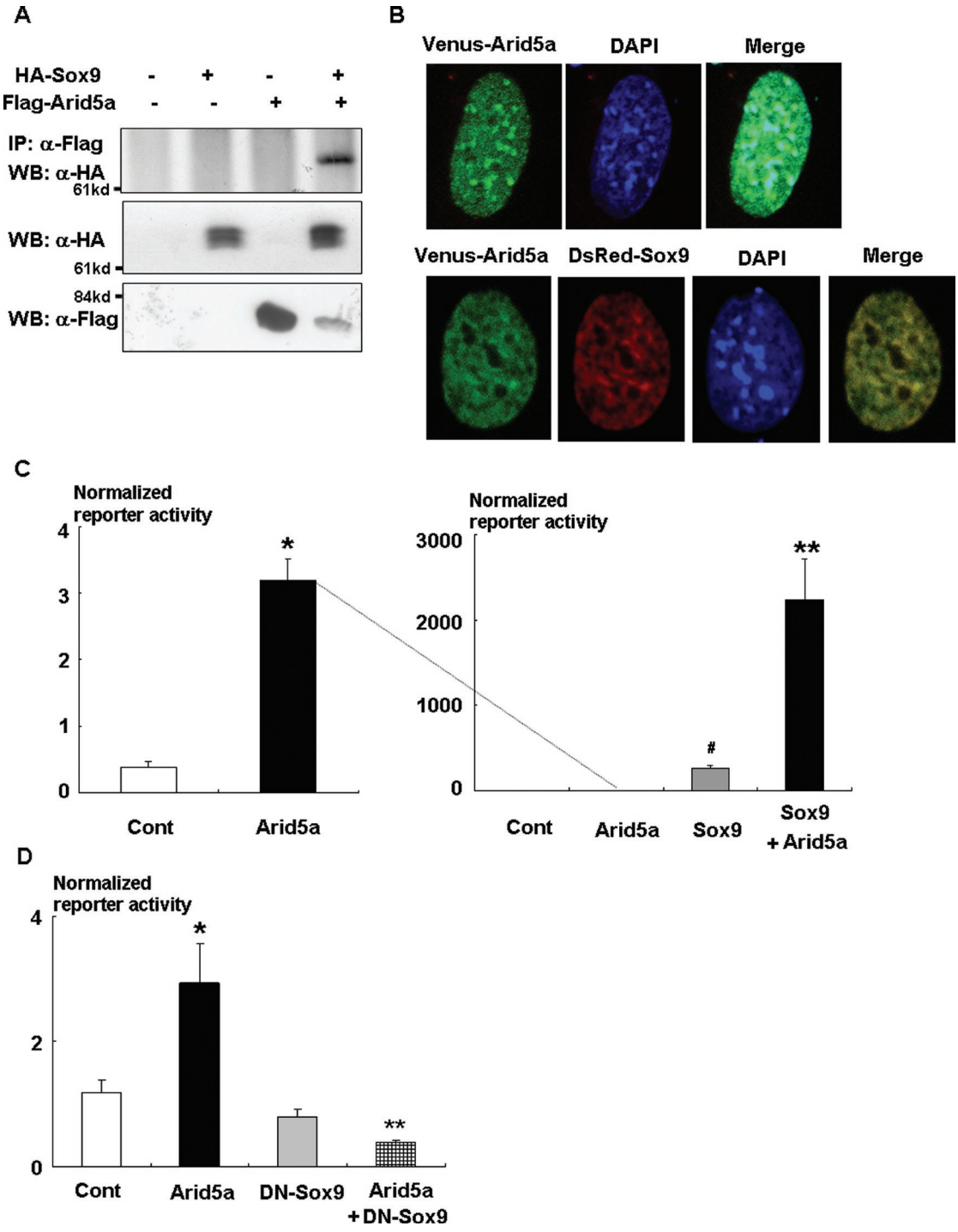

FIGURE 2: Interaction of Arid5a with Sox9. (A) BOSC23 cells were transfected with HA-Sox9 and/or Flag-tagged Arid5a (Flag-Arid5a) as indicated. Lysates of the cells were immunoprecipitated with anti-Flag antibody, and then precipitated samples were subjected to immunoblotting with anti-HA antibody (top panel). Expression of HA-Sox9 and Flag-Arid5a was determined by immunoblotting with anti-HA antibody (middle panel) or anti-Flag antibody (bottom panel). (B) Venus-Arid5a was transfected into ATDC5 cells with (bottom panels) or without (top panels) DsRed-Sox9. The cells were fixed, stained with DAPI, and visualized using a confocal laser scanning microscope. (C) ATDC5 cells were transfected with the *Col2a1* reporter and the TK-renilla luciferase constructs with an empty vector (Cont), HA-Sox9, 6xMyc-Arid5a, or both HA-Sox9 and 6xMyc-Arid5a, as indicated. After 48 h of transfection, firefly luciferase activity in the cell lysates was measured, and reporter activity was normalized to renilla luciferase activity as shown. The left and right panels were obtained in the same experiment but are shown using different scales. *$p < 0.01$ (vs. control), #$p < 0.01$ (vs. control) as determined by Student's *t* test. **$p < 0.01$ vs. Sox9, as determined by one-way analysis of variance. (D) ATDC5 cells were transfected with the Col2a1 reporter and the TK-renilla luciferase constructs with an empty vector (Cont), 6xMyc-Arid5a, DN Sox9 (DN-Sox9), or both 6xMyc-Arid5a and DN-Sox9. After 48 h of transfection, firefly luciferase activity in the cell lysates was measured. Reporter activity was normalized to renilla luciferase activity. *$p < 0.01$ (vs. control), **$p < 0.01$ (vs. Arid5a) as determined by one-way analysis of variance.

