Molecular Mechanisms Regulating Chondroblast Differentiation

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By Lisa M. Hoffman, PhD*, Andrea D. Weston, PhD*, and T. Michael Underhill, PhD

Background: Formation of the cartilage template involves a multi-step process in which prechondrogenic mesenchymal cells form condensations prior to differentiating into matrix-producing chondroblasts. Retinoids, particularly retinoic acid, are among the numerous signaling molecules that have been implicated in this process. A proper balance of retinoids is essential for normal skeletal development in that too much or too little negatively impacts skeletogenesis. During the past few years, substantial advances have been made in our understanding of the role of retinoid signaling in these processes, which is reviewed in this report.

Methods: To examine the function of retinoid signaling in skeletal development, transgenic mice that overexpressed a weak, constitutively active retinoic acid receptor (retinoic acid receptor-α) in their developing limbs were generated. The mice presented with a range of skeletal abnormalities. To examine the mechanisms responsible for these abnormalities, primary limb mesenchymal cultures from the transgenic mice were compared with cultures from wild-type mice. In addition, to address the molecular basis of retinoic acid receptor action, retinoic acid receptor activity in the primary cultures was manipulated with use of retinoic acid receptor-selective agonists and antagonists. The evaluation of the response to the manipulation of retinoic acid receptors was followed by histological studies and by the use of Northern blot analysis and reporter assays to analyze changes in the expression of chondrocytic markers and to monitor transcription factor activity, respectively.

Results: The evidence reviewed here indicates that retinoids maintain cells within condensations in a prechondrogenic, mesenchymal cell state, which prevents the cells from differentiating into chondroblasts. More recent studies have demonstrated that the inhibition of receptor-mediated retinoid signaling induces the expression of Sox9, a transcription factor that is considered a "master switch" for the differentiation of chondroblasts. These effects are largely mediated by the activation of the p38 MAPK signaling cascade.

Conclusions: These findings demonstrate that retinoid receptor-mediated repression is both necessary and sufficient for chondroblast differentiation. Moreover, retinoic acid receptor repression acts downstream of BMP signaling or in a distinct pathway to activate p38 MAPK, which in turn induces chondroblast differentiation.

Considerable progress has been made over the past few years toward our understanding of the role of retinoid signaling in cartilage development. This report reviews the mounting evidence that gene repression, mediated by retinoic acid receptors, is a requisite trigger for chondroblast differentiation. In addition, we describe the molecular events that occur upstream and downstream of retinoid signaling to mediate chondrogenesis. The assembly of a model for the network of events underlying chondrogenesis is well underway and will dramatically improve our current understanding of diseases associated with chondrogenic abnormalities. Moreover, given that many reparative processes in the adult recapitulate, to some extent, the events that take place during embryonic development, our knowledge of chondrogenesis will be invaluable for the treatment of diseases and disorders associated with a cartilage deficiency.

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Much of the vertebral skeleton develops through the endochondral ossification of a preexisting cartilaginous template. The first noticeable step leading to formation of this template is the condensation of prechondrogenic mesenchymal cells. The resulting cell-cell interactions contribute to the onset of chondroblast differentiation, which is characterized by a switch from a fibroblast-like morphology to a spherical cell shape and by the secretion of an extracellular matrix composed predominantly of type-2 collagen (Col2) and aggrecan. Substantial insight has been gained about the extracellular matrix components and the cell-cell adhesion molecules that mediate the condensation of prechondrogenic mesenchymal cells. However, until recently, little was known about the signaling pathways that regulate the differentiation of chondroprogenitors. It has been known for decades that vitamin A and its metabolites, collectively referred to as retinoids, are required for normal embryonic development. Specifically, pregnant mothers who consume too much or too little vitamin A produce offspring with skeletal abnormalities. Retinoic acid

(RA) is the main metabolite of vitamin A and is believed to be the primary cause of those abnormalities. The importance of retinoid signaling in the early stages of skeletal development, namely chondrogenesis, is highlighted by the fact that skeletal malformations induced by retinoic acid are most severe when retinoic acid is applied during the period of cartilage development. Numerous studies have subsequently demonstrated, more directly, an inhibition of cartilage differentiation by retinoic acid[1].

Retinoic Acid Receptors and Chondrogenesis

More recently, focus has shifted toward uncovering the role of RA receptors during skeletogenesis. Retinoic acid regulates gene transcription by means of nuclear receptors[2,3]. Two classes of these receptors exist: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), both of which function as ligand-inducible transcription factors[4], with the RXRs forming heterodimeric complexes with the RARs[5,6]. Im-

