Regulation of Skeletal Progenitor Differentiation by the BMP and Retinoid Signaling Pathways

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Abstract. The generation of the paraxial skeleton requires that commitment and differentiation of skeletal progenitors is precisely coordinated during limb outgrowth. Several signaling molecules have been identified that are important in specifying the pattern of these skeletal primordia. Very little is known, however, about the mechanisms regulating the differentiation of limb mesenchyme into chondrocytes. Overexpression of RARα in transgenic animals interferes with chondrogenesis and leads to appendicular skeletal defects (Cash, D.E., C.B. Bock, K. Schughart, E. Linney, and T.M. Underhill. 1997. *J. Cell Biol.* 136:445–457). Further analysis of these animals shows that expression of the transgene in chondroprogenitors maintains a prechondrogenic phenotype and prevents chondroblast differentiation even in the presence of BMPs, which are known stimulators of cartilage formation. Moreover, an RAR antagonist accelerates chondroblast differentiation as demonstrated by the emergence of collagen type II–expressing cells much earlier than in control or BMP-treated cultures. Addition of Noggin to limb mesenchyme cultures inhibits cartilage formation and the appearance of precartilaginous condensations. In contrast, abrogation of retinoid signaling is sufficient to induce the expression of the chondroblastic phenotype in the presence of Noggin. These findings show that BMP and RAR-signaling pathways appear to operate independently to coordinate skeletal development, and that retinoid signaling can function in a BMP-independent manner to induce cartilage formation. Thus, retinoid signaling appears to play a novel and unexpected role in skeletogenesis by regulating the emergence of chondroblasts from skeletal progenitors.

Key words: retinoic acid • chondrogenesis • bone morphogenetic proteins • limb development • Noggin

Introduction

Patterning of the vertebrate limb bud relies on the cooperative action of several signaling centers. Signals have been identified that emanate from the apical ectodermal ridge, dorsal ectoderm and a region within the distal posterior margin termed the zone of polarizing activity (see Johnson and Tabin, 1997; Tickle and Eichele, 1994, and references therein). The mechanism by which these patterning cues influence mesenchyme commitment is thought to occur within the progress zone. Cells within the progress zone receive signals from all three signaling centers, integrating them into a positional identity that is translated into a cell identity. With respect to skeletal formation, these signals culminate in the commitment of mesenchymal cells to the chondrocytic lineage.

Bones within the limb are formed from a cartilage precursor that forms from condensed mesoderm. These condensations represent the earliest stages of limb patterning and are considered to be the forebears of the mature limb bones (Ede, 1983). After condensation, the mesodermal cells in the interior of each condensation differentiate into chondrocytes. This differentiation occurs in concert with limb outgrowth, such that proximal mesenchymal cells (close to the body wall) that are fated to become chondrocytes differentiate before more distal cells. The spatiotemporal regulation of mesenchyme differentiation into chondrocytes is a crucial step in endochondral bone formation in that it preserves the pattern of the bone primordia established earlier in limb development and provides a suitable matrix for subsequent ossification. Despite the importance of this stage in skeletal development, mechanisms that control mesenchyme differentiation into chondrocytes are poorly understood.

During limb outgrowth, signals that promote as well as

inhibit chondrogenesis are important determinants in limb ontogeny (Wolpert, 1990). Many molecules have been identified that promote chondrogenesis in vivo and in vitro. Some of these include members of the TGF-β superfamily, including the bone morphogenetic proteins (BMPs)[1], growth differentiation factors (GDF), and TGF-β-1, -2, and -3 (Kingsley, 1994; Hogan, 1996; Moses and Serra, 1996; Wozney and Rosen, 1998). Signaling molecules that may modulate skeletal development by inhibiting chondrogenesis include vitamin A and its metabolite, retinoic acid (RA; see Underhill and Weston, 1998, and references therein). Exposure of embryos to excess RA results in a range of defects depending not only on the dose of RA, but also on the timing of its administration (Shenefelt, 1972). For instance, when administered to embryonic age (E) 11.5 to E14.5 mouse embryos, large doses of RA cause limb defects (Kochhar, 1973; Kwasigroch and Kochhar, 1980). This period during which RA treatment has the most dramatic effects on limb formation coincides with the timing of chondrogenesis in the limb bud.

RA exerts most of its biological effects primarily through receptors belonging to one of two subfamilies of the steroid hormone family of nuclear receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs; Mangelsdorf et al., 1994; Chambon, 1996). Three subtypes (α, β, and γ) exist for both the RARs and RXRs, which are expressed in distinct and overlapping regions during limb development. Between E9.5 and E11.5, RARα and RARγ are expressed throughout the limb mesenchyme (Dolle et al., 1989; Ruberte et al., 1990). Beyond this stage, RARα is downregulated within the cartilaginous areas, while RARγ expression remains localized to these areas. RARα expression becomes restricted to the interdigital region (IDR) overlapping with RARβ expression at this time, and is also present in the perichondrium. In addition to being present in the IDR, RARβ is expressed in the interior, anterior, and posterior necrotic zones (Dolle et al., 1989; Mendelsohn et al., 1991). While null mutants of either RARα, RARβ, or RARγ exhibit no limb skeletal malformations (Lohnes et al., 1993; Lufkin et al., 1993; Luo et al., 1995; Ghyselinck et al., 1997), compound homozygous null alleles of RARα and RARγ exhibit a range of severe limb abnormalities from reductions to duplications (Lohnes et al., 1994) demonstrating an important function for the RARs in skeletal development.

To further explore RAR function in limb skeletogenesis, we used an existing transgenic line of mice that overexpresses a weak constitutively active RARα1 in the developing limb bud (Cash et al., 1997). These animals present a number of limb skeletal abnormalities including polydactyly, syndactyly, ectrodactyly, fibular deficiencies, and tarsal and carpal fusions. Previously, we showed that transgene-expressing cells do not differentiate into chondroblasts. Here we report that instead, these cells continue to express a prechondrogenic phenotype. Addition of BMP-2 or -4 to in vitro cultures of transgenic mesenchyme dramatically stimulates condensation of transgene-express-

ing mesenchymal cells but fails to induce their differentiation. In contrast, addition of an RARα-selective antagonist stimulates chondroblast differentiation, and is sufficient to induce cartilage formation when BMP signaling is repressed. Taken together, these results suggest that BMP and RAR signaling play an important function in regulating both the size and shape of the skeletal primordia. Maintenance and expansion of the prechondrogenic condensations is dependent upon activation of both signaling pathways, whereas the timing of chondroblast differentiation is regulated by RAR activity.

Materials and Methods

Preparation of Cultures from Limb Mesenchyme

Cultures were prepared from murine fore and hind limb buds of E11.25 to E11.75 embryos (obtained from breedings of homozygous male transgenic animals or C57Bl6F$_1$/J males with C57Bl6F$_1$/J females) as previously described with the following modifications (Cash et al., 1997). After proteolytic digestion, cells were filtered through a Cell Strainer (40 μm; Falcon) to obtain a single cell suspension. Culture media (40% Dulbecco's modified Eagle's medium and 60% F12 supplemented with fetal bovine serum to 10%; GIBCO BRL) was changed daily. BMP-2 or -4 (Genetics Institutes), AGN 194301 (Allergan Pharmaceuticals), and/or purified *Xenopus* Noggin protein was added to culture media at a concentration of 10 ng/ml, 1 μM, and 10 ng/ml, respectively. Addition/removal experiments included either adding or removing supplemented media on the indicated culture day. 24 h after culture initiation was considered day 1. To detect transgene-expressing cells, cultures were fixed and stained as previously described, with magenta-gal (BioSynth International Inc.) being substituted for X-gal. This was followed by alcian blue staining for cartilage-specific glycosaminoglycans (Lev and Spicer, 1964). Alcian blue staining of magenta-gal–stained cultures turned the red precipitate to a purple color, as a result of incubating magenta-gal–stained cells at pH 1. This double-staining technique enables transgene-expressing cells to be localized with respect to alcian blue–stained cartilage nodules. Images were captured using a Sony DXC-950 3CCD color video camera and analyzed using Northern Eclipse image analysis software (Empix Imaging, Inc.) and composite figures were generated in CorelDraw.