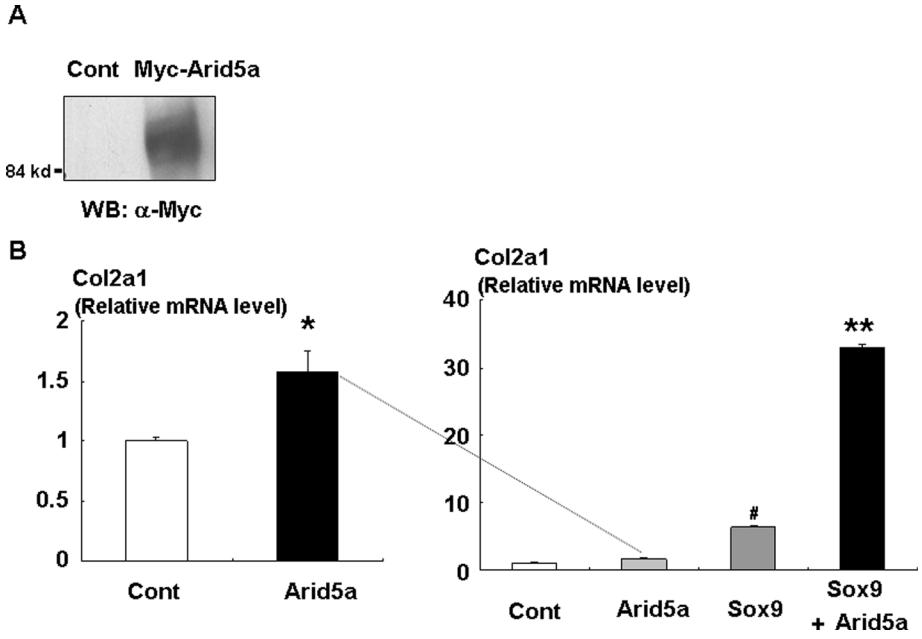

FIGURE 3: Effects of Arid5a overexpression on expression and promoter activity of the *Col2a1* gene. (A) ATDC5 cells infected with control (Cont) or 6xMyc-Arid5a adenovirus were cultured for 2 d. Expression of 6xMyc-Arid5a after the infection was determined by immunoblotting with anti-Myc antibody. (B) ATDC5 cells were infected with control (Cont), HA-Sox9, 6xMyc-Arid5a adenovirus, or both HA-Sox9 and 6xMyc-Arid5a adenovirus as indicated, and then cultured for 3 d. Total RNA isolated from the cells was subjected to real-time RT-PCR analyses to measure *Col2a1* expression. Relative mRNA levels were normalized to β-actin expression. The left and right panels of (B) were obtained in the same experiment but are shown using different scales. *$p < 0.01$ (vs. control) as determined by Student's *t* test. #$p < 0.01$ (vs. control), **$p < 0.01$ (vs. Sox9) as determined by one-way analysis of variance.

chondrocytes, we overexpressed Arid5a in primary chondrocytes isolated from mouse ribs and then cultured them with BMP2, which induces calcification of the primary chondrocytes (Figure 5H), and then we determined the extent of calcification by alizarin red staining. We found that overexpression of Arid5a abolished BMP2-induced calcification of these primary chondrocytes (Figure 5H). Similarly, overexpression of Arid5a blocked calcification of these primary chondrocytes induced by overexpression of Runx2 (Figure 5I). These results suggest that Arid5a stimulates the early-stage chondrocyte differentiation but inhibits later stage differentiation.

Regulation of histone 3 acetylation surrounding the *Col2a1* gene promoter by Arid5a

Having demonstrated that Arid5a regulated transcription and expression of the *Col2a1* gene, we further examined the relationship between Arid5a and the *Col2a1* gene promoter region. We performed chromatin IP (ChIP) experiments on the three regions of the *Col2a1* gene shown in Figure 6A in C3H10T1/2 cells. Arid5a bound directly to the promoter region (P1) containing the TATA box of the *Col2a1* gene promoter; however, Arid5a did not bind to the upstream region (P2) of the TATA box or the Sox9 enhancer region (Sox9 binding site; Sox9BS) present in the first intron (Figure 6B). We next examined whether Arid5a is involved in acetylation of the histones surrounding the region by performing ChIP experiments using antibodies recognizing acetylated histone 3 (H3) or histone 4 (H4). We found that overexpression of Arid5a enhanced acetylation of H3 surrounding the promoter region but not that of H4 (Figure 6, C and D). Thus, these results suggest that Arid5a may up-regulate the transcription and chondrocyte-specific activity of Sox9 by stimulating the acetylation of H3.

Functional analysis of domains of Arid5a

To understand the molecular function of Arid5a, we generated a series of deletion mutants of Arid5a as shown in Figure 7A and then examined the effects of these mutants on the Arid5a–Sox9 interaction, *Col2a1* reporter activity, *Col2a1* expression, and H3 acetylation at the *Col2a1* gene. Based on pull-down experiments with histidine (His)-tagged Sox9 protein in a cell-free system, and consistent with data in Figure 2A, wild-type Arid5a binds to Sox9 in a cell-free condition (Figure 7B). It is therefore likely that Arid5a directly associates with Sox9. Both the ΔC1 and ΔC2 mutants associated with Sox9 (Figure 7B). Although the ΔN2 mutant showed modest binding to Sox9, the ΔN1 mutant did not interact with Sox9 (Figure 7B). To confirm the results, we determined whether these mutants colocalized with DsRed-Sox9 using Venus-tagged Arid5a mutants. Both the ΔC1- and ΔC2-tagged mutants showed uniform colocalization with Sox9 (Figure 7C). The ΔN2 mutant also shows a localization pattern similar to that of Sox9 (Figure 7C). These results suggest that an N-terminal region is responsible for the association with Sox9. As shown in Figure 7D, all deletion mutants failed to up-regulate Col2a1 reporter activity. In addition, mutants lacking either an N- or C-terminal region did not up-regulate expression of *Col2a1* mRNA (Figure 7E). ChIP experiments indicated that ΔN1 and ΔC1 mutants failed to increase the acetylation of H3 at the *Col2a1* gene promoter (Figure 7F). It is therefore likely that both the N- and C-terminal regions of Arid5a were required for acetylation of H3 at the *Col2a1* gene.

DISCUSSION

A large body of evidence indicates that Sox9 is an important transcription factor during the development of several organs (Thomsen *et al.*, 2008; Gordon *et al.*, 2009; Hanover *et al.*, 2009). In particular, Sox9 is essential for chondrocyte lineage determination and differentiation (Kronenberg, 2006; Akiyama, 2008). Several studies show that Sox9 regulates several target genes, including *Col2a1*, *Col11a2*, *agc1*, and *Pthrp*, during chondrogenesis (Lefebvre *et al.*, 1996, 1998; Bell *et al.*, 1997; Bridgewater *et al.*, 1998; Han and Lefebvre, 2008; Amano *et al.*, 2009). In this study, we focused on understanding the transcriptional regulation of the *Col2a1* gene because *Col2a1* encodes a major cartilage matrix component (Bell *et al.*, 1997; de Crombrugghe *et al.*, 2000; Kronenberg, 2003). We showed that Arid5a, a member of the Arid family of DNA binding proteins, can regulate transcription of the *Col2a1* gene and may play a role in chondrocyte differentiation in association with Sox9. First, we demonstrated that the expression of the Arid5a protein in cartilage was similar to Sox9 protein expression. The expression of *Arid5a* mRNA was also associated with Sox9-dependent chondrocyte differentiation. Second, we found that Arid5a physically and functionally interacted with Sox9. Third, overexpression and knockdown