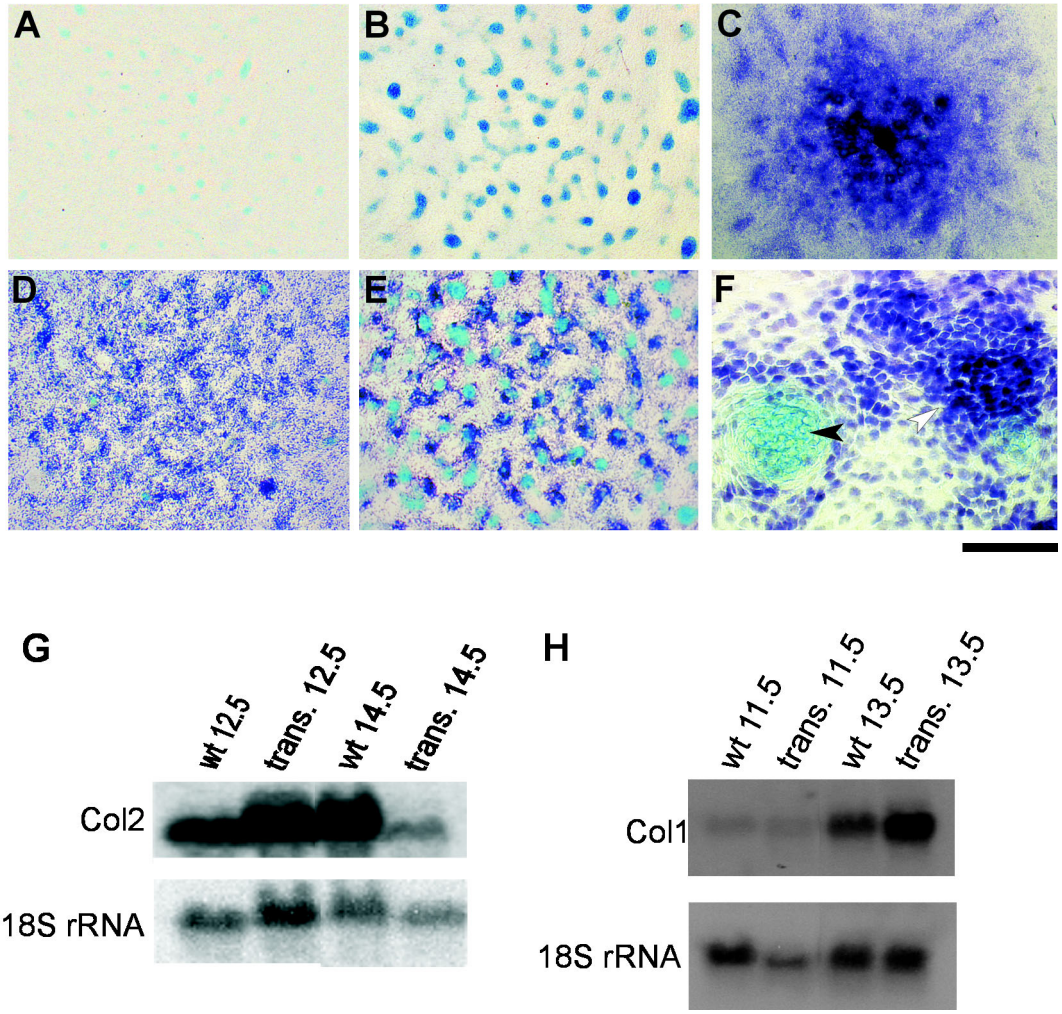


Fig. 1

Misexpression of a weak, constitutively active retinoic acid receptor-α (RARα) inhibits chondrogenesis. *A* and *B,* Wild-type forelimb cultures were fixed and stained with alcian blue on Days 2 and 4. *D* and *E,* Transgenic forelimb cultures were stained with magenta-gal on Days 2 and 4 and then were stained with alcian blue. *C,* Transgenic hindlimb cultures were stained with alcian blue and magenta-gal on Day 4. *F,* Higher-magnification of Day-4 transgenic forelimb cultures. Transgene-expressing cells condense (white arrow) but are excluded from the cartilage nodules (black arrow). *G* and *H,* Northern blot analysis was used to quantify the expression of Col2 *(G)* and Col1a2 *(H)* in the hindlimbs of wild-type and transgenic embryos. Scale bars: 1 mm *(A, B, D,* and *E)*, 2 mm *(C)*, and 0.2 mm *(F)*. (Reproduced, with modification, from: Weston AD, Rosen V, Chandraratna RA, Underhill TM. Regulation of skeletal progenitor differentiation by the BMP and retinoid signaling pathways. J Cell Biol. 2000;148:679-90. Reprinted with permission of The Rockefeller University Press.)