Synthesis of Riboprobes

Riboprobes were synthesized in the presence of UTP-digoxigenin with the appropriate RNA polymerase and linearized template DNA according to the manufacturer's directions (Roche Molecular Biochemicals). Riboprobe complementary to the *Col II* gene, was generated from BamH1 linearized pBluescript containing 1.1 kb of the c-propeptide encoding region of the *Col II* gene and transcribed in vitro with T7 RNA polymerase. *Gli-1* riboprobe was transcribed from Not1 linearized pBluescript containing a 1.6-kb fragment representing most of the zinc finger domain of *Gli-1*. A 553-bp fragment of murine *Col I* gene (Phillips et al., 1992) subcloned into pKS II (Stratagene) was linearized with Xho1 and transcribed with T7 RNA polymerase. A HindIII (bp position 605) -BamH1 (bp position 1252) fragment from the mouse *N-cad* cDNA was subcloned into pKSII. This construct was linearized with BamH1 and riboprobe synthesized with T7 RNA polymerase. A *Col II*A–specific probe was generated using RT-PCR on limb RNA with primers (5′ primer, 5′-GTGCTGCTGACGCT-GCTCAT and 3′ primer, 3′-ACCAGCCTTCTCGTCATACC) flanking exon 2 of the mouse *Col II* gene. A product of 207 bp was subcloned into pGEM-Teasy (Promega) and subsequently used to generate riboprobes. Control sense riboprobes were synthesized from the aforementioned plasmids.

Whole Mount In Situ Hybridization of Limb Mesenchyme Cultures

In situ hybridizations were carried out on cultures derived from limb mesenchyme using a technique described previously (Cash et al., 1997), with minor modifications. After permeabilization using 10 μg/ml proteinase-K in PBS supplemented with 0.05% Triton X-100, cells were post-fixed in

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4% paraformaldehyde and 2% glutaraldehyde in PBS, and hybridizations were carried out at 60°C instead of 55°C.

Transient Transfection Analysis

The ability of AGN 194301 to inhibit all trans-RA induction of an RARE-containing luciferase construct was performed in P19 embryonal carcinoma cells as previously described with some modification (Underhill et al., 1994). P19 cells were seeded at a density of 1.5×10^4 cells/well in 6-well plates. Cells were transfected using the calcium phosphate precipitation method with each well receiving 3.9 μg DNA (1.25 μg pW1RAREtk-lucif, 0.33 μg pW1ActRARα/β/γ, 0.67 μg pW1Actβ-galactosidase, and 1.65 μg pGEM9zf(−)). After transfection, cells were washed and fresh media were added that contained 1×10^{-7} M all trans-RA and various amounts of AGN 194301. 24 h later cell extracts were prepared and luciferase and β-galactosidase activity was measured. Luciferase activity was normalized with β-galactosidase activity to control for differences in transfection efficiency.

Northern Blot Analysis

Total limb bud RNA was isolated from pooled limb buds of wild-type and transgenic embryos at various gestational stages using TriPure Isolation Reagent (Roche Molecular Biochemicals). Total RNA from micromass cultures was extracted from cells pooled from 12 wells of a 24-well plate with TriPure Isolation Reagent. Cultures were established as described above. RNA samples were separated by electrophoresis of 15-μg aliquots on a 1% agarose-formaldehyde gel. RNA was then transferred to a Hybond-N nylon membrane (Amersham Life Science) and cross-linked by UV irradiation. Blots were prehybridized in Church's Buffer (7% SDS, 0.5 M NaPi pH 7.2, 1 mM EDTA, and 1% BSA) at 65°C for at least 30 min. Radiolabeled DNA probes were synthesized by random priming (Feinberg and Vogelstein, 1983) with the appropriate cDNA insert fragments. Hybridizations were carried out overnight at 60°C. After hybridization, blots were washed with wash buffer (250 mM NaPi, 10% SDS) three times for 15 min at 65°C and exposed to BioMax x-ray film at −80°C for 1–4 d.

Results

### Transgene-expressing Cells Do Not Contribute to Cartilage Nodules

RARα expression is normally downregulated during chondroblast differentiation in vitro (Cash et al., 1997) and in vivo (Dolle et al., 1989). The continued activity of RARα inhibits chondroblast differentiation leading to cessation of cartilage formation and to skeletal deficiencies that are reminiscent of those observed in RA teratogenicity. To examine the cell fate of transgene-expressing cells, limb mesenchyme from the fore and hind limbs of E11.5 transgenic embryos was used to set up high density primary limb bud cultures. Under these conditions, condensation and differentiation of limb mesenchyme to cartilage mimics those events occurring in vivo (Ahrens et al., 1977). Fig. 1 shows cartilage nodule formation at day 2 and 4 in wild-type (Fig. 1, a and b) and transgenic (Fig. 1, d and e) fore limb cultures. Consistent with previous observations, there were fewer alcian blue–stained nodules in the transgenic derived cultures than in the wild-type cultures. To precisely examine the distribution of transgene-expressing cells in cultures, a combination of magenta-gal and alcian blue staining was used to stain transgene-expressing cells and cartilage matrix, respectively. Transgene-expressing cells (purple stained) formed condensations but were mostly excluded from cartilage nodules (Fig. 1 f). These transgene-expressing condensations are still evident after 8 d in culture, suggesting that the transgene is not being downregulated during cartilage nodule formation. Fur-

thermore, by day 4 in hind limb cultures there is extensive transgene expression that almost completely precludes the formation of cartilage nodules (Fig. 1 c). This is consistent in vivo in that the transgene-expressing cells are not found within the cartilaginous elements (data not shown). In the transgenic embryos, fore limb expression of the transgene is restricted primarily to the posterior region, thus, only a portion of the cells in these cultures stain with magenta gal. In the hind limb, the transgene is expressed throughout the limb mesenchyme. This results in hind limb defects that are more dramatic than those observed in the fore limb (Cash et al., 1997) and coincides with an almost complete lack of cartilage formation in hind limb cultures (Fig. 1 c).

Transgene-expressing Cells Have a Prechondrogenic Phenotype

Cartilage formation involves two well-described steps: (1) condensation of mesenchymal cells, and (2) differentiation of condensed mesenchyme to matrix-producing chondrocytes (Hall and Miyake, 1992). Condensed prechondrogenic cells express *Col II* weakly and express *N-cad*, *Col I*, and *Gli-1* abundantly (Walterhouse et al., 1993; Oberlender and Tuan, 1994; Hall and Miyake, 1995; Marigo et al., 1996). Upon differentiation of these cells, *Col II* expression becomes much stronger, whereas expression of *N-cad*, *Col I*, and *Gli-1* is downregulated. To further characterize the phenotype of the transgene-expressing cells, whole mount in situ hybridization was carried out to examine the expression patterns of these genes in transgenic and wild-type cultures. In wild-type cultures, *Col II* expression is very strong and localized primarily to the core of the cartilage nodules with weaker expression in regions surrounding the nodules (Fig. 2 a). Expression of *N-cad*, *Col I*, and *Gli-1* in wild-type cultures is restricted to perinodular regions where condensed, undifferentiated mesenchyme is present, and is weakly expressed in the centre of the nodules (Fig. 2, c, e, and g). The expression patterns of these genes in the wild-type cultures is consistent with their expression in vivo. In transgenic cultures, however, their expression patterns indicate that various condensations in these cultures have been maintained instead of going on to form cartilage nodules. In several regions, *Col II* was only weakly expressed (Fig. 2 b), and instead of being downregulated, *N-cad*, *Col I*, and *Gli-1* were all expressed throughout the cultures (Fig. 2, d, f, and h), resembling a pattern one would expect to see in condensations but not in cartilage nodules. These observations suggest that the transgene-expressing cells condense but do not undergo chondroblast differentiation.