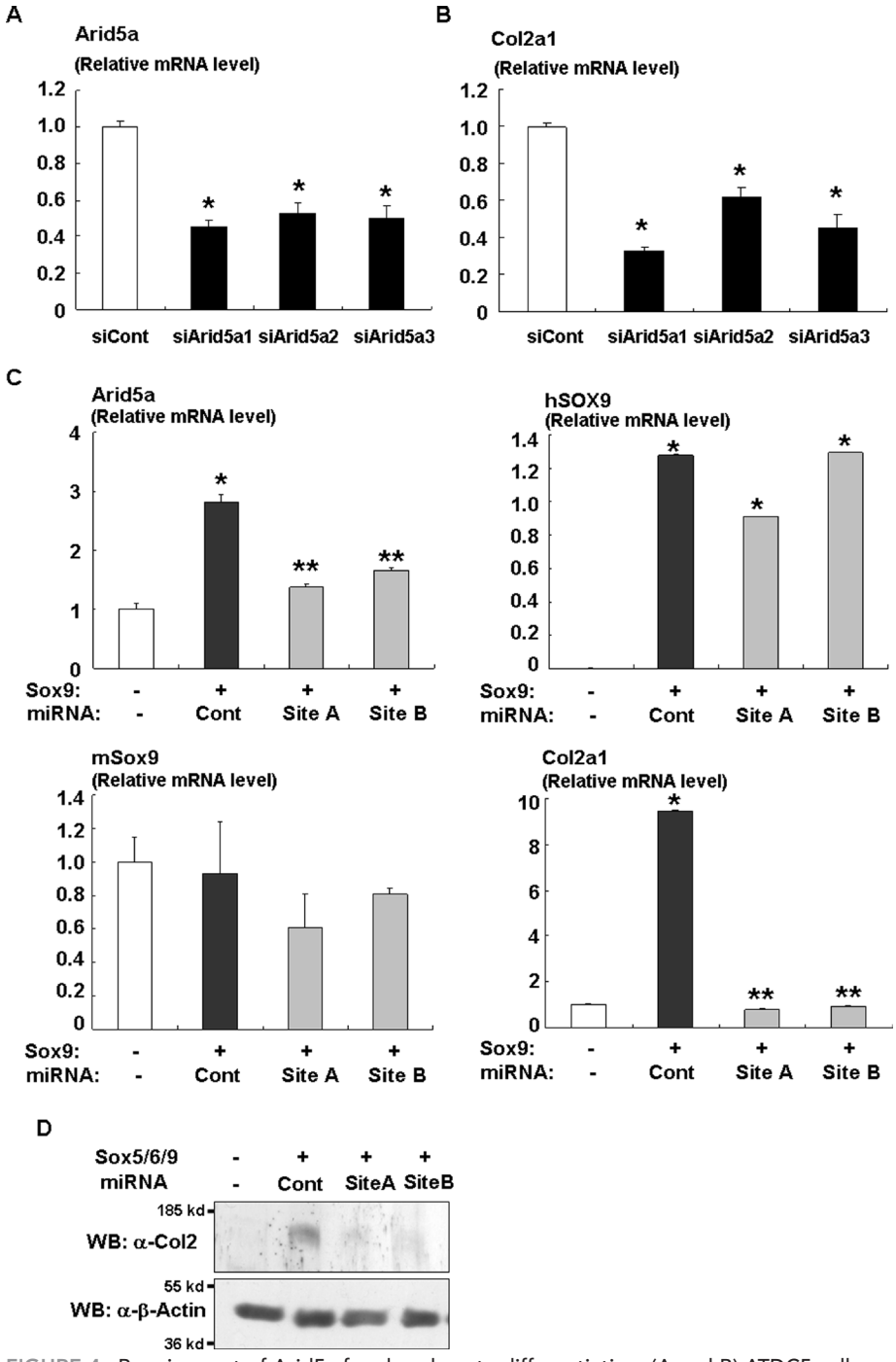

FIGURE 4: Requirement of Arid5a for chondrocyte differentiation. (A and B) ATDC5 cells transfected with control siRNA (siCont) or an siRNA for Arid5a (siArid5a1, siArid5a2, or siArid5a3) were cultured for 2 d. *Arid5a* (A) and *Col2a1* (B) mRNA levels were quantified by real-time RT-PCR of total RNA isolated from the cells. Relative mRNA levels were normalized to β-actin expression. *$p < 0.01$ (vs. control siRNA) as determined by one-way analysis of variance. (C) C3H10T1/2 cells were infected with control adenovirus, Sox9 adenovirus, and/or synthetic Arid5a miRNA adenovirus (Site A, Site B) as indicated, and then cultured for 2 d. *Arid5a* (top of left panel), human *SOX9* (hSOX9, top of right panel), endogenous *Sox9* (mSox9, bottom of left panel), or *Col2a1* (bottom of right panel) mRNA levels were quantified by real-time RT-PCR of total RNA isolated from the cells. Relative mRNA levels were normalized to β-actin expression. *$p < 0.01$ (vs. no Sox9), **$p < 0.01$ (vs. Sox9 alone) as determined by one-way analysis of variance. (D) C3H10T1/2 cells were infected with control (Cont), Sox5, Sox6, HA-Sox9, or synthetic Arid5a miRNAs (Site A, Site B) adenoviruses as indicated, and then cultured for 2 d. The lysates of the cultured cells were subjected to immunoblotting with anti-Col2 (top panel) or anti–β-actin antibodies (bottom panel).

experiments indicated that Arid5a is involved in stimulation of chondrocyte differentiation. Therefore collectively our results suggested that Arid5a might function as a transcriptional partner of Sox9 during chondrocyte differentiation.

Chondrogenesis is a unique and complex biological event that is sequentially regulated by several exogenous factors, signaling pathways, and transcription factors. It is well established that Sox9 stimulates the early stage of chondrocyte differentiation by regulating the target genes that are expressed during this early stage. Recently we and others reported that Sox9 negatively regulates the late stage of chondrocyte differentiation that involves hypertrophy and calcification (Saito *et al.*, 2007; Amano *et al.*, 2009; Hattori *et al.*, 2010). In this study, we found that Arid5a, like Sox9, has distinct roles in the early and late stages of chondrocyte differentiation (Saito *et al.*, 2007; Amano *et al.*, 2009; Hattori *et al.*, 2010). Thus, it appears that Arid5a plays a role in regulating the late stage of chondrocyte differentiation by interacting with Sox9.