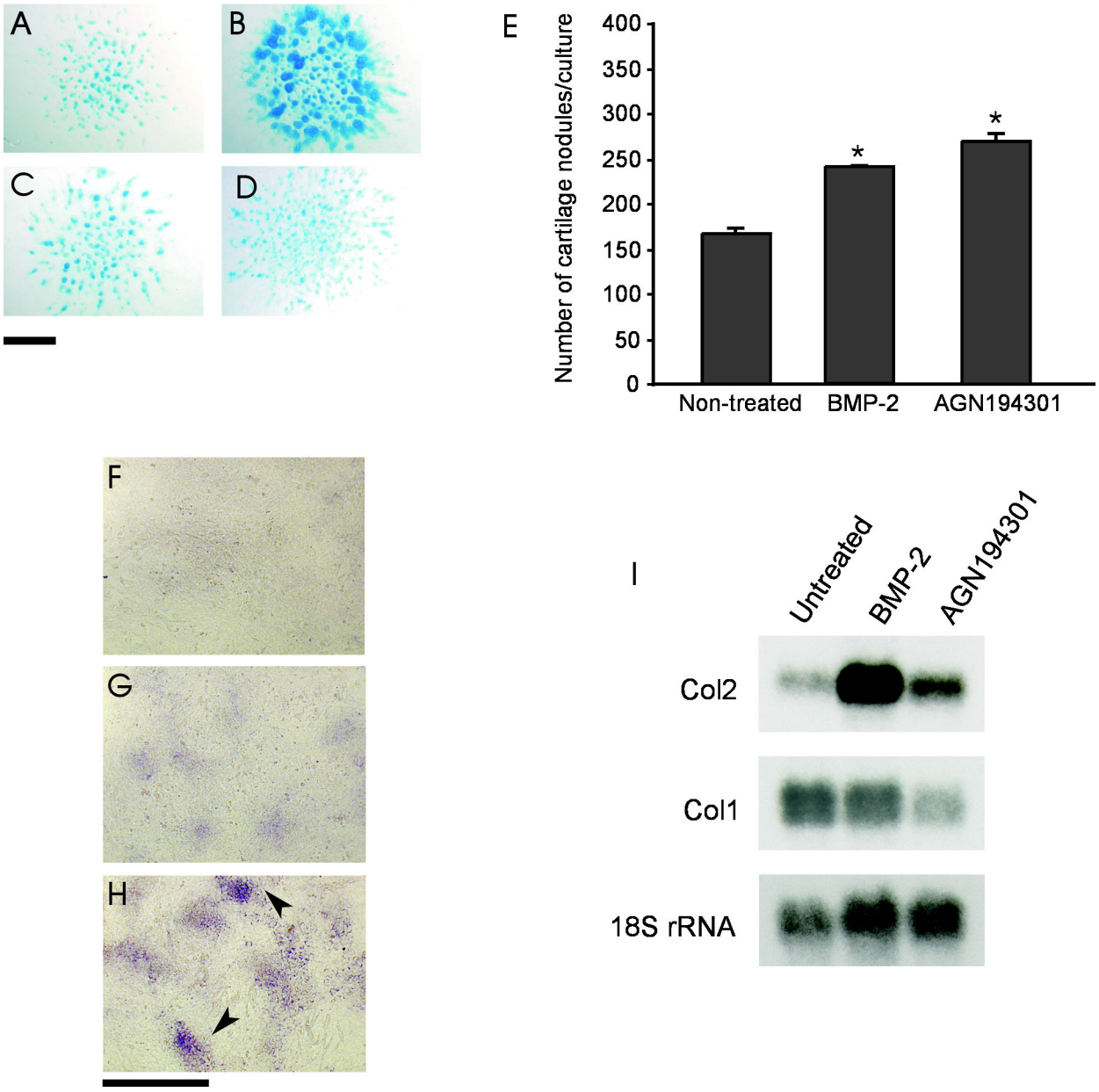


Fig. 2

Treatment of primary cultures with a retinoic acid receptor-selective antagonist stimulates chondroblast differentiation. *A* through *D,* Untreated wild-type cultures *(A* and *C)* and the corresponding cultures treated continuously with either 10 ng/mL BMP-2 *(B)* or 1 μM AGN 194301 *(D)* were stained with alcian blue on Day 8. *E,* Quantification of cartilage nodule formation in response to BMP-2 or AGN 194301. *F, G,* and *H,* Whole-mount in situ hybridization was used to follow the distribution of Col2 transcripts in wild-type cultures after twenty-four hours of treatment in a control medium *(F),* BMP-2-containing medium *(G),* or medium supplemented with AGN 194301 *(H).* Arrowheads indicate foci of Col2-expressing cells that are apparent in the AGN 194301-treated cultures. *I,* Analysis of Col1a2 and Col2 expression in Day 4 wild-type cultures treated with either BMP-2 or AGN 194301, relative to untreated controls. Scale bars: 1 mm *(A* through *D)* and 0.5 mm *(F, G,* and *H).* (Reproduced, with modification, from: Weston AD, Rosen V, Chandraratna RA, Underhill TM. Regulation of skeletal progenitor differentiation by the BMP and retinoid signaling pathways. J Cell Biol. 2000;148:679-90. Reprinted with permission of The Rockefeller University Press.)

portantly, however, unliganded receptors are also recognized as major regulators of gene expression through the recruitment of nuclear co-repressors and associated histone deacetylases[7-11]. Within the RAR and RXR subfamilies, there are three subtypes (α, β, and γ) with multiple isoforms of each[12]. These receptors exhibit dynamic expression patterns throughout skeletal development[13]. During the early development and outgrowth of mouse limbs, RARα and RARγ are expressed