Comparison of the distribution of *Col II* expression in transgenic and wild-type cultures suggests that a similar number of condensations are formed in the transgenic cultures as compared with wild-type cultures (Fig. 2, a and b). Furthermore, the *Col II*–stained regions in transgenic and wild-type cultures were similar in size indicating that transgene-mediated inhibition of chondroblast differentiation was not a consequence of insufficient numbers of prechondrogenic cells (Hall and Miyake, 1992). Moreover, during the culture period the transgene-expressing cell aggregates continued to expand in size and intensity of ma-
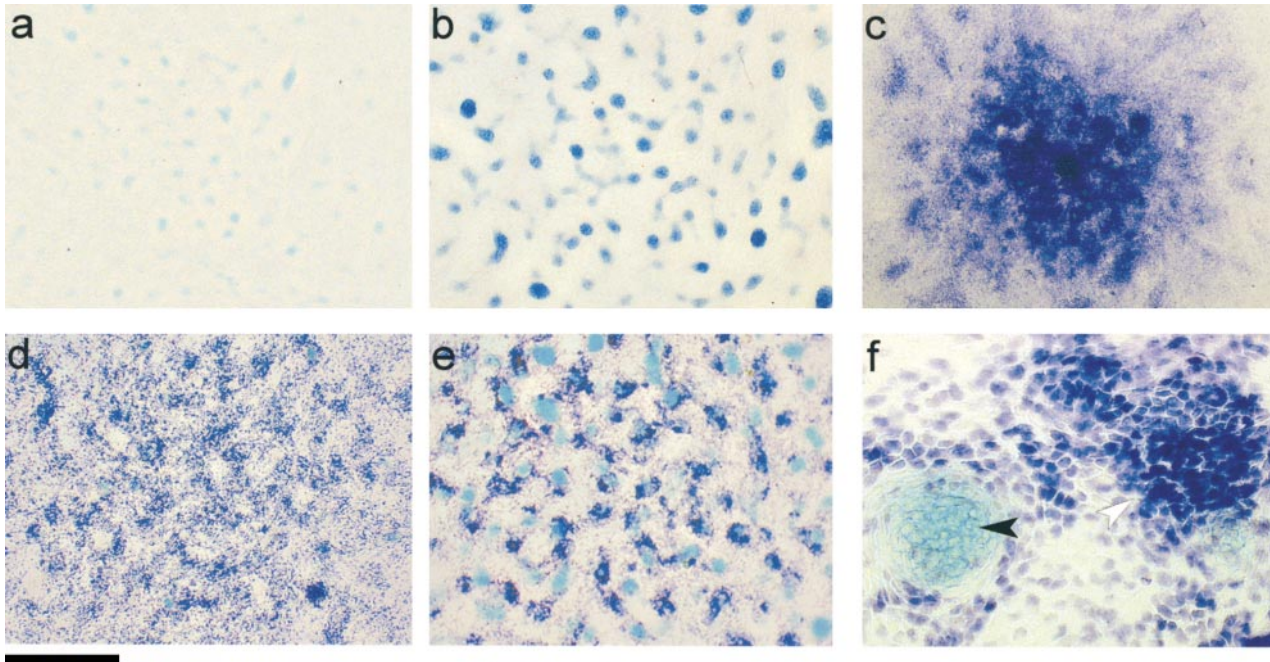
Figure 1. Transgene-expressing cells are excluded from cartilage nodules. (a and b) Wild-type fore limb cultures were fixed and stained with alcian blue on days 2 and 4. (d and e) Transgenic fore limb cultures were stained with magental-gal on days 2 and 4, followed by alcian blue staining. (c) Transgenic hind limb cultures were stained with magenta-gal on day 4, followed by alcian blue staining. (f) Higher magnification of day 4 transgenic fore limb cultures. Transgene-expressing cells condense (white arrow), but are excluded from the cartilage nodules (black arrow). Bar: (a, b, d, and e) 1 mm; (c and f) 0.2 mm.

genta-gal staining. Northern blot analysis confirmed differential expression of *Col I* and *Col II* between the hind limbs of wild-type and transgenic mice (Fig. 2, i and j). At E13.5, there is a higher level of *Col I* expression in transgenic hind limbs compared with wild-type hind limbs (Fig. 2 j). In contrast, at E 14.5, *Col II* expression is much lower in transgenic hind limbs in comparison to wild-type hind limbs (Fig. 2 i). The reduced expression of *Col II* in transgenic hind limbs is likely a reflection of the decreased size of cartilaginous elements that form and the reduced expression of the chondrogenic phenotype. Similarly, the elevated *Col I* expression in the transgenic hind limbs at E13.5 suggests a delay in chondroblast differentiation. The in situ hybridization results combined with results from Northern blot analysis strongly suggest that the phenotype displayed by transgene-expressing cells in vitro and in vivo is that of condensed prechondrogenic cells.

Transgene-expressing Cells Are Refractile to BMP-stimulated Chondroblast Differentiation

To further investigate the mechanism of transgene-mediated inhibition of chondroblast differentiation, we examined the expression of genes known to be important in chondrogenesis, namely the BMPs. Based on their expression in limb development and their well-defined chondrogenic stimulatory properties, we have focused our analysis on *Bmps-2* and *-4*. One plausible explanation for the transgenic phenotype is either the reduced expression of *Bmps* or overexpression of *Noggin* which encodes a BMP inhibitor. Northern analysis using mRNA from hind limbs of

transgenic and wild-type animals at E 11.5, 12.5, and E 14.5 demonstrated that neither *Bmp-2* nor *Noggin* were differentially expressed in transgenic animals in comparison to wild-type animals (data not shown). The defect in transgenic limb mesenchyme, therefore, is not likely a result of changes in expression of either of these two transcripts. However, these results do not preclude the possibility that transgene-expressing mesenchymal cells are unresponsive to BMPs.

To evaluate whether transgene-expressing cells are able to respond to BMPs, we treated transgenic and wild-type limb mesenchymal cultures with 10 ng/ml BMP-2 and -4. Similar to previous reports, we demonstrated that addition of BMP-2 (Fig. 3 a) and -4 (data not shown) dramatically increased the number of cartilage nodules in wild-type cultures. BMP-2 treatment for 6 d increased the number of cartilage nodules by ~125% and ~115% in wild-type and transgenic fore limb cultures, respectively (Fig. 3 a). To determine if BMP-2 completely rescues cartilage formation of transgene-expressing cells, treated cultures were stained with magenta-gal followed by alcian blue (Fig. 3, e and f). As observed in nontreated transgenic cultures, few if any transgene-expressing cells were found to be present within the alcian blue–stained cartilage nodules (Fig. 3 g). Addition of BMP-2, however, stimulated condensation of transgene-expressing cells as seen by magenta-gal staining (Fig. 3, e–g), and by the expression pattern of *Col II* in transgenic cultures (Fig. 3, j and k). In response to BMP-2, condensations of transgene-expressing cells increased in size (compare Fig. 1 e with 3 f), this is also apparent in the *Col II*–expressing condensations (Fig. 3, j and k). BMP-2
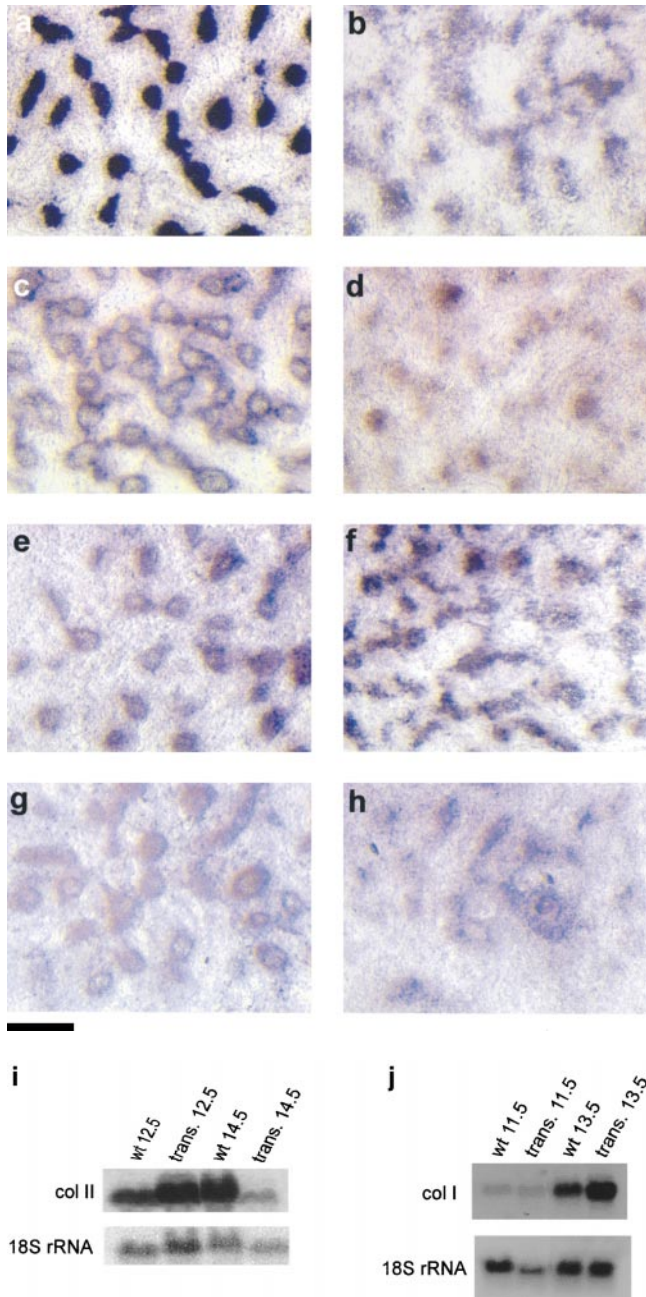
Figure 2. Transgene-expressing cells have a prechondrogenic phenotype. (a–h) Whole mount in situ hybridization of day 4 wild-type and transgenic cultures was carried out using probes characteristic of the precartilage stage (*N-cad*, *Col I*, and *Gli-1*) and chondroblast stage (*col II*). (a) *Col II* expression is abundantly expressed within discrete nodules in wild-type cultures, with weaker staining evident in the condensing mesenchyme surrounding the nodules. (b) In transgenic cultures *Col II* is weakly expressed within precartilaginous condensations, and has a more widespread distribution. (c, e, and g) In wild-type cultures, *N-cad*, *Gli-1*, and *Col I* are found predominantly in perinodular regions in condensing mesenchymal cells with less expression in the core of the nodules. (d, f, and h) In transgenic cultures, *N-cad*, *Gli-1*, and *Col I* are expressed throughout the cultures in precartilaginous condensations in a pattern similar to that of *Col II*. Bar, 1 mm. (i) Comparison of *Col II* expression in wild-type and transgenic hind limb buds indicates that *Col II* expression in wild-type and transgenic animals is similar at E11.5. At a later stage of hind limb de-

