Requirement for RAR-mediated gene repression in skeletal progenitor differentiation

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Chondrogenesis is a multistep process culminating in the establishment of a precisely patterned template for bone formation. Previously, we identified a loss in retinoid receptor–mediated signaling as being necessary and sufficient for expression of the chondroblast phenotype (Weston et al., 2000. J. Cell Biol. 148:679–690). Here we demonstrate a close association between retinoic acid receptor (RAR) activity and the transcriptional activity of Sox9, a transcription factor required for cartilage formation. Specifically, inhibition of RAR-mediated signaling in primary cultures of mouse limb mesenchyme results in increased Sox9 expression and activity. This induction is attenuated by the histone deacetylase inhibitor, trichostatin A, and by coexpression of a dominant negative nuclear receptor corepressor-1, indicating an unexpected requirement for RAR-mediated repression in skeletal progenitor differentiation.

Inhibition of RAR activity results in activation of the p38 mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) pathways, indicating their potential role in the regulation of chondrogenesis by RAR repression. Accordingly, activation of RAR signaling, which attenuates differentiation, can be rescued by activation of p38 MAPK or PKA. In summary, these findings demonstrate a novel role for active RAR-mediated gene repression in chondrogenesis and establish a hierarchical network whereby RAR-mediated signaling functions upstream of the p38 MAPK and PKA signaling pathways to regulate emergence of the chondroblast phenotype.

Introduction

Almost all skeletal elements form on a cartilage template that is established early during embryogenesis to provide a precisely patterned framework for the future skeleton. During chondrogenesis, numerous factors act together to coordinate commitment and differentiation of skeletal progenitors such that these processes occur in a spatial- and temporal-specific manner. Of these factors, the retinoids have been known for decades to have a considerable influence on cartilage formation. Specifically, an imbalance in vitamin A metabolites, such as retinoic acid (RA),* during development will result in severe skeletal defects among a multitude of other developmental anomalies (Hale, 1935; Warkany and Schraffenberger, 1946; Cohlan, 1953; Wilson et al., 1953; Kalter and Warkany, 1961). Modulation of RA availability during the time period of chondrogenesis has the most profound impact on the skeleton, suggesting that this period of skeletal development is particularly sensitive to the retinoids (Kochhar, 1973; Kwasigroch and Kochhar, 1980). Accordingly, retinoids have been shown by several groups to inhibit chondrogenesis in vivo and in vitro (for review see Underhill and Weston, 1998).

Of the intracellular retinoid binding proteins, nuclear receptors are thought to mediate most of RA’s effects on cell behavior. Two subfamilies of nuclear retinoid receptors exist: the RA receptors (RARs) and the retinoid X receptors (RXRs). Within each subfamily there are three subtypes (α, β, and γ), with multiple isoforms of each. These receptors belong to the nuclear hormone receptor superfamily and provide a level at which much of the diversity of retinoid responses is generated (for review see Leid et al., 1992).

*Abbreviations used in this paper: ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE binding element; EGFP, enhanced green fluorescent protein; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; N-CoR, nuclear receptor corepressor; PKA, protein kinase A; RA, retinoic acid; RAR, RA receptor; RXR, retinoid-X receptor; TR, thyroid hormone receptor; TSA, trichostatin A.

Key words: chondrogenesis; Sox9; p38 MAPK; protein kinase A; nuclear corepressors
Although ligand binding to the RARs followed by recruitment of transcriptional coactivators is the basic mechanism underlying RAR-mediated gene transcription, unliganded receptors are now recognized as having an equally important function by actively repressing target gene expression through the recruitment of nuclear corepressors and associated histone deacetylases (Horlein et al., 1995; Chen et al., 1996; Heinzel et al., 1997; Nagy et al., 1997; Koide et al., 2001).

The RARs and their isoforms exhibit dynamic expression patterns throughout development (Mollard et al., 2000). With respect to skeletal development in the limb, RARα is expressed throughout the limb mesenchyme early in limb development. As cells begin to differentiate into chondrocytes, RARα is downregulated, remaining highly expressed in the perichondrium and in the interdigital region, whereas RARγ expression becomes localized to the cartilaginous elements (Dolle et al., 1989; Mendelsohn et al., 1991; Cash et al., 1997; Mollard et al., 2000). Throughout limb morphogenesis, RARβ is expressed in noncartilage-forming regions such as the interdigital region (Mendelsohn et al., 1991). We demonstrated previously that the continued expression of RARs in prechondrogenic cells prevents their differentiation, resulting in severely malformed skeletal elements in transgenic mice (Cash et al., 1997; Weston et al., 2000). Moreover, inhibition of RARα activity was sufficient to induce chondrogenesis (Weston et al., 2000).

The proper size and shape of the developing skeletal elements relies on the appropriate control of chondroblast differentiation. To date, the Sox genes, L-5, -6, and -9 are the only known transcription factors through which this control is achieved. These genes contain a high mobility group domain and belong to the Sox family of proteins that are homologous to the protein encoded by Sry (sex-determining region of Y chromosome). Of this group, Sox9 is known to play an essential role in establishing the precartilaginous condensations and in initiating chondroblast differentiation (Bi et al., 1999; Smits et al., 2001). Specifically, Sox9 binds to a region within the first intron of the type II collagen gene (Col2a1) to regulate its transcription (Lefebvre et al., 1996). Mutations in Sox9 underlie the rare congenital dwarfism syndrome, campomelic dysplasia (Foster et al., 1994; Wagner et al., 1994), and Sox9-null mice are embryonic lethal, whereas Sox9<sup>−/−</sup> cells in chimeric embryos are excluded from all cartilages (Bi et al., 1999). Conversely, if ectopically expressed, Sox9 will induce ectopic Col2a1 expression and cartilage formation (Bell et al., 1997; Healy et al., 1999). Thus, Sox9 activation of Col2a1 can be considered to be a hallmark event in cartilage formation.

To date, only a handful of factors have been found to influence Sox9 expression and/or activity, all of which are known modulators of chondrogenesis (Healy et al., 1999; Murakami et al., 2000a,b). Here we demonstrate that Sox9 expression is regulated by the retinoid signaling pathway. Our findings indicate that RAR-mediated repression is required for induction of Sox9. Moreover, expression of a dominant negative RAR leads to an increase in Sox9 reporter activity that is substantially greater than that elicited by any other factor described thus far. In addition, we show that the p38 MAPK and protein kinase A (PKA) signaling cascades are required downstream of retinoid signaling for chondroblast differentiation. Earlier studies have implicated both p38 MAPK and PKA in chondrogenesis (Lee and Chuong, 1997; Nakamura et al., 1999; Huang et al., 2000; Oh et al., 2000; Yoon et al., 2000); however, for the first time our results have enabled us to begin constructing a model for the hierarchical network of events that direct chondroblast differentiation.

**Results**

**Inhibition of RAR activity enhances chondrogenesis through a Sox9-dependent mechanism**

Previously, the continued expression of RARα activity in transgenic mice was found to inhibit the chondroprogenitor-to-chondroblast transition. Likewise, inhibition of RARα using the subtype-specific antagonist, AGN194301, induced differentiation in primary limb mesenchymal cultures earlier than normal, resulting in a substantial increase in the number of cartilage nodules that form in these cultures. This induction of cartilage formation was confirmed by the AGN194301-induced increase in expression of cartilage-specific genes such as Col2a1 (Weston et al., 2000). Given that Sox9 has been shown previously to be important in regulating the expression of Col2a1, we analyzed the effects of RARα antagonism on Sox9 expression and activity in an attempt to further understand the mechanism whereby retinoid signaling regulates chondroblast differentiation.