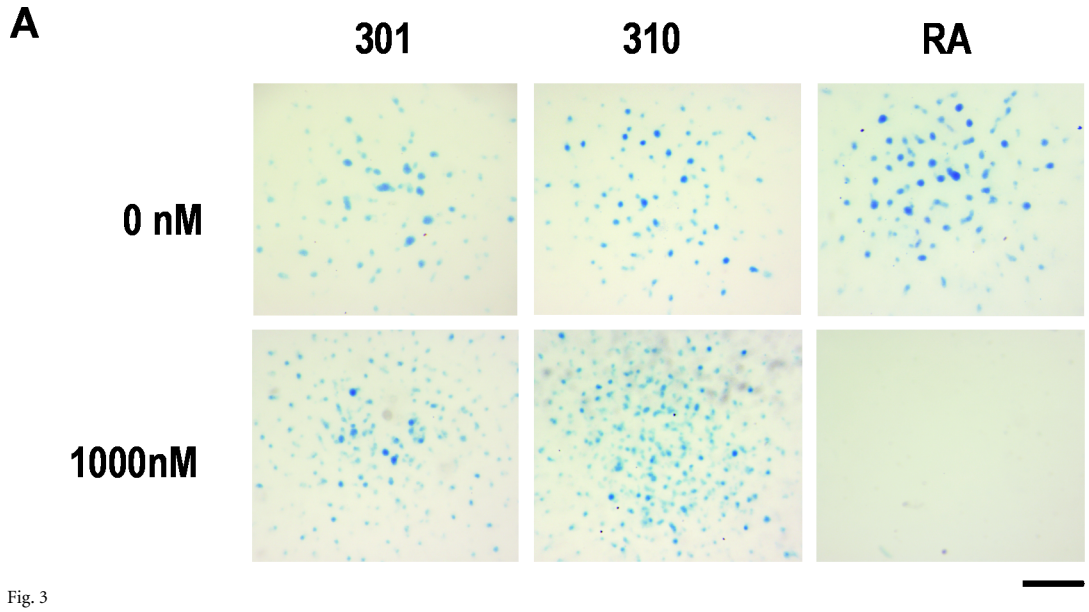


Fig. 3

Inhibition of retinoic acid receptor (RAR) activity enhances Sox9 activity and expression. *A,* The effects of an RAR agonist (RA) and RAR antagonists on the formation of cartilage nodules, assessed by staining with alcian blue. *B,* Activation of the retinoid receptors in primary limb mesenchymal cultures with all trans-RA attenuated activity of the pGL3(4X48) Sox9 reporter. In contrast, inhibition of RAR activity by the RARα-specific antagonist, AGN 194301 (301), or with the RAR pan-antagonist AGN 194310 (310) enhanced reporter activity. These results demonstrate that Sox9 reporter activity corresponds with the ability of each compound to enhance or inhibit cartilage formation in vitro, as indicated by alcian blue staining. *C,* In response to treatment with AGN 194301, there is a transient increase in Sox9 mRNA in primary cultures. *D* and *E,* To further study the influence of RAR activity during chondrogenesis, constructs containing constitutively active receptors (RARαVP16 and RXRαVP16) or dominant-negative versions of the receptors (dnRARα and dnRXRα) were used. Co-expression of RARαVP16 and RXRαVP16 attenuated reporter gene activity. In contrast, co-transfection with vectors containing dnRARα and dnRXRα substantially increased reporter activity of pGL3(4X48). Scale bar: 1.5 mm. *B, D,* and *E,* Analysis of variance, $p < 0.0001$. *B,* Bonferroni post-tests indicated significant differences in Sox9 reporter activity at concentrations of 5×10^{-9}M for 310, 1×10^{-7}M for 301, and 5×10^{-9}M for at-RA ($p < 0.05$ for all). *D, E* *$p < 0.001$ (Bonferroni post-hoc test). (Reproduced, with modification, from: Weston AD, Chandraratna RA, Torchia J, Underhill TM. Requirement for RAR-mediated gene repression in skeletal progenitor differentiation. J. Cell Biol. 2002;158:39-51. Reprinted with permission of The Rockefeller University Press.)

throughout the limb mesenchyme. However, as mesenchymal cells condense and differentiate, RARα is downregulated and becomes largely restricted to the surrounding perichondrium and interdigital region, while the expression of RARγ becomes localized to the cartilaginous elements. RARβ is expressed in regions of the developing limb that are not destined to form cartilage, including the interdigital region and necrotic zones[13-15]. RXRα is expressed weakly throughout the limb bud[16,17], although it has been shown in vitro to be expressed at high levels in chondrocytes[18]. RXRβ is similarly expressed at low levels throughout the developing limb, whereas RXRγ expression is localized to the myogenic regions within the proximal part of the limb[16,17].

In accordance with earlier studies regarding RA teratogenicity, the overexpression of RARs, particularly RARα, or the presence of ligand-activated RARs results in skeletal malformations[19-21]. In contrast, RXR agonists fail to induce skeletal anomalies. Rather, it is believed that they potentiate the effects induced by RAR agonists[22,23]. Surprisingly, homozygous null mutants of RARα, RARβ, or RARγ exhibit relatively minor skeletal abnormalities, with no noticeable skeletal defects in developing limbs[24-27]. Those observations indicate that there may be some degree of functional redundancy among members of the RAR subfamily. Indeed, compound null mutants of RARα and RARγ display a range of skeletal deformities in limbs that can essentially be overcome by the presence of a single copy of RARα[28]. Those results support the notion that one of the receptors is minimally required for normal development of the appendicular skeleton. However, RARα/RARβ compound mutants and RARβ/RARγ compound mutants do not present with any obvious skeletal malformation of the limbs[24]. These relatively mild phenotypes pale in comparison with the dramatic teratogenic effects that are caused by an imbalance of retinoic acid during development. The lack of a major phenotype is also surprising given the results of RAR overexpression. Throughout this review, we provide an explanation for this