addition stimulated the formation of *Col II*–expressing nodules in wild-type cultures (Fig. 3 i), while stimulating the formation of condensations expressing *Col II* weakly in transgenic cultures (Fig. 3 k). In the transgenic cultures, condensations were observed as early as 2 d in culture and were still evident after 6 d in culture, whereas in wild-type cultures, there were no precartilaginous condensations visible by day 6, as almost all condensed cells had differentiated by that time. Thus, although BMP-2 or -4 induces transgene-expressing cells to form precartilaginous condensations they are not sufficient to overcome transgene-mediated inhibition of chondroblast differentiation.

Inhibition of RARα Stimulates Chondrogenesis

We have demonstrated that continued RARα activity inhibits the transition of prechondrogenic cells to chondroblasts. Consequently, we would expect that the abrogation of RARα activity would stimulate chondroblast differentiation and/or cartilage formation. RAR activity as used herein refers to the level of RA-induced transcriptional activity of the RARs. To examine the possibility that inhibition of RARα activity stimulates cartilage formation, we treated wild-type cultures with the RARα-specific antagonist AGN 194301 (Teng et al., 1997). At the concentrations of AGN 194301 (1 μM) used in these experiments, RARα signaling was inhibited to ∼0.3% of 100 nM all trans-RA–treated controls, while RARβ and RARγ were inhibited to ∼18% and ∼26% of controls, respectively (Fig. 4). Addition of 1 μM AGN 194301 to wild-type cultures lead to a dramatic increase in nodule number as compared with untreated control cultures (Fig. 5, g, j, and m). After 8 d in culture there were 60% more nodules in antagonist treated cultures than in untreated cultures (Fig. 5 m). Previous studies showed that treatment of micromass cultures with anti-sense oligonucleotides to the RARs increased cartilage nodule number (Motoyama and Eto, 1994; Jiang et al., 1995), however, an RAR inhibitor to all three RARs had no effect on cartilage nodule formation (Kochhar et al., 1998). Most of the chondrogenic stimulatory properties of the antagonist appear to be mediated through inhibition of RARα, however, it cannot be entirely discounted that diminution of RARβ or RARγ signaling may have contributed to these results. Nonetheless, loss of RAR activity stimulates cartilage formation while continued RAR activity inhibits cartilage formation.

The RARα Antagonist and BMP-2 Have Different Chondrogenic Stimulatory Properties

The loss of RAR activity and addition of BMP-2 and -4 both stimulate cartilage formation. In transgenic cultures, BMP-2/-4 stimulated condensation but not differentiation of

velopment (E14.5) that is accompanied by extensive extracellular matrix deposition, *Col II* expression increases in wild-type limb buds and decreases in transgenic limb buds. (j) Examination of *Col I* expression in wild-type and transgenic hind limbs shows that *Col I* expression is elevated in transgenic hind limb buds in comparison to wild-type hind limb buds at E13.5. Blots were reprobed with an 18S rRNA to control for differences in loading and transfer.

transgene-expressing cells, whereas a loss of RARα activity induced cartilage formation. To further delineate the role of BMP-2 and RARα in cartilage formation, we used an approach that involves incubation of wild-type cultures with AGN 194301 or BMP-2 for different periods of time during culturing. To accomplish this, BMP-2 or AGN-194301 were added to cultures for the first 2 or 3 d then removed, or were added after 2 or 3 d of culturing. BMP-2 is equally effective at stimulating cartilage formation when present during the entire culture period or only after 2–3 d after culture initiation (Fig. 5, d–f). Conversely, addition of BMP-2 for the first 2 to 3 d of culture caused an increase in nodule number but this increase was not as dramatic as that observed upon adding BMP-2 later (i.e., after 2 or 3 d), and was not sustained. The size of the nodules was also noticeably increased in cultures that were either continu-

ously exposed to BMP-2 (Fig. 5 d) or treated after 2 or 3 d of culture initiation (Fig. 5, e and f). These large nodules radiate towards the outside of the culture and are, in part, a consequence of the recruitment of proliferating uncommitted cells present in the periphery of the culture, into the nodules (Duprez et al., 1996b). Hence, the consequences of BMP-2 addition are much more pronounced in cultures treated at later stages of culture and likely reflect the ability of BMP-2 to enhance commitment of mesenchymal cells to the chondrocytic lineage with subsequent recruitment into nodules.