To follow endogenous Sox9 activity in primary mesenchymal cells, a reporter-based approach was used in which cells were transiently transfected with pGL3(4X48), a reporter containing four repeats of a Sox9 binding site from Col2a1. The RARα-specific antagonist, AGN194301 (301), induced a concentration-dependent increase in Sox9 reporter activity, whereas 836, a RARα-specific agonist, AGN193836 (836), attenuated reporter activity (Fig. 1 A). Interestingly, when cells were treated with the RAR pan-antagonist, AGN194310 (310), concentrations as low as 10 nM induced Sox9 reporter activity greater than the maximal response elicited by higher doses of 301. The maximal response to the pan-antagonist was <530% induction at 50 nM, whereas the greatest induction of Sox9 reporter activity by the RARα-specific antagonist was ~280% at 1 μM, a concentration at which this antagonist affects ligand binding to other RAR subtypes (Weston et al., 2000). Similar to RAR antagonism, the reduction in reporter activity caused by a pan-agonist such as at-RA was more pronounced than that induced by the RARα-specific agonist, 836. at-RA reduces reporter activity to 53% at 5 nM, whereas in response to a much higher dose of 836 (1 μM) reporter activity is reduced only to 64% of control. Together, these results indicate that a loss in activity of at least two or more RARs is more efficient at inducing cartilage differentiation than inhibition of the RARα subtype alone.

Interestingly, the effects of RAR modulation on Sox9 activity are opposite to that of the effects of each compound on activity of a retinoic acid responsive reporter (pW1-8RARE-<sub>β</sub>-Luc) in primary limb mesenchymal cells (Fig. 1 B). For instance, at-RA activates the RARE reporter to a greater extent than 836, whereas reporter activity is attenuated by
RARs or RXRs into primary limb mesenchymal cultures to differentiation, we introduced modified versions of the same compound. The enhanced Sox9 reporter activity seen in response to the 5 mRNA, since treatment of primary cultures with 1 more effectively than by 301. Thus, activation of the retinoid receptors affects the formation of cartilage nodules in a manner that is inversely proportional to their ability to activate a luciferase reporter containing an RA response element (pW1-BRARE-Luc). The concentration-dependent effects of each compound on Sox9 reporter activity also correspond with the ability of each compound to enhance or inhibit cartilage formation in vitro as indicated by alcian blue staining (C). In response to AGN194301, there is an increase in Sox9 mRNA as early as day 2, but this increase appears to be transient (D). Bar, 1.5 mm. ANOVAs (A and B), P < .0001; Bonferroni post-tests indicate significant differences in Sox9 reporter activity at concentrations ≥ 5 × 10⁻⁹ M for 310, 1 × 10⁻⁷ M for 301, 5 × 10⁻⁷ M for 836, and 5 × 10⁻⁶ M for at-RA. Significant changes in activity of the RARE reporter are induced by concentrations ≥ 5 × 10⁻⁶ for 310, 1 × 10⁻⁷ M for 301, 5 × 10⁻⁷ M for at-RA, and 5 × 10⁻⁹ M for 836, with all P values at least < 0.05.

310 more effectively than by 301. Thus, activation of the Sox9-responsive region of Col2a1 appears to be very closely associated with the status of RAR activity. This close association is reflected in the response of primary cultures to treatment with each compound (Fig. 1 C). Treatment with either at-RA, the RARα agonist, or with the antagonists for 4 d affects the formation of cartilage nodules in a manner that would be predicted from their effects on Sox9 reporter activity. More specifically, at-RA is a more potent inhibitor of cartilage nodule formation than 836, whereas the increase in nodule formation can be observed at a lower concentration of the pan-antagonist, (10 nM 310) compared with the RARα-specific antagonist (1 μM 301). Together, these results further validate the utility of the Sox9 reporter assay to indirectly measure the status of chondroblast differentiation, and more importantly they highlight the significant role of RAR-mediated signaling in regulating expression of the chondroblast phenotype.

The enhanced Sox9 reporter activity caused by RAR inhibition is due, in part, to an increase in the expression of Sox9 mRNA, since treatment of primary cultures with 1 μM 301 results in an precocious increase in Sox9 expression (Fig. 1 D). There is a noticeable increase in Sox9 mRNA from 2-d cultures treated with 301, but this increase over control cultures is much less pronounced by days 4 and 6. Thus, inhibition of RAR activity appears to induce an early transient upregulation of Sox9 mRNA that presumably contributes to the enhanced Sox9 reporter activity seen in response to the same compound.

To confirm the influence of RAR activity on chondroblast differentiation, we introduced modified versions of the RARs or RXRs into primary limb mesenchymal cultures to follow their effect on Sox9 reporter activity. To examine the effect of RAR activation without agonist addition, constitutively active versions of RARα and RXRα were used by fusing the acidic activation domain of VP16 to the COOH terminus of RAR and RXR referred to as RARαVP16 and RXRαVP16, respectively (Underhill et al., 1994). Here we confirmed the ability of these modified receptors to potently activate an RARE reporter in the absence of an exogenous agonist, since cotransfection of RARαVP16 and RXRαVP16 induced RARE reporter activity by 15- and 17-fold, respectively, in the absence of agonist (Fig. 2 A).

In addition to modifying RAR activity with constitutively active versions of the retinoid receptors, dominant negative versions of the receptors (dnRARα and dnRXRα) were also generated and transfected into primary limb mesenchymal cultures. These dominant negative derivatives are COOH-terminal truncations of RARα and RXRα that retain their ability to bind DNA and ligand but lack the AF-2 transactivation function (Damm et al., 1993; Feng et al., 1997). When cotransfected into primary cultures, both dnRARα and dnRXRα are effective at completely blocking activity of the RARE reporter (Fig. 2 B). Cotransfection of the modified receptors affects Sox9 reporter activity in a manner that is inversely proportional to their ability to trans-activate βRARE₃-tk-Luc. Both the RARαVP16 and RXRαVP16 inhibit Sox9 reporter activity, whereas the dn-RARα and dnRXRα potentely activate this reporter (Fig. 2, C and D). Interestingly, the activation induced by cotransfection with dnRARα is more dramatic than that elicited by any other factor studied to date, including those examined by our laboratory and those reported previously. Similar to the results using receptor agonists and antagonists,
these studies demonstrate a strong influence of retinoid receptor activity on chondrogenesis.