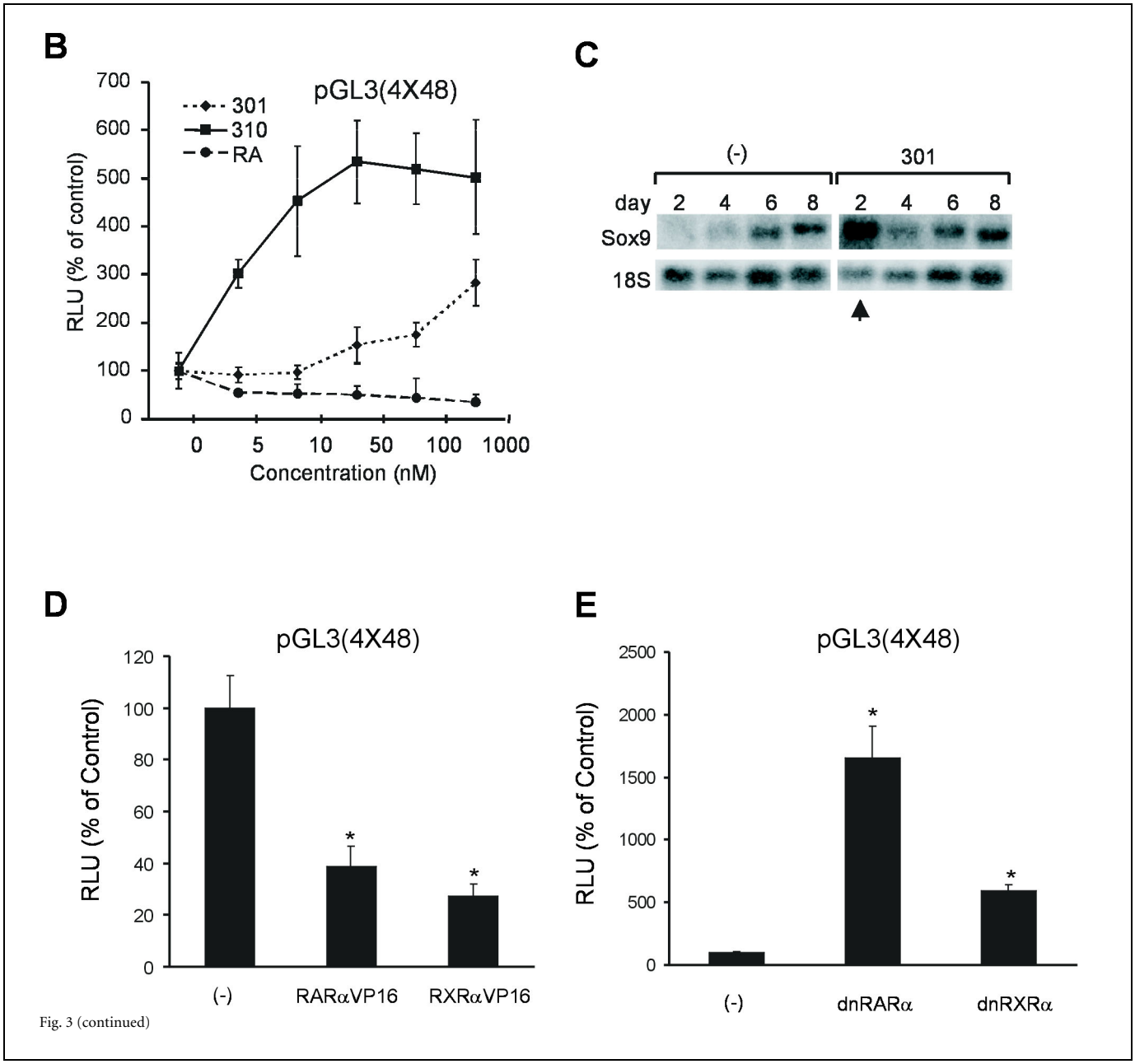


Fig. 3 (continued)

discrepancy by highlighting a major role for unliganded receptors during chondrogenesis, a role that is not directly revealed from mice knockout studies.

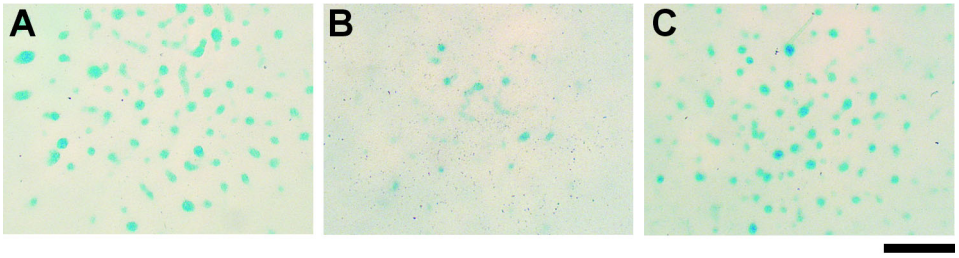
Misexpression of RARα in the Developing Limb Prevents Chondroblast Differentiation

