

BMP action in skeletogenesis involves attenuation of retinoid signaling

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The bone morphogenetic protein (BMP) and growth and differentiation factor (GDF) signaling pathways have well-established and essential roles within the developing skeleton in coordinating the formation of cartilaginous anlagen. However, the identification of bona fide targets that underlie the action of these signaling molecules in chondrogenesis has remained elusive. We have identified the gene for the retinoic acid (RA) synthesis enzyme *Aldh1a2* as a principal target of BMP signaling; prochondrogenic BMPs or GDFs lead to attenuation of *Aldh1a2* expression and, consequently, to

reduced activation of the retinoid signaling pathway. Consistent with this, antagonism of retinoid signaling phenocopies BMP4 action, whereas RA inhibits the chondrogenic stimulatory activity of BMP4. BMP4 also down-regulates *Aldh1a2* expression in organ culture and, consistent with this, *Aldh1a2* is actively excluded from the developing cartilage anlagen. Collectively, these findings provide novel insights into BMP action and demonstrate that BMP signaling governs the fate of prechondrogenic mesenchyme, at least in part, through regulation of retinoid signaling.

Introduction

Development of the vertebrate skeleton ensues with the aggregation of mesenchymal cells into condensations. In endochondral ossification, these precartilaginous condensations prefigure the elements of the adult skeleton, and alterations in this program lead to a range of congenital skeletal abnormalities. Various members of the transforming growth factor- β family figure prominently at multiple stages within the skeletogenic program. Consequently, mutation or disruption of these genes or their receptors negatively impacts growth and development of the skeleton (Kingsley, 2001; Yoon et al., 2005). In particular, many of the bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) exhibit potent prochondrogenic activity. Indeed, several of the BMPs have been shown to stimulate ectopic cartilage formation both in vivo and in vitro, whereas disruption of *Noggin*, which is a BMP antagonist (of BMP2, -4, and, to a lesser extent, -7), leads to skeletal overgrowth (Brunet et al., 1998). Thus, it appears that BMPs may

promote the expansion of cartilage, either by stimulating the proliferation of chondroprogenitors or chondroblasts, or by recruitment of cells with limited or no chondrogenic potential (Hall and Miyake, 2000). Downstream effectors of the BMP signaling cascade have been well characterized; however, the targets (direct and indirect) that underlie BMP action in chondrogenesis remain elusive.

Other signaling molecules that influence the chondrogenic program include vitamin A and its metabolites, the retinoids (Weston et al., 2003a,b). The retinoids act through the modulation of the transcriptional activity of the nuclear receptors for retinoic acid (RA), the RA receptors (RARs), and the retinoid X receptors. The transcriptional activity of these receptors is governed by RA availability, which is regulated largely by the combined actions of the enzymes involved in its synthesis and degradation, the ALDH1As and CYP26s, respectively. Indeed, the *Rars*, and other genes encoding enzymes involved in RA metabolism, are dynamically expressed during chondrogenesis. Furthermore, compound null mutants of the *Rars* and null mutants of *Aldh2a1*, *Cyp26a1*, or *b1* present with a spectrum of skeletal abnormalities (Lohnes et al., 1994; Abu-Abed et al., 2001; Niederreither et al., 2002b; Weston et al., 2003b; Yashiro et al., 2004). The formation of precartilaginous condensations and the subsequent appearance of chondroblasts require the

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Abbreviations used in this paper: atRA, all-trans RA; BMP, bone morphogenetic protein; BMPR, BMP receptor; DEAB, diethyl aminobenzaldehyde; E, embryonic age; GDF, growth and differentiation factor; IDR, interdigital region; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; Tp, distal tip; WL, whole limb.

activity of *Sox9*, which is a transcription factor belonging to the Sry-related HMG box gene family (Bi et al., 1999; Akiyama et al., 2002). In accordance with a proposed role for RA signaling in chondrogenesis, RA influences the expression and/or activity of *Sox9* (Weston et al., 2000, 2002).