**Chondrogenic response to RAR inhibition requires 48 bp enhancer elements within Col2a1 and is specific to chondrogenic cells**

To closely examine the contribution of RAR inhibition to Sox9 activity, reporters with varying sensitivities to Sox9 were used to follow their response to the dnRARα. Of four reporters analyzed, 4X48-p89 and pGL3(4X48), which demonstrate the greatest sensitivity to Sox9 (Fig. 3 A), also exhibit the greatest response to dnRARα (Fig. 3 B). In contrast, pGL3(−89+6), containing only the minimal Col2a1 promoter with no Sox9 binding sites, exhibits no activity in response to Sox9 and is unaffected by the dnRARα (Fig. 3 B). A reporter containing two tandem repeats of a larger intron-1 segment of Col2a1 (including Sox9 binding sites) along with a promoter fragment is only mildly sensitive to Sox9 (A) and is activated to a much weaker extent by dnRARα (B) compared with the 4X48-containing reporters. These results demonstrate a direct relationship between inhibition of RAR signaling and Sox9 activity.

Despite the induction of Sox9 reporter activity elicited by dnRARα in other cells with chondrogenic capacity, such as dedifferentiated rat articular chondrocytes and C5.18 chondroprogenitor cells, activity of Sox9 reporter activity is not noticeably affected in COS P7 cells (Fig. 4). Given that COS P7 cells are nonchondrogenic, these results suggest that the Sox9 reporter induction caused by RAR inhibition may be restricted to cells with chondrogenic capacity.

**Chondrogenesis requires histone deacetylase-mediated gene repression**

Transcriptional regulation by the retinoid receptors depends for the most part on ligand availability. In the absence of ligand, RAR/RXR heterodimers bind to and repress the transcription of various target genes. Receptor-mediated repression is due to association with nuclear complexes containing corepressors (nuclear receptor corepressor [N-CoR] and SMRT) and histone deacetylases (HDACs) (Nagy et al., 1997). Trichostatin A (TSA) is a Streptomyces metabolite that specifically inhibits histone deacetylases leading to hyperacetylation of histones and other proteins (Finnin et al., 1999). To date, TSA has been shown to act as a potent inducer of differentiation in many cell types, some of which are also induced to differentiate by treatment with RA. Interestingly, chondroprogenitors, which in contrast to most cell types do not differentiate in response to RA, also respond uniquely to TSA as indicated...
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by both a dose-dependent decrease in Sox9 reporter activity (Fig. 5 A) and in cartilage nodule formation (Fig. 5 C) in response to a TSA-induced increase in RARE reporter activity (Fig. 5 B). TSA also attenuates the 310-induced increase in Sox9 reporter activity and nodule formation (Fig. 5, A and C). The inhibitory effects of TSA on chondrogenesis are achieved at relatively low concentrations of TSA, since higher concentrations have been used to induce differentiation of many cell types including NIH3T3 cells and acute promyelocytic leukemia blasts (Sugita et al., 1992; Ferrara et al., 2001). Moreover, the well-characterized ability of TSA to inhibit IL-2 gene expression was found to have an IC₅₀ of 73 nM (Koyama et al., 2000), which is greater than the highest concentration (10 nM) used here. Thus, these results demonstrate an important requirement for HDAC-mediated gene repression in chondroblast differentiation.

To further examine the importance of nuclear corepressors in chondroblast differentiation, we examined the ability of a dominant negative version of N-CoR, pCMX-G/N-CoR(2174–2453), to modulate Sox9 reporter activity. This construct lacks the HDAC interaction domain and contains the nuclear hormone receptor interaction domain of N-CoR, a region similar to that of SMRT, which was shown recently to disrupt nuclear corepressor function (Koide et al., 2001). Consistent with these activities, the pCMX-G/N-CoR(2174–2453) inhibited the ability of the antagonists and the dnRAR to decrease RARE reporter activity (unpublished data). Expression of pCMX-G/N-CoR(2174–2453) alone led to an ~50% decrease in basal Sox9 reporter activity. Moreover, coexpression of pCMXG/N-CoR(2174–2453) completely inhibited the stimulatory effects of 301 and 310 and repressed the effect of the dnRAR on the Sox9 reporter (Fig. 5 D). These results suggest that active repression by RARs is required for chondroblast differentiation and that this repression requires deacetylase activity.

RAR inhibition activates the p38 MAPK and PKA pathways
To elucidate the mechanism whereby a loss in RAR activity leads to enhanced Sox9 activity, pathway profiling vectors were used to uncover signal transduction pathways that act downstream of retinoid signaling. Various reporters containing reiterated enhancer sequences were transiently cotransfected into primary cultures with a dnRAR or by treatment with AGN194301 or AGN194310 are attenuated by coexpression of pCMX-GAL4/N-CoR (D). Bar, 1.5 mm. ANOVAs (A and B), P < 0.0001 for all cases; Bonferroni post-tests (A and B), *P < .01, **P < .001, and ***P < .0001, all versus respective control cultures; Student’s t tests (D), **P < .001 and ***P < .0001.
The PKA pathway is a predominant pathway through which genes containing a cAMP-response element (CRE) are activated. When activated through various stimuli, PKA phosphorylates and activates several targets including the AP-1 component ATF2. As a positive control, MKK6E was shown to bind to AP-1 response elements, we used the pG5-Luc reporter to measure the activity of FA-ATF2, a chimeric of ATF2 and the DNA binding domain of GAL4 (pFA-CREB) was transiently transfected into cells with a luciferase reporter containing a reiterated GAL4 DNA binding element. Thus, by monitoring the activity of the pG5-Luc reporter the activation of FA-CREB was indirectly followed. Cotransfection of pCMV-PKA into the primary cultures induced an ~40-fold increase in pG5-Luc (Fig. 6 C). Cotransfection with dnRARα enhanced FA-CREB-induced transactivation of pG5-Luc by approximately sixfold.

In addition to the PKA pathway, we investigated potential mechanisms that may underlie the activation of AP-1 by dnRARα. Activating protein-1 collectively refers to dimeric transcription factors composed of Jun, Fos, or activating transcription factor (ATF) subunits. Surprisingly, a dominant negative version of Fos (A-Fos), which substantially diminishes pAP-1-TA-Luc reporter activity, was found to have no noticeable effect on activity of the Sox9 reporter (unpublished), suggesting that the induction of pAP-1-TA-Luc by dnRARα does not involve activation of Jun/Fos dimers. Moreover, constitutively active versions of kinases within the MAPK pathways were tested for their ability to modulate Sox9 transactivation. Of the kinases known to be upstream of AP-1 activation, only a constitutively active version of MKK6 (M4K6E) consistently led to increased Sox9 reporter activity. The predominant targets of MKK6 appear to be the p38 mitogen-activated protein kinase (MAPK) isoforms. When phosphorylated, p38 phosphorylates and activates several targets including the AP-1 component ATF2. As a positive control, M4K6E was cotransfected into cells and found to induce activity of pAP-1-TA-Luc (unpublished data). Given that ATF2 has been shown to bind to AP-1 response elements, we used the pG5-Luc reporter to measure the activity of FA-ATF2, a chimeric of ATF2 and the DNA binding domain of GAL4. Cotransfection of dnRARα induced an increase in FA-

Figure 6. **The p38 MAPK pathway and the PKA pathway are activated in response to RAR inhibition.** Reporters containing a cAMP response element (pCRE-TA-Luc) or activator protein-1 response element (pAP-1-TA-Luc) are both activated in response to cotransfection with dnRARα (A and B). Cotransfection with dnRARα also induces transactivation of a GAL4 reporter (pG5-Luc) by the transcription factors ATF2 and CREB, both of which are fused to the DNA binding domain of GAL4 (FA-ATF2 and FA-CREB) (C and D). The ability of PKAc and MKK6E to activate FA-CREB and FA-ATF2, respectively, was tested for positive control purposes. Student’s *t* tests (A and B), *P* < .0005; ANOVAs (C and D), *P* < .0001; Bonferroni post-tests (C and D), *P* < .0001, **P** < .001, and ***P*** < .01.