RARα transcripts are highly expressed in prechondrogenic mesenchyme but are downregulated as differentiation proceeds[13,14,19]. In transgenic mice that overexpress a weak, constitutively active RARα1 in their developing limbs, continued activity of RARα inhibits chondrogenesis, causing skeletal deficiencies that phenocopy those observed in RA teratogenicity studies[19]. Those deficiencies were later demonstrated to result from a failure of prechondrogenic cells to differentiate into chondroblasts. Specifically, in high-density micromass cultures established from the limb buds of transgenic mice, condensations of transgene-expressing cells appeared normal even though the transgene-expressing cells were excluded from cartilage nodules (Fig. 1, *A* through *F*)[19,21]. To further characterize the effect of the transgene on chondrogenesis, markers indicative of chondroblast differentiation (i.e., Col2) and precartilaginous condensations (i.e., collagen type-1, N-cadherin) were examined with use of Northern blot analysis and in situ hybridization. In transgenic mice, collagen type-1, α2 (Col1a2), is expressed at high levels in the hindlimb (Fig. 1, *H*), whereas Col2 expression is weak compared with that observed in wild-

type hindlimbs (Fig. 1, *G*). In contrast, treatment of wild-type cultures with an RAR-selective antagonist stimulates precocious chondrogenic differentiation as demonstrated by the early emergence of cartilage nodules and by an increase in Col2 and a reduction in Col1a2 expression (Fig. 2, *D, E, H,* and *I*). Taken together, these findings strongly suggest that misexpression of RARα disrupts chondrogenesis by maintaining the prechondrogenic cell phenotype.

Efforts aimed at furthering the understanding of the mechanisms whereby the continued expression of RARα attenuates chondroblast differentiation led to the discovery of a surprisingly close association between RAR activity and the transcriptional activity of Sox9. Sox9 is a major transcriptional regulator of chondrogenesis that binds to a critical consensus sequence in the Col2 promoter to activate its transcription. Inhibition of RAR signaling with RAR-selective antagonists induces Sox9 expression (Fig. 3, *C*) and activity (Fig. 3, *B*) in primary limb mesenchymal cultures, while all-trans retinoic acid substantially attenuates activity (Fig. 3, *B*). The relationship between RAR and Sox9 activity is also reflected in the ability of cultured limb mesenchyme to differentiate into chondroblasts and to secrete an extracellular cartilaginous matrix following RAR manipulation (Fig. 3, *A*). The introduction of modified versions of retinoid receptors to these cultures confirms those findings[29] as the expression of either a dnRARα or a dnRXRα substantially activates Sox9 activity, whereas constitutively active retinoid receptors inhibit it (Fig. 3, *D* and *E*). This chondrogenic response to RAR modulation appears to be restricted to cells committed to the chondrocytic lineage as Sox9 activity is unaffected in the nonchondrogenic cell line, Cos P7[29]. Moreover, and of particular interest from a clinical perspective, dedifferentiated chondrocytes that were isolated from the knees of rat pups responded similarly to limb mesenchymal cells in that there was a substantial increase in Sox9 reporter activity following RAR inhibition[29].

Given the discrepancy between the results of the manipulation of RAR activity and the results of mouse knockout studies, a potential role for unliganded RARs was considered. Interestingly, the chondrogenic-stimulatory effects of antagonizing RAR signaling are attenuated in the presence of a histone deacetylase (HDAC) inhibitor or a dominant-negative nuclear receptor co-repressor[29]. Both of these factors combine with unliganded RARs to repress gene transcription, further reinforcing the importance of RAR-mediated gene repression in chondroblast differentiation. These results suggest that differences in the approach used to ablate receptor function may



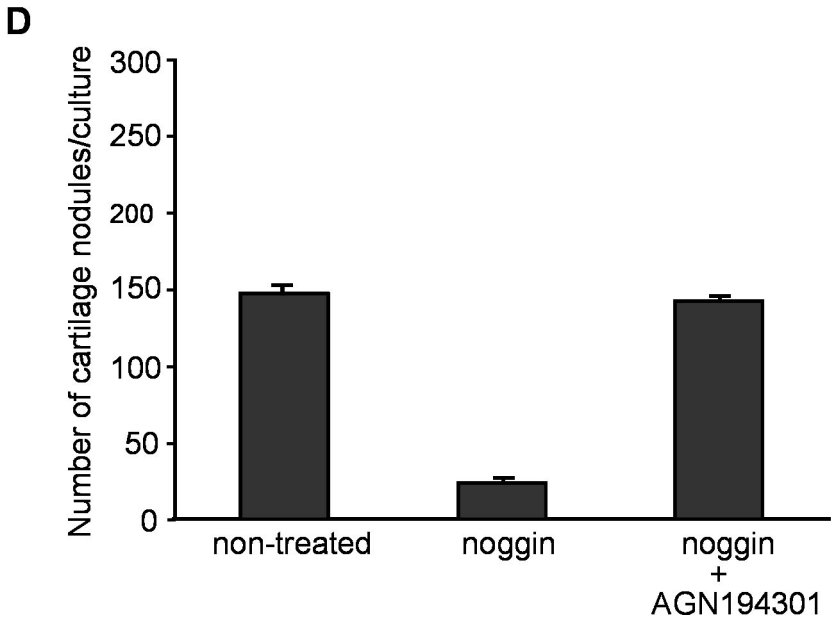


Fig. 4

Noggin inhibits the formation of cartilage nodules, and this effect is rescued by antagonism of RAR-mediated signaling. *A, B,* and *C,* Wild-type cultures incubated in the presence or absence of Noggin (10 ng/mL) and/or AGN 194301 were stained with alcian blue on Day 6. Noggin reduced cartilage nodule formation *(B)* relative to untreated controls *(A).* AGN 194301 (1 μM) stimulated nodule formation even in the presence of Noggin *(C). D,* Quantification of cartilage nodule formation in response to Noggin and AGN 194301. Scale bar: 1 mm. (Reproduced, with modification, from: Weston AD, Rosen V, Chandraratna RA, Underhill TM. Regulation of skeletal progenitor differentiation by the BMP and retinoid signaling pathways. J Cell Biol. 2000;148:679-90. Reprinted with permission of The Rockefeller University Press.)