Because Arid5a contains an Arid DNA binding domain in its N-terminal region, Arid5a is categorized into the Arid family. Members of this family play roles in growth, differentiation, and development (Wilsker *et al.*, 2002). Arid5a has been shown to repress the expression of major immediate-early genes of human cytomegalovirus (Thrower *et al.*, 1996; Huang *et al.*, 2001). A recent study also indicated that Arid5a suppresses estrogen receptor (ER)α-dependent transcriptional activation in cardiovascular cells (Georgescu *et al.*, 2005). In contrast, we demonstrated that Arid5a was a positive transcriptional regulator for chondrocyte differentiation. Similarly, another Arid family member, *Drosophila* dead ringer, has been shown to function as both a coactivator and a corepressor (Valentine *et al.*, 1998). We speculate that the diverse functions of Arid5a might be dependent on differences in the transcriptional complexes with which it associates and/or differences in its target genes.

Our analyses using Arid5a deletion mutants indicated that the N-terminal region was required for binding with Sox9. Because an Arid domain that functions as a DNA binding domain is present in the N-terminal region of Arid5a, we expected that N-terminal deletion mutants would not stimulate Col2a1 reporter activity and H3 acetylation. Interestingly, C-terminal deletion mutants also failed to stimulate the transcriptional and chondrocyte-specific activities of Sox9 and the acetylation of H3. Because Arid5a

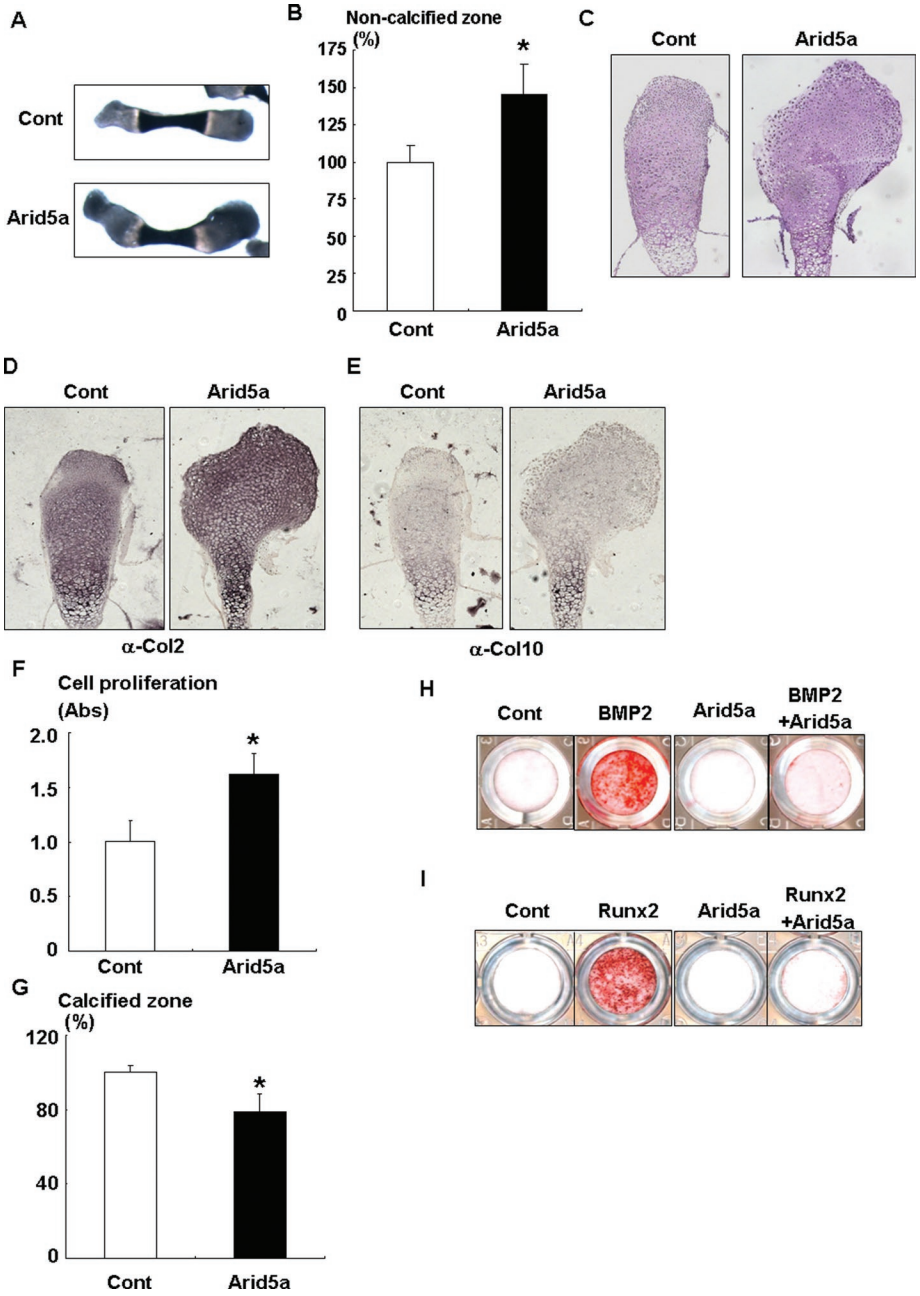

FIGURE 5: Effect of Arid5a on chondrocyte differentiation. (A) Mouse metatarsal explants isolated from 15.5-d-old mice embryos were infected with control (Cont) or 6xMyc-Arid5a adenovirus and cultured in the presence of BMP2 (250 ng/ml) for 6 d. The cultured metatarsals were photographed. Figures show representative images (n = 7). (B) The noncalcified zone of the metatarsal explants was measured (n = 7). The *y*-axis shows the fold change in the noncalcified zone relative to the control group. *p < 0.01 (vs. control) as determined by Student's *t* test. (C) The cultured metatarsals were subjected to H&E staining. Magnification: 50×. Figures show representative images (n = 4). (D and E) The cultured metatarsals were immunostained with anti-Col2 (D) and anti-Col10 antibodies (E). Magnification: 50×. Figures show representative images (n = 4). (F) C3H10T1/2 cells infected with control (Cont) or 6xMyc-Arid5a adenovirus were cultured in the presence of BMP2 (300 ng/ml) for 3 d. WST-1 cell proliferation assays were performed as described in *Materials and Methods*. *p < 0.01 (vs. control) as determined by Student's *t* test. (G) The calcified zone of the cultured metatarsal explants was measured (n = 5). The *y*-axis shows the fold change in the calcified zone relative to the control group. *p < 0.01 (vs. control) as determined by Student's *t* test. (H) Mouse primary chondrocytes infected with control (Cont) or 6xMyc-Arid5a adenovirus were cultured with or without BMP2 (100 ng/ml) for 7 d, and the results were determined by alizarin red staining. Figures show representative images (n = 5). (I) Mouse primary chondrocytes infected with control (Cont), 6xMyc-Arid5a, or Runx2 adenoviruses were cultured for 7 d and then stained with alizarin red. Figures show representative images (n = 5).

does not contain a domain predicted to have histone acetyl transferose (HAT) activity, the C-terminal region might recruit HAT-related molecules to the Sox9-assembled transcriptional complex. Identification of the molecule(s) associated with the C-terminal region of Arid5a would contribute to further understanding of the molecular basis by which Sox9 regulates chondrocyte differentiation.