...tained the reiterated response elements upstream of a TATA box and the luciferase gene. Interestingly, when cotransfected with a dnRARα the only reporters appreciably affected (greater than twofold increases) were pCRE-TA-Luc and pAP-1-TA-Luc. Cotransfection with dnRARα enhanced activity of these reporters by greater than fourfold (Fig. 6, A and B), indicating that RAR inhibition may result in activation of pathways upstream of CRE and AP-1 responses.

The PKA pathway is a predominant pathway through which genes containing a cAMP-response element (CRE) are activated. When activated through various stimuli, PKA phosphorylates CRE binding protein (CREB), which binds to and activates genes containing cAMP response elements. Accordingly, cotransfection of pCMV-PKA dramatically enhances activation of pCRE-TA-Luc (unpublished data). Given that a pCRE-TA-Luc is activated in cells transfected with a dnRARα, we tested the ability of this modified receptor to induce activation of CREB. A chimeric trans-activator protein containing CREB fused to the DNA binding domain of the yeast transcriptional activator GAL4 (pFA-CREB) was transiently transfected into cells with a luciferase reporter containing a reiterated GAL4 DNA binding element. Thus, by monitoring the activity of the pG5-Luc reporter the activation of FA-CREB was indirectly followed. Cotransfection of pCMV-PKA into the primary cultures induced an ~40-fold increase in pG5-Luc (Fig. 6 C). Cotransfection with dnRARα enhanced FA-CREB-induced transactivation of pG5-Luc by approximately sixfold.

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ATF2 activation of pG5-Luc that was almost as robust as the induction by MKK6E (Fig. 6 D).

Further support for the role of p38 MAPK and PKA in chondroblast differentiation comes from the reduction in Sox9 reporter activity caused by the p38 MAPK inhibitor SB202190 and the PKA inhibitor H89 (Fig. 7, A and B). These inhibitors also attenuated the induction of Sox9 reporter activity by dnRARα and by 301 (Fig. 7, A and B). Consistent with this, the inhibitors at 10 μM inhibited the formation of cartilage nodules in untreated (Fig. 7, D and F) and 301-treated cultures (unpublished data) compared with untreated cultures (Fig. 7, C and E).

**Activation of ATF2 and CREB induces Sox9 transactivation response**

The studies described above suggest that the suppression of RAR activity leads to activation of the p38 MAPK and PKA signaling pathways. Phosphorylation of ATF2 and CREB is reflective of activation of p38 MAPK and PKA signaling pathways, respectively. To further investigate a possible role for these signaling pathways in the activation of Sox9, factors involved in these pathways were transiently transfected into the mesenchymal cells along with the Sox9 reporter. Transient transfection of a constitutively active version of MKK6 (MKK6E) induces an approximate threefold activation of FA-ATF2 (Fig. 8 A). When transfected along with p38α or p38β, MKK6E is able to induce FA-ATF2 activity by ~13- and 14-fold, respectively, and even more so with the two isoforms together. However, p38α and p38β alone or in combination have no noticeable effect on Sox9 activity. The ability of each expression plasmid to induce activation of FA-ATF2 is directly proportional to their influence on Sox9 reporter activity (Fig. 8 B), with a >4.5-fold activation by cotransfection with MKK6E along with p38α and p38β. Similarly, Sox9 is activated by the catalytic subunit of PKA, which potently enhances FA-CREB activity. However, the induction of Sox9 activity by PKA is relatively mild given the level of FA-CREB activation elicited by PKA. These results demonstrate the relevance of activation of the p38 and PKA pathways by dnRARα, since each pathway has the potential to induce Sox9 transactivation of Col2a1.

Sox9 DNA binding and hence its transcriptional activity has been shown to be induced by PKA-mediated phosphorylation of serines 64 and 181 (Huang et al., 2000). Specifically, PKA phosphorylation of serine 181 in Sox9 was found to occur in chondrocytes of the prehypertrophic zone in response to parathyroid hormone-related peptide (Huang et al., 2000, 2001). To determine if the same phosphorylation event occurs here in response to RAR antagonism, we compared the ability of dnRARαs to induce Sox9 reporter activity in the presence of a cotransfected vector containing wtSox9 versus a mutant Sox9 in which serine 181 was replaced with alanine (Sox9-181A). In the absence of exogenous Sox9, Sox9 reporter activity is increased ~4.5 fold by activation of the PKA pathway using pCPT-cAMP (500 μM) in comparison to an ~9-fold increase by coexpression of dnRARα. Cotransfection with wt Sox9 or Sox9-181A increased reporter activity ~100 fold, and this was further increased, albeit slightly in each case (<1.5 fold), by the addition of pCPT-cAMP or by coexpression of PKAc or a dnRARα and decreased by the addition of H89 (Fig. 9 A; unpublished data). There is no significant difference in the activity of Sox9 versus Sox9-181A, suggesting that phosphorylation of serine 181 is not required for Sox9 activity during chondroblast differentiation. As mentioned, immunolocalization studies detected Ser181-phosphorylated Sox9 in prehypertrophic chondrocytes. However, the cells used here are chondroprogenitors, and thus, Sox9 activity may be...
regulated through distinct posttranslational modifications within each cell type. To ensure that the mutant Sox9 functions in a manner consistent with that reported previously (Huang et al., 2000), wtSox9 and Sox9-181A were transfected into COS P7 cells in the presence or absence of an expression vector for the catalytic subunit of PKA. COS P7 cells were originally used to identify Ser181 as the PKA phosphorylation site, and as expected the ability of PKAc to activate Sox9 in these cells is almost completely blocked by the Ser181 mutation (Fig. 9 B). Thus, similar to earlier reports Ser181 of Sox9 appears to be required for increased activation of Sox9 by PKA in these cells, but this is clearly not the case in the limb mesenchymal cells used in this study.

To determine if modulation of PKA activity affects the expression of Sox9 and Col2a1 transcripts, real-time quantitative PCR was used to measure their relative expression levels in comparison to rRNA. Sox9 and Col2a1 expression were increased by more than twofold in response to a 2-d treatment with pCPT-cAMP (500 μM) and decreased by more than twofold in response to H89 (10 μM) (Fig. 9 C). A similar increase in Col2a1 expression by activation of PKA in limb mesenchymal cultures has been reported previously (Kosher et al., 1986). Therefore, our results suggest that PKA regulates Sox9 activity during chondroblast differentiation by influencing Sox9 expression levels and not through phosphorylation of Ser181. However, a posttranslational role for PKA modulation of Sox9 cannot be entirely excluded, since...
Discussion

The precise timing of differentiation during development is critical for the establishment of a properly patterned embryo. Previously, we identified an important role for RAR activity in the timing of chondroblast differentiation (Weston et al., 2000), prompting us to investigate the mechanism underlying retinoid receptor regulation of the pre-chondroblast-to-chondroblast transition. Of the three Sox genes identified to date as chondrogenic regulators, Sox9 has emerged as a central player in regulating the initial stages of the chondrogenic process. Moreover, to reproduce the pattern of Col2a1 expression in the cartilage elements with a reporter gene only a small (48 bp) enhancer element from the first intron of Col2a1 containing Sox9 binding sites is required. For these reasons, Sox9 is commonly referred to as a “master regulator” of chondrogenesis, and as such, analysis of Sox9 expression and activity was an obvious starting point for identifying factors downstream of RAR activity that may mediate the effects of RAR activation or inhibition.