In contrast to BMP-2, the response to AGN 194301 was more pronounced when it was present early in the culture period. When 1 μM AGN 194301 was added to cultures for only the first 2 or 3 d, then removed, more cartilage nodules were formed compared with untreated cultures (Fig. 5, g–i) or to cultures exposed for longer periods of time but untreated for the first 2 or 3 d (Fig. 5, k and l). Interestingly, the increase in nodule number caused by early treatment of the antagonist was maintained even after six or more days of its removal and was comparable to the increase in nodule number observed with continuous treatment (Fig. 5, h–j). Thus, AGN 194301 caused an increase in nodule formation, however the nodules were ∼51% smaller when compared with control cultures, but were ∼22% larger in cultures receiving antagonist at later culture times. When BMP-2 and AGN 194301 were added together to wild-type cultures for 8 d at a concentration of 10 ng/ml and 1 μM, respectively, there was a ∼100% increase in nodule number compared with untreated controls (Fig. 5 m). BMP-2 addition alone to cultures initiated from the same limb buds caused a ∼45% increase, whereas AGN
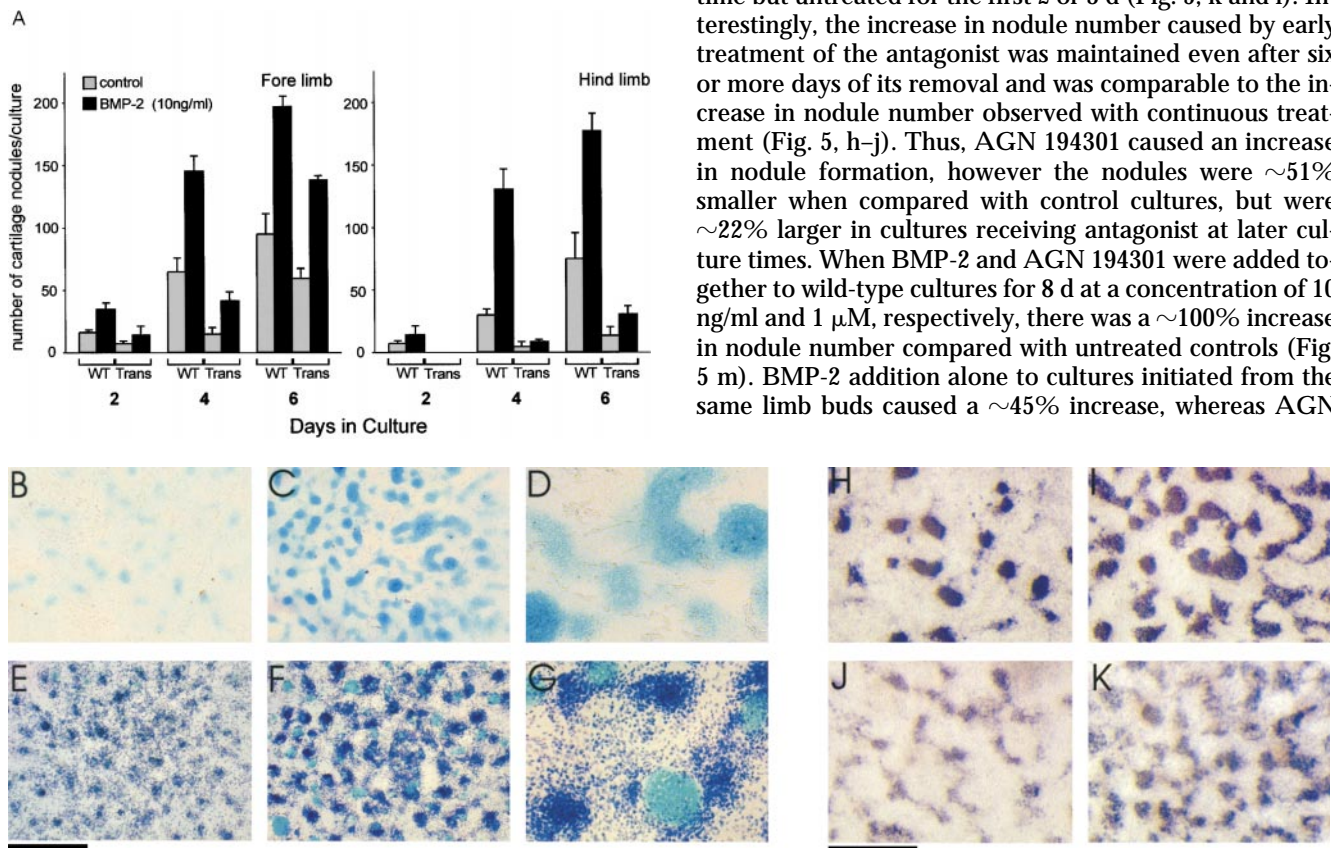
Figure 3. Transgene-expressing cells condense but do not differentiate into chondroblasts in response to BMP-2. (a) Cartilage nodule formation in BMP-2–treated and untreated, wild-type and transgenic cultures. Addition of BMP-2 (10 ng/ml) stimulates cartilage nodule formation in all cultures in comparison to untreated cultures, with the exception of day 2 transgenic hind limb cultures. Within all treatment groups there are fewer cartilage nodules in the transgenic cultures as compared with the corresponding wild-type culture. (b and c) Wild-type cultures treated with BMP-2 were stained with alcian blue at days 2 and 4. In response to BMP-2, there is a noticeable increase in nodule number and in nodule size (compare to Fig. 1, a–b). (d) Higher magnification of day 4 wild-type cultures treated with BMP-2. (e–f) Transgenic cultures treated with BMP-2 were stained with magenta-gal at days 2 and 4 followed by alcian blue staining. In response to BMP-2 there is an increase in nodule number and in nodule size. BMP-2 treatment also enhanced the formation and expansion of transgene-expressing precartilaginous condensations. (g) Higher magnification of day 4 transgenic cultures treated with BMP-2. BMP-2 stimulates condensation of transgene-expressing cells, however, similar to that observed in untreated cultures (Fig. 1 f), transgene-expressing cells are mostly excluded from the nodules. (h and j) Analysis of *Col II* expression in wild-type and transgenic day 4 cultures, respectively, with whole mount in situ hybridization. In wild-type cultures *Col II* is abundantly expressed within cartilage nodules, whereas expression is much weaker and less distinct in transgenic cultures. (i) Addition of BMP-2 to wild-type cultures increases the number of cartilage nodules that abundantly express *Col II*. (k) In transgenic cultures, treatment with BMP-2 results in more extensive *Col II* expression, with most of the increase in expression present in weaker staining precartilaginous condensations. A few regions exhibit higher levels of expression consistent with nodule formation. Bars: (b, c, e, and f) 1 mm; (d and g) 0.2 mm; (h–k) 0.5 mm.
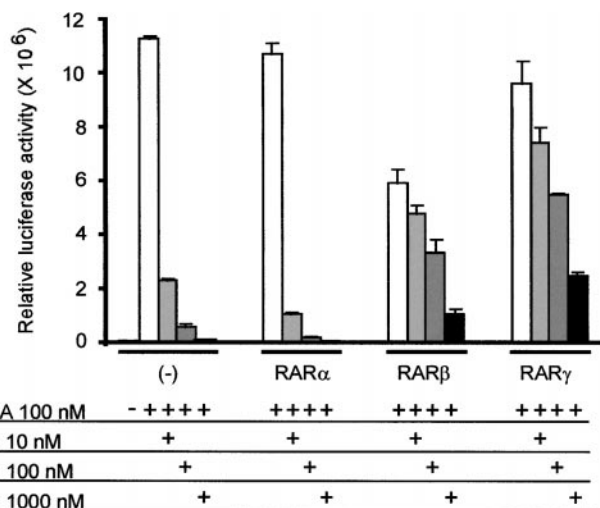
Figure 4. The RARα-selective antagonist AGN 194301 inhibits stimulation of an RARE-reporter gene by all-trans RA in a dose-dependent manner. Low concentrations of AGN 194301 selectively inhibit RARα-mediated transactivation of the reporter gene in cultures cotransfected with an RARα expression construct. At higher concentrations of AGN 194301, the compound exhibits less selectivity, in that there is also decreased reporter gene activity in cultures cotransfected with RARβ or RARγ expression plasmids.

194301 treatment caused an increase of ∼60% (Fig. 5 m). In contrast to antagonist-treated cultures, BMP-2 treatment increased nodule size by approximately eightfold over that of untreated cultures. In accordance with these results, an increase in *Col II* expression was observed in response to treatment of cultures with either BMP-2 or AGN 194301 for 4 d (Fig. 5 n), with the effect of BMP-2 being more pronounced. In addition, *Col I* expression was decreased in BMP-2– and AGN 194301–treated cultures in comparison to untreated cultures, albeit to a greater extent in AGN 194301–treated cultures. Together, these results suggest that the antagonist was operating to stimulate chondroblast differentiation.

To further define the mechanism whereby cartilage formation is enhanced by the antagonist, the ability of the AGN 194301 to stimulate *Col II* expression in wild-type cultures was assayed 24 h after culture initiation. There was little to no detectable expression of *Col II* in untreated cultures this early in the culture period (Fig. 5 o). Addition of the antagonist, however, was sufficient to induce intense *Col II* expression suggesting the presence of chondroblasts (Fig. 5 q). In cultures treated with BMP-2, *Col II* expression induced after 24 h was weak in comparison to the antagonist-treated cultures, but was localized to distinct regions indicative of precartilaginous condensations (Fig. 5 p). Together, these results suggest that although loss of RAR activity and the presence of a BMP signal augment cartilage formation, they appear to do so through distinct mechanisms.

### Cartilage Formation Is Rescued in Noggin-treated Cultures by Addition of an RARα Antagonist

Previous experiments showed that the antagonist was able to stimulate chondroblast differentiation before that observed with BMP treatment. To further address the requirement for BMPs in chondrogenesis, we treated wild-type limb mesenchyme cultures with Noggin, a secreted inhibitor of BMP-2, and -4. Addition of 10 ng/ml Noggin to wild-type cultures dramatically reduced cartilage nodule formation by ∼84% in comparison to untreated controls (Fig. 6, a, b, and d). In an attempt to define the chondrogenic stage affected by Noggin, cultures treated with Noggin were examined for the expression of *Col II* and *Col IIA*, an alternatively spliced form of *Col II* expressed in prechondrogenic cells (Sandell et al., 1991, 1994). In limb bud–derived cultures *Col IIA* is expressed in the condensing mesenchyme surrounding cartilaginous nodules and in precartilaginous condensations (Fig. 6, e and f). If Noggin delays or inhibits chondroblast differentiation but does not affect condensation, then the decrease in *Col II*–expressing nodules should be accounted for by *Col II*A–expressing condensations. In contrast, however, comparison of expression of these two genes by in situ hybridization in Noggin-treated cultures indicates there are no additional condensations present (Fig. 6, e and f). Hence, Noggin appears to interfere with formation of precartilaginous condensations.