In earlier studies, we demonstrated that mesenchymal cells isolated from a transgenic animal overexpressing a weak, constitutively active *Rara* transgene exhibit skeletal defects, and this, in part, results from delayed or inhibited chondroblast differentiation (Cash et al., 1997). Subsequent studies have indicated that the chondrogenic defect within the transgene mesenchyme is not rescued by the addition of BMP2 or -4, leading to the postulation that retinoid signaling may operate downstream of the BMP signaling pathway within this program (Cash et al., 1997; Weston et al., 2000). Further series of experiments demonstrated that skeletal progenitor differentiation requires RAR-mediated repression, and that antagonism of RAR signaling in primary cultures of limb mesenchyme is accompanied by increased *Sox9* expression and activity (Weston et al., 2002). In this study, we demonstrate that BMPs decrease RA availability, and that this is required for their prochondrogenic function. Furthermore, during autopod development, *Aldh1a2* expression is dynamically expressed and influenced by BMP signaling. During this process, *Aldh1a2* expression becomes progressively restricted to nonchondrogenic regions, where it likely serves to inhibit or suppress expression of a chondroblast phenotype.

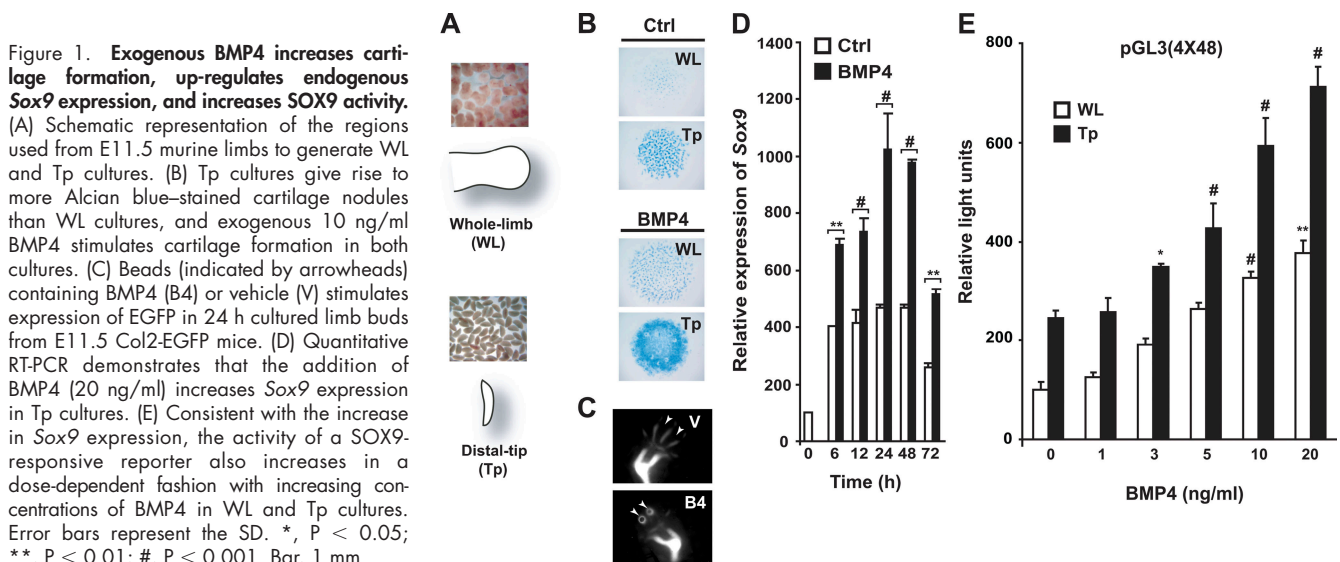
Results

To effectively model the chondrogenic program, we have used high-density primary cultures derived from the mesenchyme of embryonic age (E) 11.5 murine limb buds. For these purposes, two culture models have been used: (1) whole-limb (WL) cultures, which give rise to numerous cartilage nodules within 4 d (Fig. 1, A and B), and (2) distal tip (Tp) cultures, which contain a more homogenous chondroprogenitor population and, thus, produce a much greater number of cartilage

nodules (Fig. 1 B; Gay and Kosher, 1984). Consistent with this, Tp cultures exhibit a higher level of *Sox9* expression (not depicted) and increased basal SOX9 activity (Fig. 1 E), relative to WL cultures. In accordance with previous studies, the addition of BMP4 to either of these cultures results in an increase in cartilage formation both in WL and Tp cultures and in limb-bud organotypic cultures (Fig. 1, B and C). Accordingly, treatment with 10 ng/ml of exogenous BMP4 leads to a more than twofold increase in *Sox9* expression in both Tp cultures and WL cultures (Fig. 1 D; not depicted). Moreover, the addition of BMP4 generates a dose-dependent increase in endogenous SOX9 activity, as measured with a SOX9-responsive reporter gene (Fig. 1 E).