Association between retinoid receptor activity and Sox9 transactivation

Using soluble compounds in combination with expression plasmids, we have demonstrated a surprisingly tight correlation between retinoid receptor activity and Sox9 transactivation of Col2a1. The extent of RAR inhibition was found to be inversely proportional to the level of Sox9 induction. Moreover, manipulations affecting all three RARs were more influential than those affecting only the RAR subtype, indicating the importance of at least two RAR subtypes in chondrogenesis. Of particular interest was the dramatic induction of Sox9 activity upon cotransfection with dnRARs. To date, no single factor has been shown to induce Sox9 to a similar extent, including numerous factors known to potently stimulate chondrogenesis such as BMPs-2 and -4 either in soluble form or after cotransfection of expression plasmids containing activated BMP receptors (unpublished data). The ability of the dnRARs to stimulate Sox9 activity to a much greater extent than 301 or 310 is likely due to the constitutive repressive activity of the truncated receptor. Consistent with this, the dnRARs have been shown previously to exhibit enhanced association with nuclear corepressors (Wong and Prylaksky, 1998). Interestingly, the ability of the dnRXR to significantly enhance Sox9 activity also suggests that the absence of the RXR AF-2 domain may facilitate formation of an RAR/dnRXR–nuclear corepressor complex.

The more pronounced effects of AGN 194310 and at-RA compared with the more specific compounds, AGN 194301 and AGN 193836, respectively, implicates the involvement of at least two receptor subtypes in regulating chondroblast differentiation. These results are further supported by the effects of the dnRARx, which broadly inhibits RAR-mediated signaling (Damm et al., 1993). Together, these studies suggest that a loss of the ligand-induced activity of multiple RARs can initiate chondroblast differentiation. Accordingly, these findings explain the failure of cells expressing a weak constitutively active RAR in transgenic mice to differentiate into chondroblasts and contribute to cartilage nodules. Continued RAR activity would result in a reduction of Sox9 expression and/or activity, thereby preventing chondroblast differentiation, similar to that observed in Sox9-null cells. Interestingly, addition of RA was shown recently to cause a downregulation of Sox9 in chondrocytes (Sekiya et al., 2001). However, it is unclear from this study whether this reduction is due to a loss of the chondrocyte phenotype or by a transition to hypertrophic chondrocytes, both of which are induced by RA and are associated with a reduction in Sox9 expression (Horton et al., 1987; Koyama et al., 1999).

Importance of RAR-mediated repression in chondroblast differentiation

Our results clearly demonstrate a critical role for retinoid receptors in chondrogenesis. Surprisingly, homozygous mutant mice lacking individual RARs develop essentially normal skeletons with the exception of RARY knockout mice in which homeotic transformations of the cervical vertebra and occipital region of the skull are observed along with other minor irregularities such as fusion of the first and second ribs (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1994; Luo et al., 1995; Kastner et al., 1996; Krezel et al., 1996; Ghyselinck et al., 1997; Iulianella and Lohnes, 1997). The minor phenotypes observed in single RAR KO mice, for the most part, have been attributed to the existence of functional redundancy between members of the RAR family. Indeed, embryos in which multiple RARs are deleted exhibit more extensive skeletal defects (Lohnes et al., 1994). However, these effects are less severe than expected based on the dramatic teratogenic effects of RA and the effects observed in mice deficient in enzymes involved in RA synthesis or degradation (Niederreither et al., 1999; Abu-Abed et al., 2001; Sakai et al., 2001).

The discrepancy between our results and the RAR knock-out studies may be the result of distinct approaches used in manipulating receptor function. In the present study, we used complimentary approaches to examine the role of RAR repression in skeletal progenitor differentiation. Overexpression of a dnRARx or the addition of soluble RAR antagonists effectively abolishes transcriptional activation while at the same time maintaining or enhancing active repression. However, in the knockout mice the receptors are entirely absent and cannot function as transcriptional activators or re-
pressors. Consequently, genes that would normally be repressed by the receptors in the absence of ligand may exhibit increased expression.

The important role, demonstrated herein, for RAR-mediated repression is consistent with the recently described requirement for RAR-mediated repression in *Xenopus* head formation (Koide et al., 2001). Specifically, activation of the retinoid-signaling pathway was found to adversely affect the development of anterior structures, which could be mimicked by overexpression of a dominant negative SMRT lacking its repression domains. In contrast, repression of RAR-mediated signaling by RAR antagonists was shown to promote the expression of anterior neural markers and to enhance the formation of anterior head structures.

Direct evidence for the importance of unliganded nuclear hormone receptors also comes from studies in which the thyroid hormone receptor (TR) of mice was mutated to abolish the ligand binding function of the receptor (Hashimoto et al., 2001). Despite TR knock-out animals having completely normal structure and function of the central nervous system, mice containing the mutated TR exhibit severe neurological development and dysfunction (Hashimoto et al., 2001). These latter effects are more consistent with the severe CNS dysfunction exhibited in cases of congenital hypothyroidism and thyroid hormone resistance syndrome.

Collectively, these results combined with our findings, which demonstrate a requirement for RAR-mediated repression during skeletal development, form an emerging theme whereby retinoid receptors have an important biological role during development in the absence of ligand.

### The mechanisms of retinoid regulation of chondroblast differentiation

In addition to establishing a critical role for RAR-mediated repression in skeletal development, our findings provide a framework to describe the molecular regulation of chondroblast differentiation. Previously, we provided evidence to suggest that BMP signaling operates upstream of the retinoid signaling pathway in chondrogenesis (Weston et al., 2000). Here, we show that repression of RAR-mediated signaling results in the activation of p38 MAPK and PKA signaling pathways and that the effects of RAR antagonism on chondrogenesis can be blocked by inhibition of p38 MAPK and PKA activity. P38 MAPK and PKA have both been implicated previously in cartilage formation (Kosher et al., 1986; Lee and Chuong, 1997; Nakamura et al., 1999; Yoon et al., 2000). Specifically, we have found that inhibition of MAPK p38α and/or β prevents cartilage formation and attenuates Sox9 reporter activity. Conversely, activation of the p38α or p38β pathways is sufficient to promote Sox9 activity. Moreover, activation of p38α/β or PKA signaling are the only means identified to date, with the exception of expression of Sox9, that rescue the negative effects of activation of the RAR-signaling pathway on chondrogenesis. Together, these results suggest that activation of the p38 MAPK and PKA pathways upon RAR-mediated gene repression is necessary for acquisition of the chondroblast phenotype.