Earlier experiments in which addition of BMP-2 was unable to rescue cartilage in transgene-expressing cultures coupled with the accelerated appearance of *Col II*–expressing cells in antagonist-treated cultures suggests that loss of RAR activity alone is sufficient to initiate the chondrogenic differentiation program. To test this, Noggin-treated cultures were exposed to the RAR antagonist. The addition of 1 μM AGN 194301 to Noggin-treated cultures, stimulated cartilage nodule formation such that these cultures resembled untreated cultures (Fig. 6, a, c, and d). In contrast, addition of 10 or 20 nM all trans-RA further diminished nodule formation in Noggin-treated cultures (data not shown). These results indicate that suppression of RAR–mediated signaling not only stimulates expression of the chondrogenic phenotype, but can do so independently of a BMP signal.

Discussion

The appropriate timing of chondroblast differentiation is critical to proper formation of the appendicular skeleton. We have demonstrated that the status of RAR activity determines when prechondrogenic cells in the limb differentiate into chondroblasts. Continued expression of an RARα transgene maintains the prechondrogenic phenotype of condensed mesenchyme and prevents their differentiation, whereas cessation of RAR-mediated signaling induces chondroblast differentiation. The importance of RARs in this process is further exemplified by the observations that addition of BMP-2 or -4 is not sufficient to initiate chondroblast differentiation of transgene-expressing cells. Furthermore, addition of an RAR antagonist results in cartilage formation in the presence of Noggin, a BMP inhibitor. Thus, BMP- and RAR-mediated signals have distinct roles that together coordinate the growth and differentiation of skeletal progenitors.
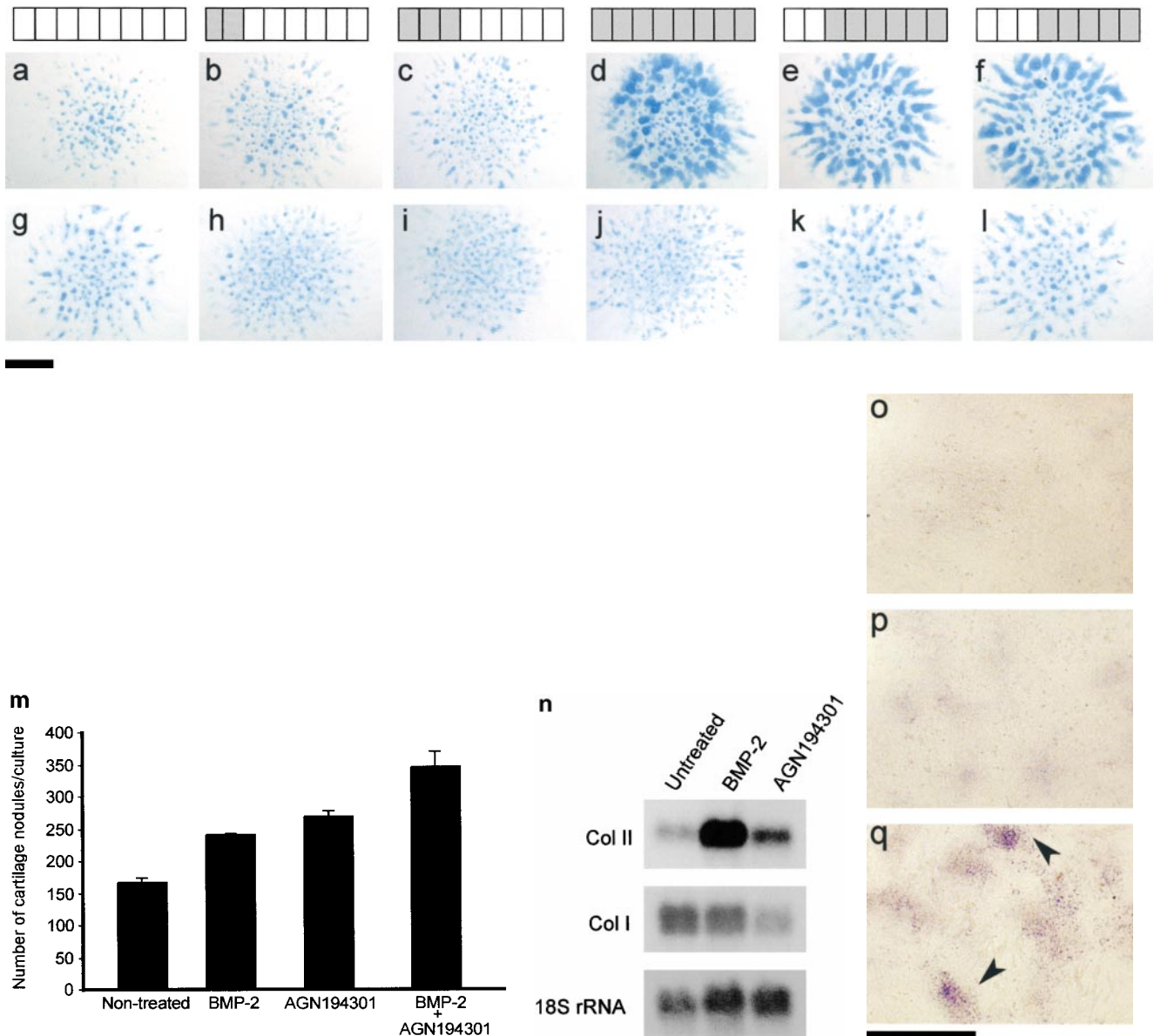
Figure 5. BMP-2 and AGN 194301 stimulate chondrogenesis at different stages of the chondrogenic sequence. (a) Untreated wild-type cultures were stained with alcian blue on day 8. (b and c) Wild-type cultures were treated with BMP-2 (10 ng/ml) for the first 2 and 3 d of culture, respectively, and stained with alcian blue on day 8. This treatment regiment results in a small increase in nodule number. (d) Wild-type cultures were treated continuously with BMP-2 (10 ng/ml) and stained with alcian blue on day 8. (e and f) Wild-type cultures were treated after 2 or 3 d, respectively, and stained with alcian blue on day 8. These cultures resemble cultures that were treated continuously with BMP-2, in that there is a substantial increase in both nodule number and size. (g) Untreated wild-type cultures were stained with alcian blue on day 8. (h and i) Wild-type cultures were treated with AGN 194301 (1 μM) for the first 2 and 3 d of culture, respectively, and stained with alcian blue on day 8. These cultures resemble cultures treated continuously with AGN 194301 (j) in that there are significantly more nodules than in control cultures. (j) Wild-type cultures were treated continuously with AGN 194301 and stained with alcian blue on day 8. (k and l) Wild-type cultures were treated with AGN 194301 after two or three days respectively and stained with alcian blue at day 8. There are fewer cartilage nodules compared with cultures treated continuously, but they are larger. Shaded boxes above the figure panels represent the days cells were treated during the 8-d culture period. (m) Quantification of cartilage nodule formation in response to BMP-2 and AGN 194301. In response to BMP-2, or AGN 194301 alone, there is a significant increase in the number of cartilage nodules over control cultures. Simultaneous addition of BMP-2 and AGN 194301 leads to a further increase in the number of nodules that form. (n) Analysis of *Col I* and *Col II* expression in day 4 wild-type cultures treated with AGN 194301 (1 μM) or BMP-2 (10 ng/ml) compared with untreated controls. Both treatments were found to increase *Col II* expression and to decrease *Col I* expression to varying degrees. The blots were rehybridized with an 18S rRNA probe to control for differences in RNA abundance. (o–q) Analysis of *Col II* mRNA expression in wild-type cultures after a 24-h treatment period. (o) *Col II* expression is not detectable in untreated cultures. (p) In BMP-2 (10 ng/ml)–treated cultures, *Col II* is weakly expressed. (q) In cultures treated with AGN 194301 (1 μM) there is much more extensive *Col II* expression than in control or BMP-2–treated cultures, with a few distinct regions of more intense *Col II* expression (arrowheads) being present that are not observed in control or BMP-2–treated cultures. Bars: (a–l) 1 mm; (n–p) 0.5.
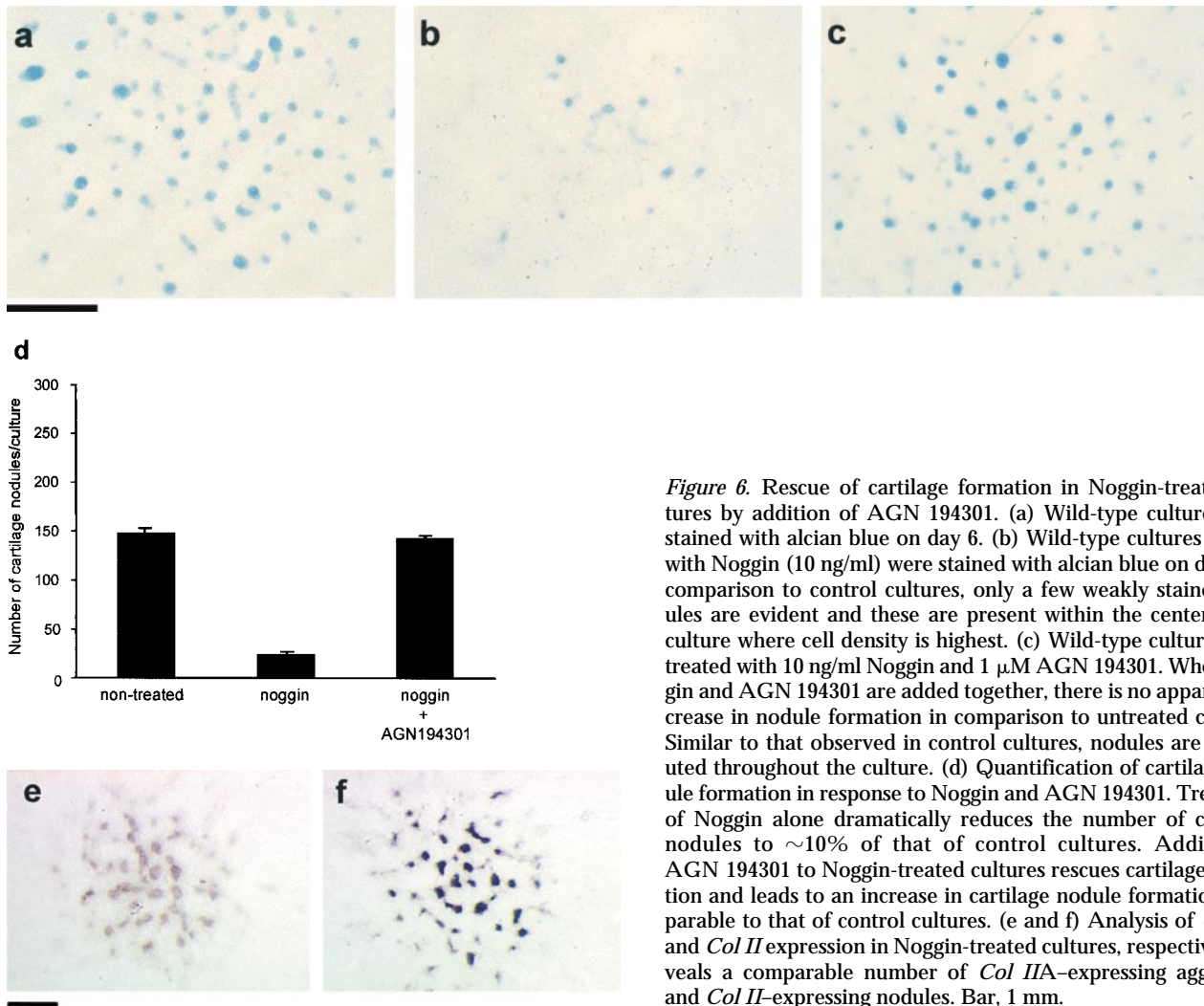
Figure 6. Rescue of cartilage formation in Noggin-treated cultures by addition of AGN 194301. (a) Wild-type cultures were stained with alcian blue on day 6. (b) Wild-type cultures treated with Noggin (10 ng/ml) were stained with alcian blue on day 6. In comparison to control cultures, only a few weakly stained nodules are evident and these are present within the center of the culture where cell density is highest. (c) Wild-type cultures were treated with 10 ng/ml Noggin and 1 μM AGN 194301. When Noggin and AGN 194301 are added together, there is no apparent decrease in nodule formation in comparison to untreated cultures. Similar to that observed in control cultures, nodules are distributed throughout the culture. (d) Quantification of cartilage nodule formation in response to Noggin and AGN 194301. Treatment of Noggin alone dramatically reduces the number of cartilage nodules to ~10% of that of control cultures. Addition of AGN 194301 to Noggin-treated cultures rescues cartilage formation and leads to an increase in cartilage nodule formation comparable to that of control cultures. (e and f) Analysis of *Col II*A and *Col II* expression in Noggin-treated cultures, respectively, reveals a comparable number of *Col II*A–expressing aggregates and *Col II*–expressing nodules. Bar, 1 mm.