account for the discrepancies between the results reported in our study, in which disruption of RAR activity enhanced chondrogenesis, and the results from knockout studies, in which complete removal of individual RARs produced no obvious skeletal phenotype. In knockout mice, not only are receptors unavailable to transduce a retinoid signal but their normal role as gene repressors in the absence of ligand is also removed. Current efforts are aimed toward identifying targets directly regulated by RAR repression.

Retinoid Signaling in the Context of Other Factors Modulating Chondrogenesis

Since the establishment that RAR-mediated gene repression is both necessary and sufficient for chondroblast differentiation, much effort has been spent to place retinoid signaling within the context of numerous other factors known to regulate chondrogenesis. To this end, we have obtained sufficient evidence to suggest that retinoid signaling functions downstream of, or at least in parallel to, the BMP signaling pathway, and that p38 MAPK and PKA, both activated in response to RAR repression, are also important regulators of chondrogenesis. BMP signaling is required for chondrocyte differentiation as members of this family have been shown both in vivo and in vitro to induce cartilage formation[30-35]. Moreover, expression of dominant-negative or constitutively active BMP receptors inhibits or stimulates chondrogenic differentiation[35], respectively, whereas misexpression of the BMP antagonist, Noggin[36], inhibits chondrogenesis of embryonic limb mesenchyme in vitro (Fig. 4, *A, B,* and *D*) and in vivo[37]. Interestingly, the overexpression of RARα in developing limbs modifies the response of prechondrogenic mesenchymal cells to BMPs[21]. Specifically, in the presence of exogenous BMPs, condensation of transgene-expressing cells is dramatically enhanced, but these condensed cells still fail to differentiate into chondroblasts, indicating that the RARα-mediated inhibition of chondroblast differentiation cannot be rescued by BMPs. In contrast, RAR-selective antagonists can induce differentiation even in the presence of Noggin (Fig. 4, *C* and *D*) indicating that RARs act downstream of BMP signaling or in a distinct pathway.

Downstream of RARs, activation of the p38 mitogen-activated protein kinase (MAPK) has been shown to mediate cartilage differentiation[29]. Signaling through the p38 MAPK intracellular pathway has previously been shown to regulate cartilage formation[38,39]. Accordingly, the addition of pharmacological p38 MAPK inhibitors to the primary cultures of mouse limb mesenchyme inhibits both Sox9 activity and cartilage formation (Fig. 5, *A* and *C*), whereas the abrogation of RARα signaling activates the p38 MAPK signaling cascade[29]. In contrast, p38 inhibitors attenuate the induction of Sox9, which is normally caused by RAR inhibition (Fig. 5, *A*). Moreover, activation of the p38 MAPK pathway is sufficient to restore Sox9 activity in cultures transfected with a constitutively active RAR[29]. Together, these results suggest that activation of p38 MAPK signaling functions downstream of RAR-mediated gene repression to induce chondroblast differentiation.

Overview and Perspectives

Retinoid signaling is required for chondroblast differentiation; however, unlike many other developmental systems, it is the absence of ligand that triggers differentiation. The inhibition of RAR-mediated signaling leads to increased expression of chondroblast markers and is associated with a dramatic increase in Sox9 expression and activity. The ability to modulate chondroblast differentiation by the manipula-