Expression profiling of BMP action reveals the retinoid pathway as a central target

To better understand the mechanisms underlying BMP action, we used transcriptional profiling with Affymetrix U74 v2 chips A and B to identify genes in Tp cultures whose patterns of expression change in response to BMP4. Tp cultures were chosen for these analyses, as they are more homogenous and lack myogenic progenitors (Gay and Kosher, 1984; Weston et al., 2003c). Initially, the array data was filtered to identify genes that exhibit at least a fourfold change in expression between any of the treatment groups, generating a list of 364 genes (Fig. 2 A). Further inspection of this group revealed that, with progressive culture time and/or the addition of BMP4, the expression of several cartilage ECM genes (Fig. 2, A and B, blue) and transcription factors with proscribed roles in chondrogenesis, such as *Sox5*, *6*, and *9*, and *Dlx5* (Fig. 2, A and B; Smits et al., 2001; Robledo et al., 2002), increases significantly. Importantly, these trends in gene expression are consistent with expression of the chondroblast phenotype. To identify those genes that were specifically up- or down-regulated in response to BMP4, individual time points with and without treatment were analyzed and a stringent fourfold expression change cutoff was once again used. Genes from the 24- and 72-h time points were pooled, resulting in a list of 41 genes, 4 of which (*Aldh1a2*, *Rbp1*, *Nog*,



RA status influences the expression of many of its binding proteins, receptors, and catabolism enzymes (de The et al., 1989; Durand et al., 1992; White et al., 1996). Thus, it is not entirely surprising that decreased expression of *Aldh1a2* may lead to widespread changes in the expression of these genes. Consistent with this, changes in *Aldh1a2* expression precede changes in the expression of these genes (unpublished data). For these reasons, we focused our attention on the analysis of *Aldh1a2* expression in response to BMP4. Quantitative PCR confirmed that BMP4 addition reduces *Aldh1a2* expression at 24 h (~12-fold reduction); further analysis revealed that *Aldh1a2* expression is substantially reduced as early as 6 h (an approximately sixfold reduction), and at 12 h there is an ~38-fold differential between treated and untreated cultures (Fig. 3 A). As discussed earlier, BMP has the potential to recruit cells with low chondrogenic potential to the chondrocytic lineage. Based on this information, we sought to determine the distribution of *Aldh1a2*-expressing cells within limb mesenchymal cultures. Whole-mount in situ hybridization revealed that in WL cultures, *Aldh1a2* is expressed in discrete

foci in a pattern reminiscent of *Col2a1* expression (Fig. 3 B). Furthermore, addition of BMP4 abolishes *Aldh1a2* expression, whereas, consistent with previous observations, *Col2a1* expression is intensified and its domain of expression expanded (Fig. 3 B). Thus, it appears that BMP4 expands cartilage formation through down-regulation of *Aldh1a2*.

BMPs function predominantly through modulation of Smad 1, 5, and 8 activity, and through activation of the p38 MAPK signaling pathway. To gain insights into the mechanisms underlying BMP regulation of *Aldh1a2*, a series of experiments were performed to address the requirement for protein synthesis and the role of mRNA stability in this process. 20 ng/ml BMP4 was added either shortly after culture initiation (2 h) or during the peak of *Aldh1a2* expression (~18 h), and *Aldh1a2* transcript abundance was quantified thereafter at 4 h intervals for 12 h. To determine if BMP4 decreased *Aldh1a2* mRNA stability, BMP4-treated and untreated cultures were incubated with 5 μ g/ml of the transcription inhibitor actinomycin D. In the presence of actinomycin D, *Aldh1a2* mRNA levels decline in control cultures, and the presence of BMP4 does not accelerate this decline (Fig. 3 C).