Function of RARs in Skeletal Development

Manipulation of the RA signaling cascade during skeletal morphogenesis leads to a number of characteristic malformations. The developing skeleton is especially sensitive to RA, as embryos exposed to RA between E10.5 and E14 present with a number of limb malformations, including skeletal elements that are missing, fused, thickened, or reduced in size (Kochhar, 1973; Kwasigroch and Kochhar, 1980). While RARα and γ single null mutants do not have appendicular defects (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Subbarayan et al., 1997), compound mutant mice exhibit a number of defects (Lohnes et al., 1994). Overexpression of a weak constitutively active RARα in the limbs also contributes to the development of skeletal defects, with severity being proportional to the level of transgene expression (Cash et al., 1997). These manipulations of either RA or its receptors modify formation of the skeleton by affecting chondrogenesis, however, the molecular and cellular basis of these effects is not well understood.

To better understand the function of the RARs in chondrogenesis the aforementioned transgenic mice were further investigated. Transgene-expressing cells have a pre-chondrogenic phenotype, demonstrated by expression of *N-cad*, *Gli-1*, *Col I*, and weak *Col II*, a pattern of expression that is seen in precartilaginous condensations and the perichondrium. The normal expression pattern of RARα in the developing limb is consistent with its absence being essential for the transition from a chondroprogenitor to a chondroblast. RARα is expressed in the prechondrogenic condensations, the perichondrium, and in the interdigital region, but is downregulated in newly formed cartilaginous elements in vivo (Dolle et al., 1989) and cartilages in vitro (Cash et al., 1997). Moreover, the abrogation of RARα through the use of a specific antagonist stimulates cartilage formation in vitro. Taken together, these results suggest that a loss of RARα activity regulates the expression of the chondroblastic phenotype, and hence is a pivotal step in coordinating the timing of chondroblast differentiation. Downregulation of RAR activity during chondroblast differentiation is accompanied by an increase in the expression of RA 4-hydroxylase (referred to as P450RA) in precartilaginous condensations (de Roos et al., 1999), which catalyzes 4-hydroxylation of RA (White et al., 1996, 1997). In this manner, downregulation of RARα combined with increased degradation of RA

would result in an even greater decrease in RAR activity and the subsequent stimulation of chondroblast differentiation.