The ability of gene repression by the RARs to induce chondroblast differentiation suggests that retinoid signaling directly regulates the expression of a gene(s) whose function is to inhibit chondroblast differentiation. This novel role for gene repression in chondroblast differentiation is opposite to that of myogenic cells in which gene repression prevents differentiation. TSA stimulates differentiation of muscle progenitors, consistent with the recently described requirement for HDAC export from the nucleus before expression of the myoblastic phenotype (McKinsey et al., 2000). Thus, although the status of histone acetylation represents a general mechanism underlying cell differentiation the outcome of such modifications is cell type dependent. In this context, opposite roles for RAR-mediated gene repression in different progenitors may provide a means whereby a common local signal (RA) can differentially regulate cell differentiation. In chondroprogenitors, this repression can clearly direct changes in Sox9 activity; thus, identifying the genes affected directly by RAR repression in the chondrogenic sequence will undoubtedly further enhance our current understanding of the molecular networks underlying chondroblast differentiation.

### Materials and methods

#### Expression plasmids and reporter constructs

The Sox9-responsive reporter (herein referred to as the Sox9 reporter) was generated by subcloning a fragment containing a reiterated (4X48) Sox9 binding sequence coupled to the mouse Col2a1 minimal promoter (−89 to +6) into pGL3. The fragment containing the 4X48 repeat and minimal promoter was isolated as a BamHl/HindIII fragment from the original 4X48-pGLuc reporter plasmid described previously (Lefebvre et al., 1997) and was subcloned into the BglII and HindIII sites of pGL3-basic (Promega) to generate pGL3(4X48). The reporter pW1-Col2-Luc was generated from the original p309(182X2)ggeoCol2a1 (Zhou et al., 1995) by subcloning the regulatory region (consisting of a 309-bp promoter region and two tandem repeats of a 182-bp intron–1 fragment) into pW1 (Balkan et al., 1992) as an EcoRI/BamHl fragment. A BglII fragment containing the luciferase gene isolated from pjd205 (de Wet et al., 1987) was subcloned into the BamHl site of pW1-Col2 to generate pW1-Col2-Luc. The pcDNA3-Sox9 reporter vector was as described (Lefebvre et al., 1997). To generate a mutated form of Sox9, hSox9 was subcloned into pcDNA(Straganen), and serine 181 was replaced with alanine using the Quick-Change XL system (Stratagen) with the following overlapping primers: 5′-GCCGCCGCGGAG-GAAGGCCTGAAGAAAGGGACG-3′ and 5′-CTCTCCCCGTTCA-CGGCCTTTCCGCAGGGCCG-3′. After mutagenesis, the Sox9 mutant and wt Sox9 were subcloned into pcDNA3, and the serine-alanine conversion was confirmed by sequencing.

The dominant negative versions of RARα and RXRa were generated as enhanced green fluorescent protein (EGFP) fusions containing COOH-terminal truncations at amino acid positions 403 and 449, respectively (Damm et al., 1993; Feng et al., 1997). A BglII restriction endonuclease site was incorporated into the primers to facilitate cloning and to allow for an in-frame fusion to pEGFP-N1 (CLONTECH Laboratories, Inc.). Internal primers used for truncation of the receptors were as follows: 5′-AGA-TCTGGGATCTCCATCTTCAATG-3′ for RARα and 5′-CAGATCTGCCATGAAGAA-3′ for RXRa. For expression in cells, receptor–EGFP fusion constructs were cloned into the mammalian expression plasmid pSG5 (Stratagen). EGFP-N1 was initially subcloned into the pcSG5 vector followed by the corresponding truncated receptor to generate pSG5-dnRARα and pSG5-dnRXRaEGFP. Constitutively active versions of RARα and RXRa were subcloned into pSG5-HS as Hind III/SpeI fragments isolated from the constructs described (Underhill et al., 1994). These receptors contain COOH-terminal fusions to the acidic activation domain of VP16 (Underhill et al., 1994). The constitutively active version of RARα used here was the previously described pcDNA3-HA-MKK6E (Han et al., 1996). Expression plasmids, pcDNA3-p38α-Flag, and pcDNA3-p38β2-Flag were used to express p38α and p38β2 in mesenchymal cells (Ensl et al., 1998). To activate the p38 pathway, pCMV-PKA (CLONTECH Laboratories, Inc.), which contains the catalytic subunit of PKA was used. To follow activation of ATF2 and CREB, constructs containing the transactivation domain of these transcription factors fused to the DNA binding domain of GAL4 (pFA-ATF2 and pFA-CREB) were used (Stratagen). The pFA-ATF2 and pFA-CREB plasmids were cotransfected with pGL3-Luc, a reporter containing five copies of a GAL4 recognition sequence.
DNA binding element upstream of a TATA box and the luciferase gene (Strategene). PMCX-N-CoR and pCMX-GAL4/N-CoR (2174–2453) (referred to as pG/N-CoR[2,174–2,453] herein) consists of the DNA binding domain of GAL4 fused to the 3’ region of N-CoR encompassing amino acids 2,174–2,453 as described (Heinzel et al., 1997).

Reporter vectors from Systems 1 and 2 of CLONTECH Laboratories Inc.’s Profiling System were used to identify pathways downstream of retinoic signaling. These systems are sets of vectors that contain distinct cis-acting enhancer elements upstream of a TATA box and the luciferase gene.

Establishment and transient transfection of primary limb mesenchymal cultures Limb mesenchymal cells were harvested from embryonic age 11.25–11.75 mouse embryos as described previously (Weston et al., 2000). The cells were resuspended at a density of ∼2.5 × 10^6 cells/ml before transfection; otherwise, they were resuspended at ∼1.5 × 10^6 cells/ml. For transfection purposes, cells were mixed with a DNA/FuGene6 mixture in a 2:1 ratio. FuGene6-DNA mixtures were prepared according to the manufacturer’s instructions (Roche Biomolecular). Briefly, 1 µg of reporter, 1 µg of expression vector, and 0.05 µg of pRLSV40 (Promega) were mixed for a total of ∼2 µg DNA in 100 µl of media and FuGene6. Fifty microliters of the DNA mixture was transferred into a sterile 1.5 ml Eppendorf tube followed by 100 µl of cells. Cells were gently trituated, and 10 µl was used to seed each single well of a 24-well culture dish. After 1.5 h in a humidified CO2 incubator, 1 ml of media containing compounds of interest was added to each well and subsequently replaced 24 h after transfection. All trans RA (at-RA; Sigma-Aldrich), AGN193836 (Teng et al., 1996), AGN194301 (Teng et al., 1997), and AGN194310 (Johnson et al., 1999) (Allergen Inc.) were dissolved in 15% FBS and antibiotics. Articular chondrocytes were maintained in DME containing 10% FBS (GIBCO-BRL) and AGN193836 (Teng et al., 1996), AGN194301 (Teng et al., 1997), and AGN194310 (Johnson et al., 1999) (Allergen Inc.) were dissolved in 95% ethanol. SB202190 and SB203580 (Calbiochem) and H89 (Sigma-Aldrich; Sigma-Aldrich) were dissolved in DMSO (BDH). 8-4-chlorophenylthio-cAMP (pCPT-cAMP; Sigma-Aldrich) was dissolved in water just before use.

Analysis of reporter gene activity using the Dual Luciferase Assay System was done following the manufacturer’s instructions (Promega). Briefly, ∼48 h after the transfections cells were washed once with PBS and lysed in 100 µl of Passive lysis buffer for 20 min. Firefly and renilla luciferase activities were determined using 40 µl of lysate in a 96-well plate-reading luminometer (Molecular Devices). Aclian blue staining of cultures was performed as described previously (Cash et al., 1997).