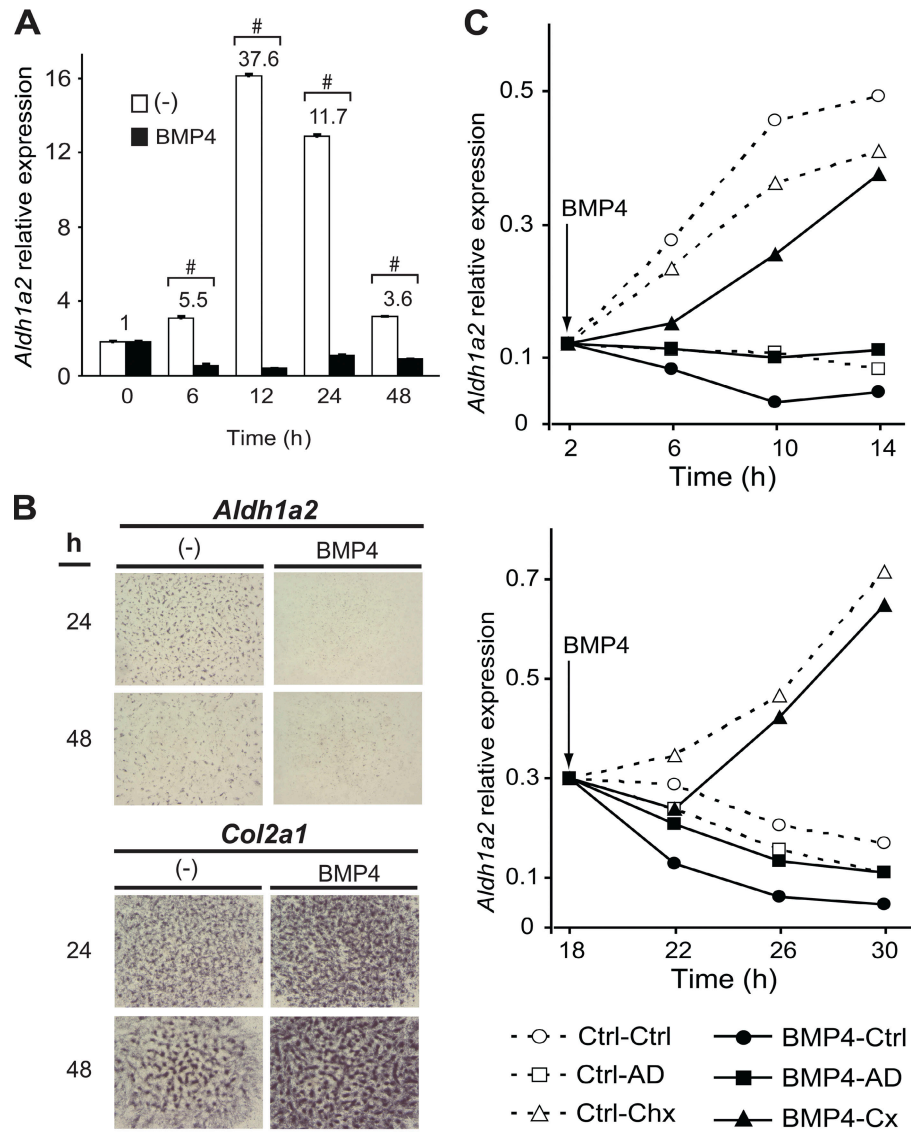


Figure 3. BMP4 decreases *Aldh1a2* expression and this requires protein synthesis. (A) Addition of BMP4 to WL cultures leads to an immediate and sustained decrease in transcripts of *Aldh1a2*, as determined by quantitative PCR (normalized to rRNA expression). The numbers above the bars represent the ratio of control over treated RNA. Error bars represent the SD. (B) Analysis of *Aldh1a2* and *Col2a1* in WL cultures via in situ hybridization. In untreated cultures, *Aldh1a2* and *Col2a1* are expressed in foci. Exogenous BMP4 leads to a loss of *Aldh1a2* expression, and increased and more extensive expression of *Col2a1*. Bar, 0.3 mm. (C) Inhibition of protein synthesis with 10 μ g/ml cycloheximide (Chx) interferes with BMP4-mediated reduction of *Aldh1a2*. BMP4 does not modify *Aldh1a2* mRNA stability as determined by measurement of *Aldh1a2* mRNA abundance after inhibition of transcription with actinomycin-D (5 μ g/ml). 20 ng/ml BMP4 was added at either 2 or 18 h after culture initiation (top and bottom, respectively). #, $P < 0.001$.