RARα has an important role in chondroblast differentiation, however, the involvement of RARγ cannot be precluded. In contrast to RARα, RARγ expression is upregulated during chondroblast differentiation (Cash et al., 1997; Dolle et al., 1989). RARα and RARγ null mutants do not exhibit appendicular skeletal defects, whereas limb skeletal abnormalities are observed in RARα−/− γ−/− embryos (Lohnes et al., 1994). Thus, under certain circumstances RARγ is able to, in part, compensate for the absence of RARα and vice versa, the skeletal defects in the RARα/γ null mutants can be rescued, for the most part by, a single allele of RARα2 (Lohnes et al., 1994).
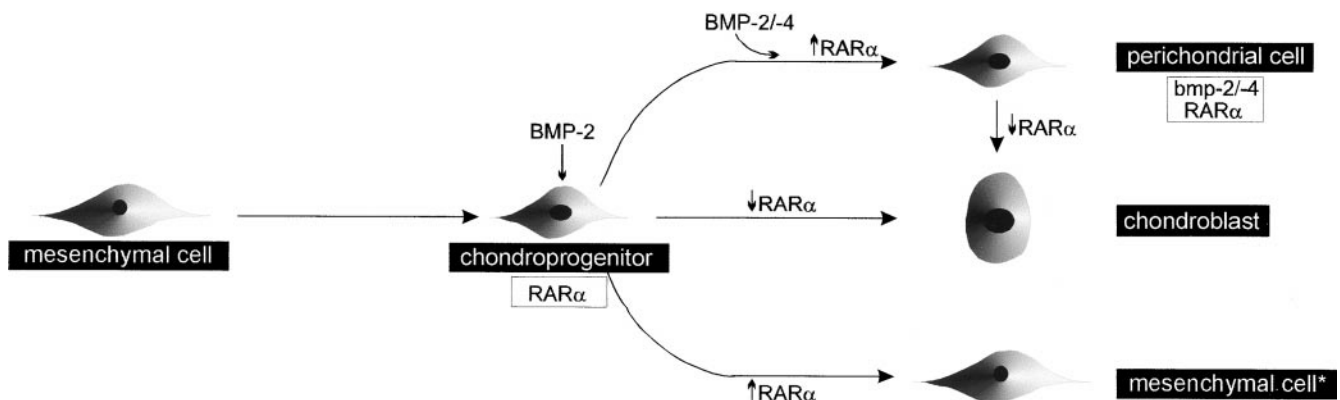
BMP and RAR Signaling in Limb Skeletal Development

RARα and RARγ are expressed in condensed mesenchyme and perichondrial cells, which are both sites of action of BMP-2 and BMP-2/4, respectively (Jones et al., 1991; Duprez et al., 1996a; Rosen et al., 1996; Macias et al., 1997). Support for the importance of BMP signaling in cartilage formation comes from studies using dominant-negative or constitutively active BMP type IB receptors in vitro and in vivo to inhibit and stimulate chondrogenesis, respectively (Kawakami et al., 1996; Zou et al., 1997). Furthermore, loss or gain of function studies with Noggin, an inhibitor of BMP-2 and -4 (Zimmerman et al., 1996), have shown that BMP-2 and -4 are important in skeletal development and that regulation of BMP signaling is required for delineation of the various skeletal elements (Brunet et al., 1998; Capdevila and Johnson, 1998; Pizette and Niswander, 1999). BMP signaling may function in the specification of mesenchyme to the chondrocytic lineage, expansion of prechondrogenic cell populations and/or chondroblast differentiation. Transgene expression inhibits chondroblast differentiation, even in the presence of BMP-2 or -4. Both BMP-2 and -4 enhance the expression of the prechondrogenic phenotype by stimulating the condensation of transgene-expressing cells as confirmed by magenta-gal staining and by in situ hybridization with probes for *Col II* (Fig. 3, j and k), *N-cad*, *Gli-1* and *Col I* (data not shown). This shows that BMPs are able to stimulate expansion of prechondrogenic cell populations, but, are unable to circumvent the requirement for abrogation of RAR activity in chondroblast differentiation.

As described herein, exogenous added Noggin inhibits cartilage formation of limb mesenchyme in culture. The decrease in nodule formation observed in response to Noggin appears to be due to reduced formation of precartilaginous condensations. While Noggin may not bind to all BMP/GDF family members it appears to interact with those BMP species important in the early stages of skeletal development within the limb. These results suggest that BMPs have an important function early on in chondrogenesis at the condensation stage or preceding it. Manipulation of the retinoid signaling pathway is important in regulating chondroblast differentiation. Premature chondroblast differentiation is induced by the antagonist after only 24 h of culture, before that observed with BMP addition. Hence, both BMP and retinoid signaling pathways make fundamental contributions to skeletal development. BMP signaling is required early in skeletal development for the establishment and possibly maintenance of cells in precartilaginous condensations, while retinoid signaling acts later to control their differentiation.

The proposed role for RAR-signaling in chondroblast differentiation can account for the skeletal defects observed upon their manipulation in animals. Loss of RAR signaling is expected to cause precocious differentiation of skeletal progenitors and lead to a reduction in size or complete absence of skeletal elements as observed in some compound RAR and RAR/RXR mutant animals. If differentiation occurs before prechondrogenic populations reach a critical size, then the resultant cartilage elements would not form or would be reduced in size (Hall and Miyake, 1992). This is also consistent with the smaller, hypoplastic nodules observed herein in limb mesenchyme cultures in response to abrogation of RAR signaling. Conversely, inappropriate activation of the RAR signaling pathway would inhibit cartilage formation by prevent-

Figure 7. Model for the role of the BMP- and RA-signaling pathways in limb skeletal development. Mesenchymal cells become committed to a chondrogenic cell fate by signals that are not well defined. Stabilization and expansion of this committed cell population appears to require the action of the BMPs. The availability of BMPs along with RA is important for the maintenance of prechondrogenic cell populations present in precartilaginous condensations and the perichondrium. Loss of RAR activity induces skeletal progenitor differentiation and emergence of the chondroblast phenotype. In the absence of BMP signals, chondroprogenitors that continue RARα expression loose their prechondrogenic phenotype. Genes within boxes represent their expression in those cell types in vivo.

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ing chondroblast differentiation, thereby resulting in a loss or reduction in the size of the skeletal elements as observed in RA teratogenicity or in transgenic animals overexpressing a weak constitutively active RARα.

Diminution of RAR activity was sufficient to stimulate cartilage formation in the presence of Noggin. Noggin blocks cartilage formation at the condensation stage or earlier, suggesting that attenuation of retinoid signaling not only stimulates chondroblast differentiation, but may also be sufficient to initiate the chondrogenic sequence in a BMP-independent manner. This possibility is supported by the presence of ectopic cartilages at a number of sites in RARα−/− γ−/− mice. In these animals cartilages appear in the meninges, peritoneum, diaphragm, and at the base of the semi-lunar cusps in the heart (Lohnes et al., 1994; Mendelsohn et al., 1994). In addition, loss of RAR activity can lead to the formation of interdigital cartilages as observed in some RARα/γ compound knockouts (Lohnes et al., 1994) and by application of an RAR antagonist to the interdigital region (Rodriquez-Leon et al., 1999). Hence, this suggests that, in some instances, cartilage formation can proceed, through a BMP-independent mechanism.

A proposed model for the action of BMP and RAR signaling in early limb skeletogenesis, those stages involved with establishment of chondrogenic elements, is illustrated in Fig. 7. During limb outgrowth chondroprogenitors do not spontaneously differentiate into chondroblasts. Instead, their differentiation is temporally regulated during limb bud outgrowth, such that proximal cells within the limb bud differentiate before distal cells. Similarly, appositional growth of the cartilages is mediated by the spatiotemporal differentiation of perichondrial cells to chondrocytes (Bairati et al., 1996). RAR activity in conjunction with a BMP signal in prechondrogenic condensations and the perichondrium in vivo are required for the expression of the prechondrogenic phenotype. The maintenance of a population of prechondrogenic cells such as those in the perichondrium may therefore be attributed to the continued activity of these gene products in this region (von Schroeder and Heersche, 1998; Koyama et al., 1999). Loss of RAR activity in limb precartilaginous condensations or perichondrial cells leads to their differentiation, contributing to longitudinal and appositional cartilage growth, respectively.

RAR and BMP signaling may act in a similar manner to coordinate other developmental processes. During heart formation BMP-2 and -4 appear to be important in specifying ventricular cardiomyocytes (Schultheiss et al., 1997), whereas the RARs regulate the differentiation of this population of cells. Loss of RAR activity through inactivation of RARα, along with either RXRα, RXRβ, or the activation function 2 of RXRα causes precocious differentiation of ventricular myocytes leading to a hypoplastic ventriculum (Kastner et al., 1997; Mascrez et al., 1998). Thus, an important function for the loss of a retinoid-mediated signal in controlling the timing of progenitor cell differentiation is emerging in various morphogenetic processes.

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