Because Arid5a mutations have not been reported in humans and mice, the physiological role of Arid5a in these organisms is currently unknown. Thus generation and dissection of Arid5a-deficient mice would further advance our understanding of the role of Arid5a in vivo. Because Arid5b appears to be involved in skeletal development (Lahoud *et al.*, 2001; Whitson *et al.*, 2003; Schmahl *et al.*, 2007), it is possible that both Arid5a and Arid5b share similar functions in skeletal development. Further investigation is necessary to address these points.

In conclusion, our study indicated that Arid5a was a transcriptional partner of Sox9 that regulated chondrocyte differentiation, probably through stimulation of the acetylation of H3 at chondrocyte-specific genes, such as *Col2a1*. These findings advance our understanding of the molecular mechanisms by which Sox9 regulates chondrocyte lineage determination and differentiation.

MATERIALS AND METHODS

Cells and reagents

The mouse chondrocytic cell line ATDC5 was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured in DMEM/F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS). The mouse mesenchymal cell lines C3H10T1/2, Cos7, and BOSC23 were purchased from RIKEN Cell Bank and cultured in α-MEM (Sigma-Aldrich) supplemented with 10% FCS.

Luciferase assay

The firefly luciferase reporter construct contained the human Col2a1 gene promoter (–89 to +16) and four 48-base pair tandem-repeats of the Sox9 binding element present in the first intron of the Col2a1 gene (Muramatsu *et al.*, 2007). The Col2a1 reporter construct and thymidine kinase (TK)-renilla luciferase construct (Promega, Madison, WI) were cotransfected with the expression vectors, as described in the figures, into ATDC5 cells using Fugene6 reagent (Roche, Basel, Switzerland). After 48 h of transfection, the cells were lysed, and firefly and renilla luciferase

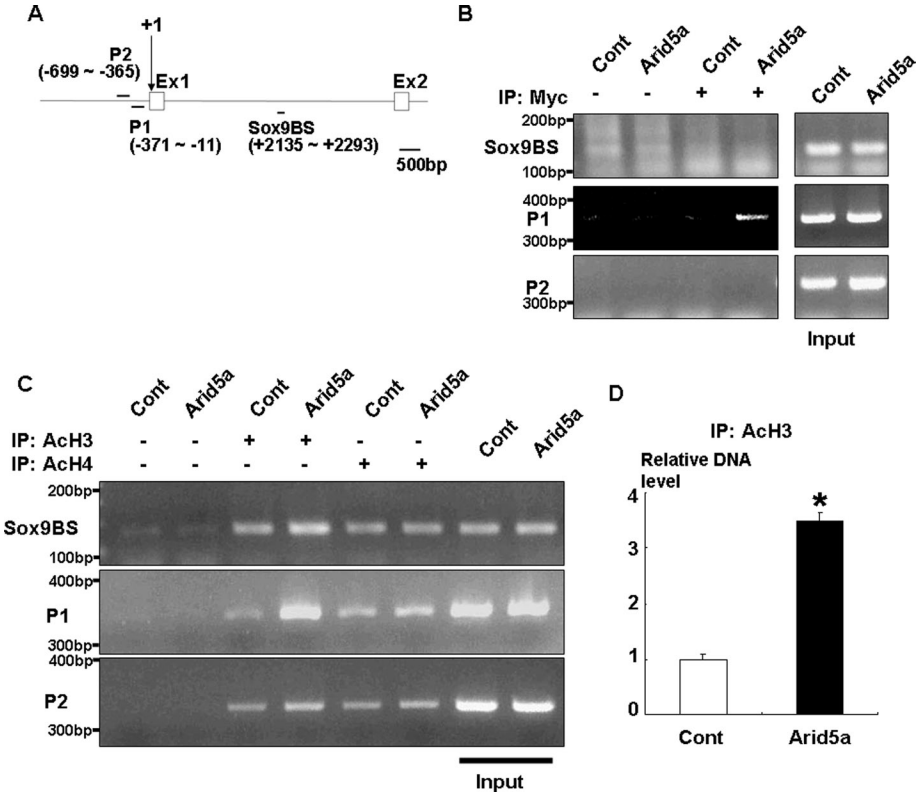

FIGURE 6: Stimulation of H3 acetylation surrounding the *Col2a1* gene promoter region by Arid5a. (A) Schematic diagram of the regions of the *Col2a1* gene targeted in the ChIP assays. P1, the region containing TATA box; P2, the upstream region of P1; Ex1, exon1; Ex2, exon2. (B) C3H10T1/2 cells infected with control (Cont) or 6xMyc-Arid5a adenoviruses were cultured for 3 d. Chromatin prepared from the cells was immunoprecipitated with anti–mouse IgG or anti-Myc antibody. DNA fragments were precipitated with protein-G agarose beads and then amplified by PCR using specific primers corresponding to the three *Col2a1* gene regions. Input DNA in chromatin was amplified, as shown in the right panel. Reproducible results were obtained from three independent experiments. (C) C3H10T1/2 cells infected with control (Cont) or 6xMyc-Arid5a adenoviruses were cultured for 3 d. Chromatin prepared from the cells was immunoprecipitated with anti–rabbit IgG, anti–acetyl H3 (AcH3), or anti–acetyl H4 (AcH4) antibody. DNA fragments precipitated with protein-A agarose beads and input DNA were amplified by PCR using specific primers corresponding to the three *Col2a1* gene regions, as indicated. Reproducible results were obtained from three independent experiments. (D) Precipitated DNA fragments and input DNA were subjected to real-time PCR for the P1 region in the *Col2a1* gene. Relative DNA levels were normalized to the amount of input DNA. Reproducible results were obtained from three independent experiments. *$p < 0.01$ (vs. control) as determined by Student's *t* test.

activities were determined using the appropriate substrates and a luminometer (Promega), according to the manufacturer's instructions. Renilla luciferase activity was measured to normalize transfection efficiency. Data represent mean ± SD (n = 4).

Isolation of Arid5a cDNA

Arid5a was isolated by performing mammalian expression cloning as previously described (Muramatsu *et al.*, 2007; Hata *et al.*, 2008). Briefly, a full-length cDNA library was generated from ATDC5 cells and engineered into the pME18S expression vector. cDNA clones from the library were individually transfected along with the Col2a1 reporter construct, and positive clones, as determined by firefly luciferase activity, were isolated and subjected to DNA sequence analysis.