In contrast, treatment of cultures with the protein synthesis inhibitor cycloheximide abolished down-regulation of *Aldh1a2* by BMP4 (Fig. 3 C). There is an initial slight downward trend in the BMP4 and cycloheximide-treated cultures at the 4-h time point, but by 8 and 12 h there is no significant difference between the cycloheximide-treated control and BMP4 cultures. Thus, down-regulation of *Aldh1a2* by BMP4 requires protein synthesis, indicating that BMP4 probably acts indirectly through the regulation of factors to down-regulate *Aldh1a2*.

Endogenous RA signaling regulates expression of the chondroblast phenotype

To further demonstrate the potential importance of ALDH1a2 in BMP4 action, we first examined the role of endogenous RA synthesis and degradation in chondrogenesis. Catabolic inactivation of RA is mediated by the CYP26 subfamily of cytochrome P450 enzymes (Niederreither et al., 2002a). Inhibition of RA degradation with an inhibitor of cytochrome P450 enzymes, ketoconazole (Osanai and Petkovich, 2005), leads to increased endogenous RA signaling, as evaluated with an RA-responsive reporter gene. Under these conditions, even small changes in endogenous RA availability (approximately fourfold) lead to a

>10-fold reduction in SOX9 activity and a decrease in the formation of cartilage nodules (Fig. 4 A, insets). Importantly, the effects of ketoconazole on the retinoid and SOX9-responsive reporter genes can be completely reversed through addition of a pan-RAR antagonist, demonstrating that the observed effects of ketoconazole in this system reside in its ability to inhibit CYP26 enzyme activity (Fig. 4 B). Conversely, RA degradation increases through heterologous expression of *Cyp26a1*, and this is accompanied by a decrease in RA response element (RARE) reporter gene activity and an approximately fourfold increase in SOX9 activity (Fig. 4 C). Consistent with these observations, inhibition of ALDH1a2 activity with the ALDH1 inhibitor DEAB (Perz-Edwards et al., 2001) results in an approximately sevenfold reduction in “RA availability” and an approximately fivefold increase in SOX9 activity; this is associated with increased cartilage nodule formation (Fig. 4 D, insets). To follow *Col2a1* expression in live cultures, WL cultures were established from transgenic embryos that contain a 6.3-kb portion of the *Col2a1* promoter, driving expression of EGFP (Grant et al., 2000). In control cultures, weak transgene expression is apparent within 24 h, and by 96 h an abundance of EGFP-positive nodules can be observed (Fig. 4 E). Modulation of endogenous