Cell culture and transient transfection of cell lines COS P7 cells were maintained in DME containing 10% FBS (GIBCO-BRL) and antibiotics. C5.18 cells, subcloned from the parental chondroblast clone RCl 3.1C5 (Grigoriadis et al., 1996), were maintained in α-MEM supplemented with 15% FBS and antibiotics. Articular chondrocytes were derived from the knee joints of 1-d-old Sprague-Dawley rats. Briefly, 1–2 mm cartilage fragments from the femoral condyles were isolated, washed three times in sterile PBS, and digested with 0.3% Collagenase P (Worthington Biochemical Corporation) at 37°C for 4 h, adding fresh collagenase P after the first 30 min. After digestion, cells were filtered through a 70 µm cell strainer (70 µm cell strainer) and resuspended at a density of 1–2 × 10^6 cells/ml before transfections. Cells were resuspended at a density of 1.5 × 10^6 cells/ml before transfections; otherwise, they were resuspended at ∼1.5 × 10^6 cells/ml. For transfection purposes, cells were mixed with a DNA/FuGene6 mixture in a 2:1 ratio. FuGene6-DNA mixtures were prepared according to the manufacturer’s instructions (Roche Biomolecular). Briefly, 1 µg of reporter, 1 µg of expression vector, and 0.05 µg of pRLSV40 (Promega) were mixed for a total of ∼2 µg DNA in 100 µl of media and FuGene6. Fifty microliters of the DNA mixture was transferred into a sterile 1.5 ml Eppendorf tube followed by 100 µl of cells. Cells were gently trituated, and 10 µl was used to seed each single well of a 24-well culture dish. After 1.5 h in a humidified CO2 incubator, 1 ml of media containing compounds of interest was added to each well and subsequently replaced 24 h after transfection. All trans RA (at-RA; Sigma-Aldrich). AGN193836 (Teng et al., 1996), AGN194301 (Teng et al., 1997), and AGN194310 (Johnson et al., 1999) (Allergen Inc.) were dissolved in 95% ethanol. SB202190 and SB203580 (Calbiochem) and H89 (Sigma-Aldrich; Sigma-Aldrich) were dissolved in DMSO (BDH). 8-4-chlorophenylthio-cAMP (pCPT-cAMP; Sigma-Aldrich) was dissolved in water just before use.

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Northern blot analysis and real-time quantitative PCR Northern blots were performed using total RNA from limb mesenchymal cultures as described previously (Weston et al., 2000). Briefly, total RNA was extracted from cells cultured for 2, 4, 6, or 8 d. Cells were treated with media alone or with AGN194301. Synthesis of the Col2a1 cDNA fragment used was as described previously (Weston et al., 2000). The Sox9 CDNA probe was made using an EST clone (GenBank/EMBL/DDJB under accession no. AI594348 [Research Genetics]). The Sox9 fragment was released from pT73 using EcoRI and NotI. To monitor changes in transcript levels of Sox9 and Col2a1, quantitative real-time PCR was performed using the 7900HT Sequence Detection System.
Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7:725–737.

Dolle, P., E. Ruberte, P. Kastner, M. Petkovich, C.M. Stoner, L.J. Gudad, and P. Chambon. 1989. Differential expression of genes encoding alpha, beta and gamma retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature.* 342:702–705.

Enslen, H., J. Raingeaud, and R.J. Davis. 1998. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J. Biol. Chem.* 273:1741–1748.

Feng, X., Z.H. Peng, W. Di, X.Y. Li, C. Rochette-Egly, P. Chambon, J.J. Voorhees, and J.H. Xiao. 1997. Suprabasal expression of a dominant-negative RXR alpha mutant in transgenic mouse epidermis impairs regulation of gene transcription and basal keratinocyte proliferation by RAR-selective retinoids. *Gene Dev.* 11:59–71.

Ferrara, F.F., F. Fazi, A. Bianchini, F. Padula, V. Gelmetti, S. Minucci, M. Mancini, P.G. Pellicci, F. Lo Coco, and C. Nervi. 2001. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. *Cancer Res.* 61:2–7.

Finnin, M.S., J.R. Donigian, A. Cohen, V.M. Richon, R.A. Rifkind, P.A. Marks, R. Breslow, and N.P. Pavletich. 1999. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature.* 401:188–193.

Foster, J.W., M.A. Dominguez-Steglich, S. Guioli, G. Kowk, P.A. Weller, M. Stevanovic, J. Weisshenk, S. Mansour, I.D. Young, P.N. Goodfellow, et al. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature.* 372:525–530.

Ghyselinck, N.B., V. Dupe, A. Dietrich, N. Messaddeg, J.M. Garnier, C. Rochette-Egly, P. Chambon, and M. Mark. 1997. Role of the retinoic acid receptor beta (RARbeta) during mouse development. *Int. J. Dev. Biol.* 41:425–447.

Grigoradis, A.E., J.N. Heersche, and J.E. Aubin. 1996. Analysis of chondrogenitor lineage frequency and cartilage differentiation in a novel family of clonal chondrogenic rat cell lines. *Differentiation.* 60:299–307.

Hale, F. 1935. The relation of vitamin A to anophthalmos in pigs. *Am. J. Pathol.* 18:1087–1093.

Han, J., J.D. Lee, Y. Jiang, Z. Li, L. Feng, and R.J. Ulevitch. 1996. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem.* 271:2886–2891.

Hashimoto, K., F.H. Curry, P.P. Borges, C.E. Lee, E.D. Abel, J.K. Elmslie, R.N. Cohen, and F.E. Wondisford. 2001. An unliganded thyroid hormone receptor causes severe neurological dysfunction. *Proc. Natl. Acad. Sci. USA.* 98:3998–4003.

Healy, C., D. Uwamungo, and P.T. Sharpe. 1999. Regulation and role of Sox9 in cartilage formation. *Dev. Dyn.* 215:69–78.

Heinzel, T., R.M. Lavinsky, T.M. Mulllen, M. Soderstrom, C.D. Laherty, J. Torchia, W.M. Yang, G. Brand, S.D. Ngo, J.R. Davie, et al. 1997. A complex containing N-CoR, mSin3, and histone deacetylase mediates transcriptional repression. *Nature.* 387:43–48.

Horlein, A.J., A.M. Naar, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Soderstrom, C.K. Glass, et al. 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature Rev.* 377:397–404.

Horton, W.E., Y. Yamada, and J.R. Hasseil. 1987. Retinoic acid rapidly reduces cartilage matrix synthesis by altering gene transcription in chondrocytes. *Dev. Biol.* 123:508–516.

Huang, W., X. Zhou, V. Lefebvre, and B. de Crombrugghe. 2000. Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. *Proc. Natl. Acad. Sci. USA.* 97:725–729.

Huang, W., U.I. Chung, H.M. Kronenberg, and B. de Crombrugghe. 1996. A 47-bp sequence of the first intron of the mouse pro alpha 1(II) collagen gene is sufficient to direct chondrocyte expression. *Ann. NY Acad. Sci.* 785:284–287.