Construction of vectors

Arid5a and Arid5a mutants were tagged with a 6× Myc (6xMyc), a Flag, or a Venus epitope, and these constructs were ligated into pcDNA3 (Invitrogen, Carlsbad, CA) at the *Eco*R1 and *Xba*1 sites. The expression vectors encoding hemagglutinin-tagged Sox9 (HA-Sox9) or 6xMyc-tagged DN Sox9, Sox5, and Sox6 were used as previously described (Amano *et al.*, 2009).

Real-time RT-PCR

Total RNA was isolated using a total RNA isolation kit (Macherey-Nagel, Duren, Germany). After denaturation of total RNA at 70°C for 10 min, cDNA was synthesized with oligo dT primer and reverse transcriptase (TaKaRa, Shiga, Japan). Real-time RT-PCR amplification was performed using the Taqman PCR protocol and the ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA). Taqman primers and probes used for amplification are listed in Table 1. The expression level of the mRNA was normalized by β-actin mRNA expression. Data represent mean ± SD (n = 3).

Western blotting

The cells were rinsed twice with phosphate-buffered saline (PBS) and solubilized in lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, aprotinin at 10 μg/ml, leupeptin at 10 μg/ml, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.2 mM sodium orthovanadate). The lysates were centrifuged for 10 min at 4°C at 15,000 × *g* and boiled in SDS sample buffer containing 0.5 M β-mercaptoethanol for 5 min. The supernatant was separated by SDS–PAGE, transferred to nitrocellulose membranes, immunoblotted with primary antibodies, and visualized with horseradish peroxidase–coupled anti–mouse or –rabbit immunoglobulin (Ig)G antibody using enhanced chemiluminescence detection kits (GE Healthcare, Buckinghamshire, UK). Anti-HA, -Sox5, -Sox6, -Myc, and –β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag and -Sox9 antibodies were purchased from Sigma-Aldrich. Anti-Col2 antibody was purchased from Cosmo Bio (Tokyo, Japan).

Immunohistochemical analysis

The anti-Arid5a rabbit polyclonal antibody was generated with the CRHGARSPNKDIQD peptide as both the antigen and the peptide on an affinity-purification column. The specificity of the Arid5a polyclonal antibody was determined by immunoblotting using lysates of the cells overexpressing 6xMyc-Arid5a. The samples from bone or organ-cultured metatarsals were fixed with 4% buffered paraformaldehyde, decalcified in 5% EDTA, embedded in paraffin, and cut into 5-μm-thick sections. Hematoxylin and eosin (H&E) staining was done according to the standard procedure. Immunohistochemistry was performed using the following primary polyclonal rabbit antibodies: anti-Col2 antibody (LSL Biolafitte, St. Germain en Laye, France) at a 1:500 (vol/vol) dilution, anti-Col10 antibody