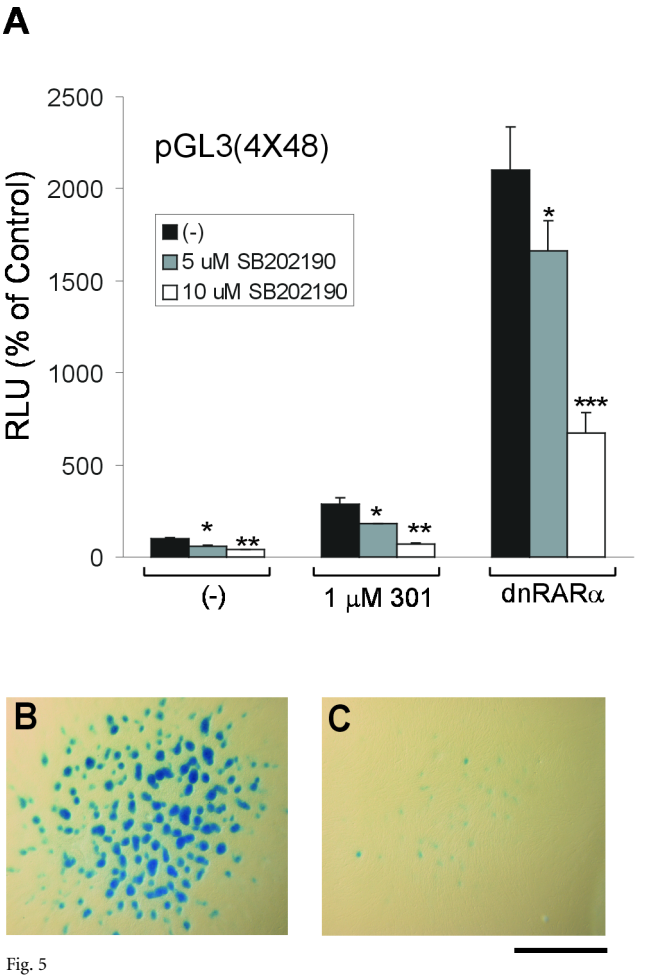


Fig. 5

Modulation of chondrogenesis by the inhibition of p38 MAPK signaling. *A,* In the presence of 5 μM or 10 μM SB202190, there was a decrease in Sox9 reporter activity compared with untreated controls. SB202190 also attenuated the chondrogenic response to AGN 194301 and the dnRARα. *B* and *C,* The inhibition of Sox9 activity following treatment with SB202190 was reflected in vitro by an absence of cartilage nodules stained with alcian blue relative to untreated controls. Scale bar: 1.5 mm. Analysis of variance, $p < 0.0001$. Bonferroni post-tests, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in comparison with respective non-SB202190-treated controls. (Reproduced, with modification, from: Weston AD, Chandraratna RA, Torchia J, Underhill TM. Requirement for RAR-mediated gene repression in skeletal progenitor differentiation. J. Cell Biol. 2002;158:39-51. Reprinted with permission of The Rockefeller University Press.)

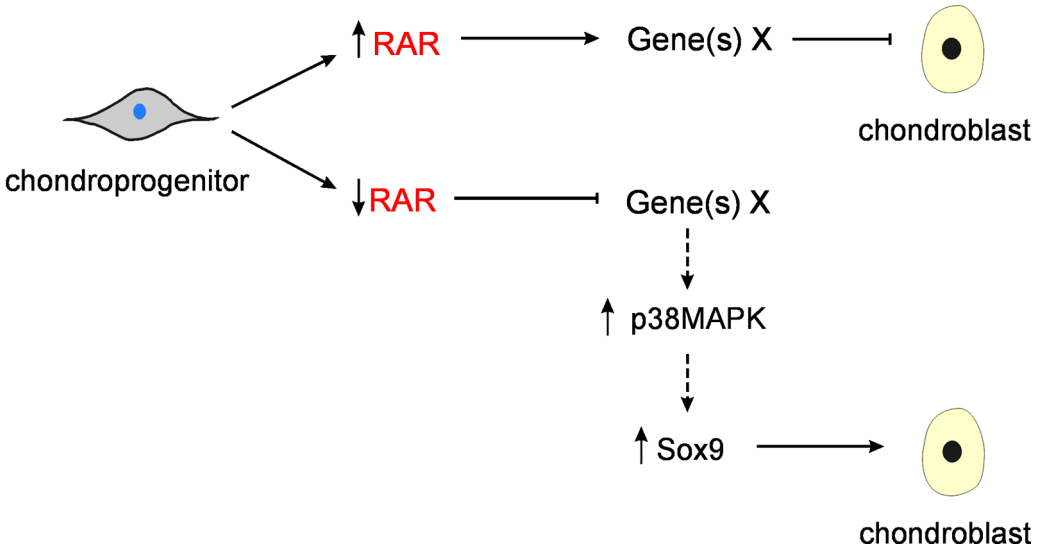


Fig. 6

Model for the molecular mechanisms underlying retinoid function in chondrogenesis. In vivo, RARα is highly expressed in chondroprogenitors and remains abundant in perichondrial cells but is downregulated as cells differentiate into chondroblasts. In contrast, RARγ is upregulated during chondroblast differentiation and becomes abundantly expressed in chondrocytes. Activation of the retinoid signaling pathway maintains the prechondrogenic phenotype, whereas RAR-mediated repression is required for chondroblast differentiation. In this manner, RARs may act by repressing the expression of a gene (or genes) that negatively influences activation of downstream pathways, such as the p38 MAPK signaling pathway. Through mechanisms that are not yet understood, activation of these pathways culminates in an increase in Sox9 expression and perhaps Sox9 activation during chondroblast differentiation.

tion of retinoid signaling has enabled the delineation of pathways that appear to function upstream and downstream of the RARs. In this regard, the BMP pathway appears to function either upstream or in a separate pathway to the retinoid signaling pathway within the chondrogenic program, whereas the p38 MAPK pathway appears to operate downstream of RARs (Fig. 6). The approaches described in this review are being used alongside current technologies for expression profiling, namely microarray analysis, to further define the molecular pathways underlying cartilage formation.

Understanding the framework of molecular events underlying the chondrogenic program has clear benefits from a clinical perspective. First, the demonstration that RAR repression is a major requirement for chondrogenesis may enable us to overcome a major barrier to the treatment of diseases associated with cartilage loss, simply by mimicking the repression of RARs. In addition, beginning with our understanding of RAR function, we have uncovered the roles of various other factors during chondrogenesis, and we are beginning to assemble a model that explains the hierarchy of events directing chondroprogenitor differentiation. Knowledge of these events is critical for understanding the genetic disorders that cause skeletal malformations and for identifying putative therapeutic targets for the treatment of diseases such as osteoarthritis. ■

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