Lefebvre, V., W. Huang, V.R. Harley, P.N. Goodfellow, and B. de Crombrugghe. 1997. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha 1(II) collagen gene. *Mol. Cell. Biol.* 17:2356–2366.

Leid, M., P. Kastner, and P. Chambon. 1992. Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends Biochem. Sci.* 17:427–433.

Li, E., H.M. Sucov, K.P. Lee, R.M. Evans, and R. Jaenisch. 1993. Normal development and growth of mice carrying a targeted disruption of the alpha 1 retinoic acid receptor gene. *Proc. Natl. Acad. Sci. USA.* 90:1590–1594.

Lohnes, D., P. Kastner, A. Dietrich, M. Mark, M. LeMeur, and P. Chambon. 1993. Function of retinoic acid receptor gamma in the mouse. *Cell.* 73:643–658.

Lohnes, D., M. Mark, C. Mendelsohn, P. Dolle, A. Dietrich, P. Gorry, A. Gansmuller, and P. Chambon. 1994. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development.* 120:2723–2748.

Lufkin, T., D. Lohnes, M. Mark, A. Dietrich, P. Gorry, M.P. Gaub, M. LeMeur, and P. Chambon. 1993. High postnatal lethality and tests degeneration in retinoic acid receptor alpha mutant mice. *Proc. Natl. Acad. Sci. USA.* 90:7225–7229.

Luo, J., P. Paerici, R.A. Conlon, J. Rosinant, and V. Giguerre. 1995. Mice lacking all isofoms of retinoic acid receptor beta develop normally and are susceptible to the teratogenic effects of retinoic acid. *Mech. Dev.* 53:61–71.

McKinsey, T.A., C.L. Zhang, J. Lu, and E.N. Olson. 2000. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature.* 408:106–111.

Mendelsohn, C., E. Ruberte, M. LeMeur, G. Morris-Kay, and P. Chambon. 1991. Developmental analysis of the retinoic acid-inducible RAR-beta 2 promoter in transgenic animals. *Development.* 113:723–734.

Mendelsohn, C., M. Mark, P. Dolle, A. Dietrich, M.P. Gaub, A. Kurst, C. Lampron, and P. Chambon. 1994. Retinoic acid receptor beta 2 (RAR beta 2) null mutant mice appear normal. *Dev. Biol.* 166:246–258.

Mollard, R., S. Viville, S.J. Ward, D. Decimo, P. Chambon, and P. Dolle. 2000. Tissue-specific expression of retinoic acid receptor isoforms in the mouse embryo. *Mech. Dev.* 94:223–232.

Murakami, S., M. Kan, W.L. McKeehan, and B. de Crombrugghe. 2000a. Up-regulation of the chondrogenic Sox9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc. Natl. Acad. Sci. USA.* 97:1113–1117.

Murakami, S., V. Lefebvre, and B. de Crombrugghe. 2000b. Potent inhibition of the master chondrogenic Sox9 gene by interleukin-1 and tumor necrosis factor-alpha. *J. Biol. Chem.* 275:3687–3692.

Nagy, L., H.Y. Kao, D. Chakravarti, R.J. Lin, C.A. Hassig, D.E. Ayer, S.L. Schreiber, and R.M. Evans. 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell.* 89:373–380.
Nakamura, K., T. Shirai, S. Morishita, S. Uchida, K. Saeki-Miura, and F. Maki-shima. 1999. p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. *Exp. Cell Res.* 250:351–363.

Niederreither, K., V. Subbarayan, P. Dolle, and P. Chambon. 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* 21:444–448.

Oh, C.D., S.H. Chang, Y.M. Yoon, S.J. Lee, Y.S. Lee, S.S. Kang, and J.S. Chun. 2000. Opposing role of mitogen-activated protein kinase subtypes, erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymes. *J. Biol. Chem.* 275:5613–5619.

Sakai, Y., C. Meno, F. Fujii, J. Nishino, H. Shiratori, Y. Saijoh, J. Rossant, and H. Hamada. 2001. The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the antero-posterior axis within the mouse embryo. *Genes Dev.* 15:213–225.

Sekiya, I., P. Koopman, K. Tsuji, S. Mertin, V. Harley, Y. Yamada, K. Shinomiya, A. Niguji, and M. Noda. 2001. Transcriptional suppression of Sox9 expression in chondrocytes by retinoic acid. *J. Cell. Biochem.* (Suppl):71–78.

Smits, P., P. Li, J. Mandel, Z. Zhang, J.M. Deng, R.R. Behringer, B. de Crombrugghe, and V. Lefebvre. 2001. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev. Cell.* 1:277–290.

Sugita, K., K. Koizumi, and H. Yoshida. 1992. Morphological reversion of vitamin A-deficient NIH3T3 cells by retinoid. *Cancer Res.* 52:168–172.

Teng, M., T.T. Duong, E.S. Klein, M.E. Pino, and R.A. Chandraratna. 1996. Identification of a retinoic acid receptor alpha subtype specific agonist. *J. Med. Chem.* 39:3035–3038.

Teng, M., T.T. Duong, A.T. Johnson, E.S. Klein, L. Wang, B. Khalifa, and R.A. Chandraratna. 1997. Identification of highly potent retinoic acid receptor alpha-selective antagonists. *J. Med. Chem.* 40:2445–2451.

Underhill, T.M., and A.D. Weston. 1998. Retinoids and their receptors in skeletal development. *Micro. Res. Tech.* 43:137–155.

Wagner, T., J. Wirth, J. Meyer, B. Zabel, M. Held, J. Zimmer, J. Pasantes, F.D. Bricarelli, J. Keutel, E. Hustert, et al. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell.* 79:1111–1120.

Warkany, J., and S. Schraffenberger. 1946. Congenital malformations induced in rats by maternal vitamin A deficiency. I. Defects of the eye. *Arch. Ophthal.* 35:150–169.

Weston, A.D., V. Rosen, R.A.S. Chandraratna, and T.M. Underhill. 2000. Regulation of skeletal progenitor differentiation by the BMP and retinoid signaling pathways. *J. Cell Biol.* 148:679–690.

Wilson, J.G., C.B. Roth, and J. Warkany. 1953. An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. *Am. J. Anat.* 92:189–217.

Wong, C.W., and M.L. Privalsky. 1998. Transcriptional silencing is defined by isomorph- and heterodimer-specific interactions between nuclear hormone receptors and corepressors. *Proc. Natl. Acad. Sci. U. S. A.* 95:7022–7027.

Yoon, Y.M., C.D. Oh, D.Y. Kim, Y.S. Lee, J.W. Park, T.L. Huh, S.S. Kang, and J.S. Chun. 2000. Epidermal growth factor negatively regulates chondrogenesis of mesenchymal cells by modulating the protein kinase C-alpha, Erk-1, and p38 MAPK signaling pathways. *J. Biol. Chem.* 275:12353–12359.

Zhou, G., S. Garofalo, K. Mukhopadhyay, V. Lefebvre, C.N. Smith, H. Eber-pachter, and B. de Crombrugghe. 1995. A 182 bp fragment of the mouse pro alpha 1(II) collagen gene is sufficient to direct chondrocyte expression in transgenic mice. *J. Cell Sci.* 108:3677–3684.