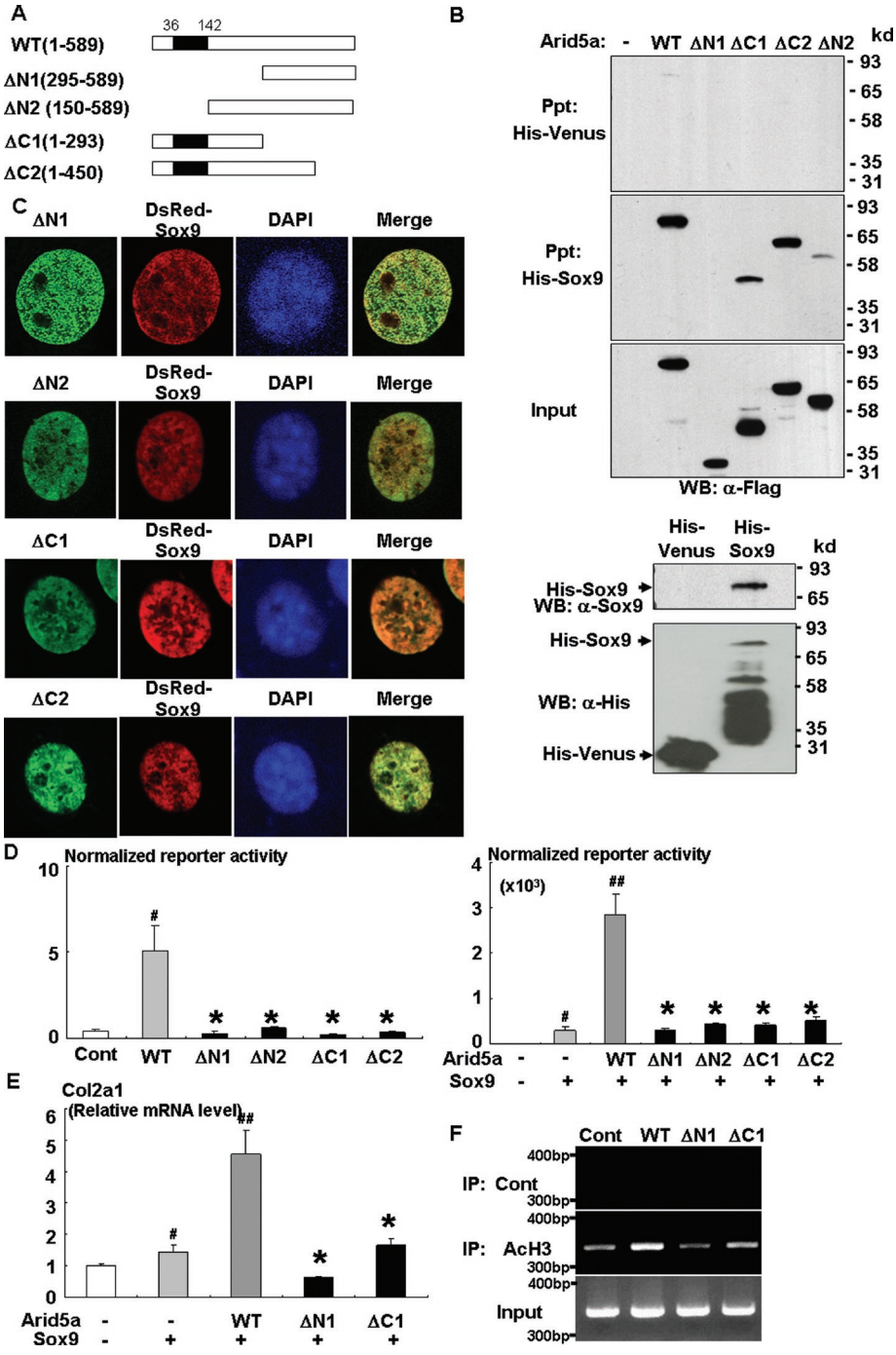

FIGURE 7: Functional regions of Arid5a. (A) Schematic diagram of a series of constructs of Arid5a mutants. The Arid domain is filled with black. The amino acids present in each mutant are indicated. (B) His-tagged Venus (His-Venus) or Sox9 (His-Sox9) protein was incubated with lysates of BOSC23 cells transfected with wild type or mutants of Flag-Arid5a. His-Venus (top panel) or His-Sox9 (second panel) proteins precipitated by Talon-Beads were subjected to immunoblotting with anti-Flag antibody. Input proteins were determined by immunoblotting with anti-Flag (third panel), anti-Sox9 (fourth panel), or anti-His (fifth panel) antibody. (C) ATDC5 cells transfected with Venus-tagged mutants of Arid5a (ΔN1, ΔN2, ΔC1, ΔC2) together with DsRed-Sox9 were cultured for 2 d. The cells were counterstained with DAPI and visualized using a confocal microscope. (D) ATDC5 was transfected with the *Col2a1* reporter and TK-renilla luciferase constructs and with empty vector (Cont), wild-type 6xMyc-tagged Arid5a (WT), or mutant 6xMyc-tagged Arid5a (ΔN1, ΔN2, ΔC1, ΔC2) constructs, as indicated, together with (right panel) or without (left panel) Sox9. After 48 h of transfection, firefly luciferase activity of the cell lysates was measured. Luciferase reporter activity was normalized to renilla luciferase activity. #p < 0.01 (vs. control), ##p < 0.01 (vs. Sox9 alone group), *p < 0.01 (vs. wild-type group) as determined by one-way analysis of variance. (E) ATDC5 cells infected with control (Cont), HA-Sox9, wild-type (WT) Arid5a, or an Arid5a mutant (ΔN1or ΔC1) adenovirus as indicated were cultured for 3 d. *Col2a1* mRNA levels were assayed by real-time RT-PCR of total RNA isolated from the cells. Relative mRNA levels were normalized to β-actin expression. #p < 0.05 (vs. no Sox9 group) as determined by Student's *t* test. ##p < 0.01 (vs. Sox9 alone group), *p < 0.01 (vs. Sox9 and wild-type Arid5a group) as determined by one-way analysis of variance. (F) C3H10T1/2 cells infected with control (Cont), wild-type Arid5a (WT), or an Arid5a mutant (ΔN1or ΔC1) adenovirus was cultured for 3 d. Chromatin was immunoprecipitated with anti-rabbit IgG (Cont) or anti-acetyl H3 (AcH3) antibodies. DNA fragments precipitated with protein-A agarose beads and input DNA were amplified by PCR using specific primers corresponding to the P1 region in the *Col2a1* gene.

(LSL Biolafitte) at a 1:500 (vol/vol) dilution, anti-Arid5a antibody at a 1:1000 (vol/vol) dilution, and anti-Sox9 antibody (H-90; Santa Cruz Biotechnology) at a 1:500 (vol/vol) dilution. Antigen retrieval was treated using 2.5% hyaluronidase for anti-Col2 and anti-Col10. Immunoreactivity was visualized with a biotinylated anti–rabbit IgG secondary antibody using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA) and the peroxidase substrate DAB kit (Vector Laboratories), according to the manufacturer's protocol.

Generation of adenoviruses

The recombinant adenoviruses carrying wild-type and mutant Arid5a constructs, HA-Sox9, Sox5, Sox6, and Runx2 were generated by homologous recombination between the expression cosmid cassette (pAx-CAwt) and the parental virus genome in 293 cells (RIKEN Cell Bank) using an adenovirus construction kit (TaKaRa) as previously described (Shimoyama *et al.*, 2007). The viruses showed no proliferative activity because of a lack of E1A-E1B (Shimoyama *et al.*, 2007). Titers of the viruses were determined by modified point assay. The infection of recombinant adenoviruses into cell lines was carried out by incubation with adenoviruses at 40 multiplicity of infection (m.o.i.).

Coimmunoprecipitation assay

Lysates were prepared from BOSC23 transfected with or without HA-Sox9 or Flag-Arid5a. The lysates were incubated with anti-Flag antibody overnight and then incubated with protein-G agarose beads (Santa Cruz Biotechnology) for 2 h. After five washes with lysis buffer, the precipitated samples

Source	Primer/ probe	Sequence
Mouse Col2a1	Sense primer	5′-CCTCCGTCTACTGTCCACTGA-3′
	Anti-sense primer	5′-ATTGGAGCCCTGGATGAGCA-3′
	Probe	5′-CTTGAGGTTGCCAGCCGCT-TCGTCC-3′
Mouse β-actin	Sense primer	5′-TTAATTTCTGAATGGCCCAG-GTCT-3′
	Anti-sense primer	5′-ATTGGTCTCAAGTCAGTGTA-CAGG-3′
	Probe	5′-CCTGGCTGCCTCAACACCT-CAACCC-3′
Mouse Arid5a	Sense primer	5′-CTGTCCTACGCAACAGACTGG-3′
	Anti-sense primer	5′-GAAGTGAGGTGCCGCATAGG-3′
	Probe	5′-AGCTTCGTCTGCCTGGCACAT-GCC-3′
Mouse Sox9	Sense primer	5′-CCTTCAACCTTCCTCACTA-CAGC-3′
	Anti-sense primer	5′-GGTGGAGTAGAGCCCTGAGC-3′
	Probe	5′-CCGCCCATCACCCGCTCG-CAATAC-3′
Human SOX9	Sense primer	5′-ACTCGCCACACTCCTCCTC-3′
	Anti-sense primer	5′-CCTCTCGCTTCAGGTCAGC-3′
	Probe	5′-CGAGCACTCGGGGCAATC-CCAGG-3′

TABLE 1: Sequences of Taqman probe sets for real-time RT-PCR experiments.

were boiled in 20 µl of sample buffer and subjected to Western blotting analyses using anti-HA antibody.

Fluorochrome staining

C3H10T1/2 cells transfected with Venus- or DsRed-tagged expression vectors were washed in PBS twice, fixed with 4% buffered paraformaldehyde, and treated with 0.5% Triton X for 30 min. The blocking reagent used was 5% bovine serum albumin in PBS. The nuclei were counterstained with DAPI at 0.1 µg/ml (Invitrogen). Then the slides were mounted with Vectashield (Vector Laboratories) and examined with an LSM510META confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany); images were analyzed with LSM5 image browser software (Carl Zeiss). More than five cells were recorded, and the data in our figures are representative of typical cells shown at 600× magnification.

Knockdown of Arid5a

Control and Arid5a siRNA were purchased from Invitrogen. The target sequences of Arid5a siRNA are listed in Table 2. Transfection of siRNA was performed using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol.

Source	Sequence
siArid5a-1:	5′-CGGUGUCCUCUAAACUUCACCGGUA-3′
siArid5a-2:	5′-CAAGGCAUCCAGGUUGGCCAUGAAA-3′
siArid5a-3:	5′-CAAGGCAUCCAGGUUGGCCAUGAAA-3′

TABLE 2: Target sequences of siRNA for Arid5a.

Synthetic microRNAs for Arid5a were generated by a BLOCK-iT Pol II miR RNAi Expression kit (Invitrogen). Two sets of miArid5a were generated using the primers listed in Table 3. The cassettes containing miRNA were ligated into adenovirus expression vector pAxCAwt, and the corresponding adenoviruses were generated as described earlier in the text.

Organ culture

Three central metatarsal rudiments were isolated from each hind limb of 15.5-d-old DDY mouse embryos, placed into 24-well plates, and cultured in organ culture medium as described previously (Yasoda *et al.*, 1998). The metatarsals were cultured at 37°C in a humidified 5% CO_2 incubator for 12 d. Infection of adenoviruses (80 m.o.i.) was performed 1 d after dissection (Nifuji *et al.*, 2004). For morphometric analysis, metatarsal explants were photographed under a dissecting microscope (microscope, Stemi 2000-C; camera, AxioCam MRc; acquisition software, AxioVision AC Rel. 4.5, Carl Zeiss). The length and dark calcified zone in the diaphysis and epiphysis were measured as described previously (Yasoda *et al.*, 1998). For histological analysis, paraffin sections of metatarsals were prepared and then subjected to H&E staining and immunohistochemical analyses.

WST-1 cell proliferation assay

The proliferation of the cells was examined using WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benezene disulfonate) reagent according to the manufacturer's protocol (Roche). The WST-1 reagent was added to cultured cells at a 1:10 (vol/vol) final dilution. The samples were incubated at 37°C for 2 h, and the absorbance of the samples was measured against a background control as a blank using a microplate reader (MODEL 550; Bio-Rad, Hercules, CA) at 450 nm. Data represent mean ± SD (n = 4).

Culture of mouse primary chondrocytes

Primary chondrocytes were isolated from the ribs of 4-wk-old DDY mice by repetitive digestion with 0.2% collagenase at 37°C (Amano

Primer	Sequence
Site A	
Sense	5′-TGCTGTTAATCTGCTTGAAGCCAAGAGTTTTG-GCCACTGACTGACTCTTGGCTAAGCAGATTAA-3′
Anti-sense	5′-CCTGTTAATCTGCTTAGCCAAGAGTCAGTCAGT-GGCCAAAACTCTTGGCTTCAAGCAGATTAAC-3′
Site B	
Sense	5′-TGCTGTCTTTAAGGCCGGAGAAGAAGGTTTTG-GCCACTGACTGACCTTCTTCTGGCCTTAAAGA-3′
Anti-sense	5′-CCTGTCTTTAAGGCCAGAAGAAGGTCAGT-CAGTGGCCAAAACCTTCTTCTCCGGCCTTAAA-GAC-3′

TABLE 3: Sequences of synthetic microRNA for Arid5a.

Source/ region	Primer	Sequence
Sox9BS	Sense primer	5′-ttccagatgggctgaaac-3′
	Anti-sense primer	5′-ctgtgcattgtgggagag-3′
P1	Sense primer	5′-cttatgcctctgcaaacaa-3′
	Anti-sense primer	5′-gcagaggctccagttatatg-3′
P2	Sense primer	5′-cctctgtgtaggaattcacc-3′
	Anti-sense primer	5′-gcataagcctggagttttag-3′

TABLE 4: Sequences of primers for ChIP experiments.

et al., 2008). The isolated cells were filtered through a 40-μm nylon mesh (BD Biosciences, Franklin Lakes, NJ), collected by centrifugation, and (with the exception of the cells isolated following the first digestion) cultured (Kamiya *et al.*, 2002; Gartland *et al.*, 2005). Primary chondrocyte cell cultures were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FCS, ascorbic acid at 0.1 mg/ml, and 5 mM β-glycerophosphate (Sigma-Aldrich) at 37°C in a humidified 5% CO_2 incubator. Adenoviruses were infected at 40 m.o.i.

Alizarin red staining

The cultured mouse primary chondrocytes were rinsed twice with PBS, fixed in 4% buffered paraformaldehyde and in 95% ethanol, and stained with 1% alizarin red solution (WAKO, Osaka, Japan) for 10 min. The stained samples were scanned by a GT-9500 scanner (Epson, Tokyo, Japan). Data shown represent the mean of four experiments.

ChIP analysis

ChIP analysis was performed using a ChIP assay kit (Upstate, Billerica, MA) according to the manufacturer's instructions. C3H10T1/2 cells infected with adenovirus were cultured for 3 d. Chromatin was prepared and immunoprecipitated with anti–rabbit or anti–mouse IgG (Santa Cruz Biotechnology), -Myc (Santa Cruz Biotechnology), -acetyl histone 3 (Upstate), and -acetyl histone 4 (Upstate) antibodies. The DNA fragments precipitated with protein-A or protein-G agarose beads and salmon sperm DNA (Upstate) were amplified by PCR using primer pairs specific to the Col2a1 gene: enhancer region of Sox9 present in the first intron of Col2a1 gene (Sox9BS), upstream from first exon in Col2a1 gene (P1 and P2). The sequences of these primers are listed in Table 4. Quantitative analysis in the locus of P1 primer pairs was performed with real-time PCR. The sequences of the primers are listed in Table 5. Data represent mean ± SD (n = 3).

Pull-down assay using His-tagged Sox9

His-tagged Venus or Sox9 proteins expressed in *Escherichia coli* using the Cold Shock Expression System (TaKaRa) were incubated with the lysates prepared from BOSC23 transfected with the expression vectors of wild-type or mutants of Flag-tagged Arid5a. His-Venus or -Sox9 was purified by TALON beads (Clontech, Mountain View, CA). The precipitated samples were boiled in 20 μl of sample buffer and subjected to Western blotting analyses using anti-Flag antibody.

Region	Primer/probe	Sequence
P1	Sense primer	5′-CCGGTTTGCCAGCCTTTGG-3′
	Anti-sense primer	5′-AGCGGGTCCGGGTCTCTAC-3′
	Probe	5′-CCCTCATGCAGGAGGCCCTAG-GAGC-3′

TABLE 5: Sequences of Taqman probe set for ChIP experiments.

Statistical analysis

The data were statistically analyzed by using the Student's *t* test or a multiple comparison of one-way analysis of variance (Tukey procedure), as appropriate for each case.

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