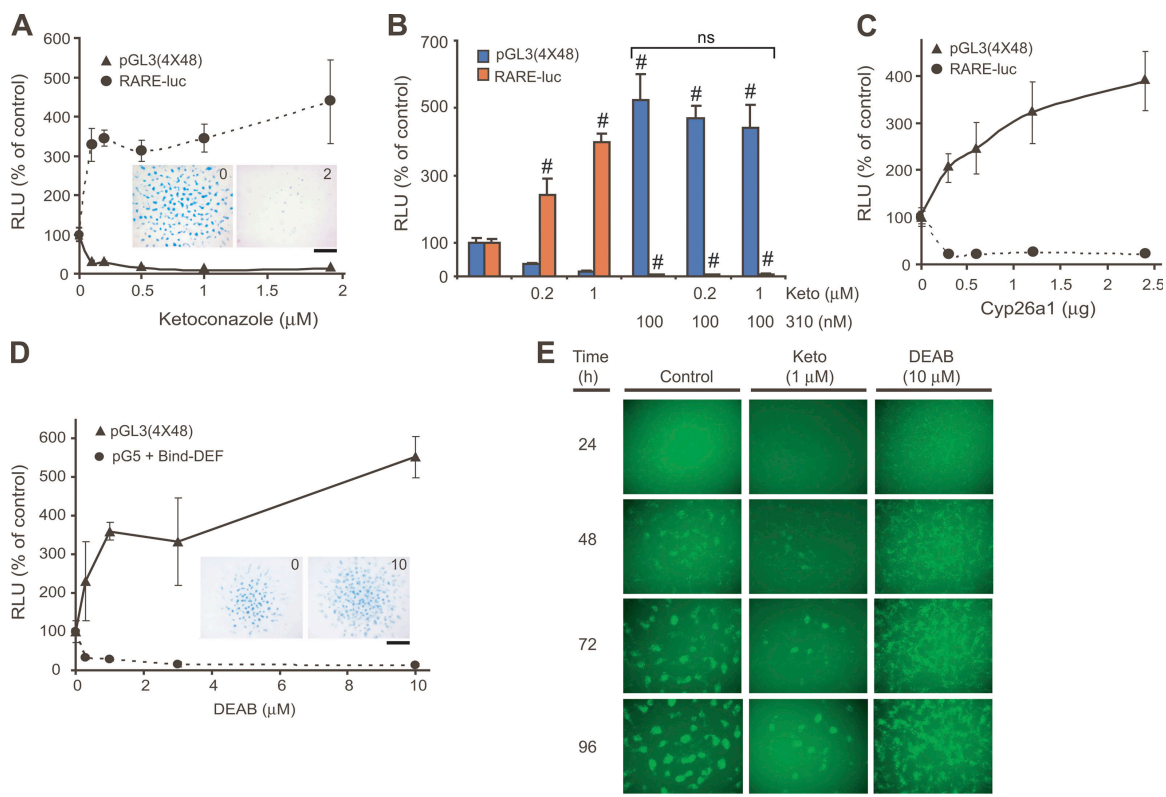


Figure 4. **Modulation of endogenous retinoid signaling impacts SOX9 activity and cartilage formation.** (A) Inhibition of CYP26 activity in WL cultures with ketoconazole (added at day 0) leads to an increase in retinoid signaling, as measured with a retinoid-responsive reporter gene (RARE-luc). Conversely, this treatment leads to a dose-dependent decrease in SOX9 activity, as determined with the SOX9-responsive reporter pGL3(4X48), as well as decreased cartilage formation (insets). (B) The effects of ketoconazole on SOX9 activity can be efficiently rescued by the addition of a pan-RAR antagonist (310). (C) Overexpression of *Cyp26a1* decreases endogenous retinoid levels and increases SOX9 activity. (D) Attenuation of ALDH activity with DEAB (added at day 0) decreases retinoid levels (pG5 + Bind-DEF), increases SOX9 activity, and increases cartilage formation (insets). (E) Ketoconazole and DEAB treatments (both added at day 0) lead to substantive changes in the expression of a *Col2* transgene in WL cultures; ketoconazole treatment leads to fewer and smaller EGFP-positive nodules. In contrast, DEAB causes precocious expression of the transgene (24 h) and expansion of EGFP-positive cells. Under these conditions, EGFP cells no longer appear in well-defined nodules, but are distributed throughout the cultures. Error bars represent the SD. #, $P < 0.001$. Bars: (A) 0.5 mm; (D) 1 mm; (E) 0.25 mm.

retinoid signaling via treatment with ketoconazole significantly impacts transgene expression, with fewer EGFP-positive cells being evident in these cultures. In contrast, attenuation of RA synthesis with DEAB leads to precocious and widespread transgene expression in comparison to control cultures (Fig. 4 E). Together, these results demonstrate that small changes in endogenous retinoid status profoundly influence expression of the chondroblast phenotype.

BMP4 action is potentiated by and requires RAR-mediated repression

We have previously shown in transgenic mice that cells expressing a weak, constitutively active *Rarα1* transgene present with skeletal abnormalities; of particular interest was the finding that transgene-expressing cells are generally absent from cartilages (Cash et al., 1997; Weston et al., 2000).

Moreover, the addition of BMP2 or -4 fails to rescue the chondrogenic defect within the transgenic mesenchyme, leading to the postulation that the BMP signaling pathway operates upstream or in parallel to the retinoid signaling pathway within the chondrogenic program (Weston et al., 2000). To further delineate this relationship, we examined the ability of BMP4 signaling to modulate retinoid signaling directly. The addition of BMP4 to WL cultures leads to a dose-dependent decrease in RARE reporter gene activity, correlating with an increase in SOX9 activity (Fig. 5 A). In previous studies, we demonstrated that expression of the chondroblast phenotype relies on RAR-mediated repression (Weston et al., 2002). RAR-mediated repression involves recruitment of nuclear-corepressor complexes to target genes (Weston et al., 2003a). As such, we hypothesized that if BMP4 functions through the attenuation of retinoid signaling, then the chondrogenic-stimulatory

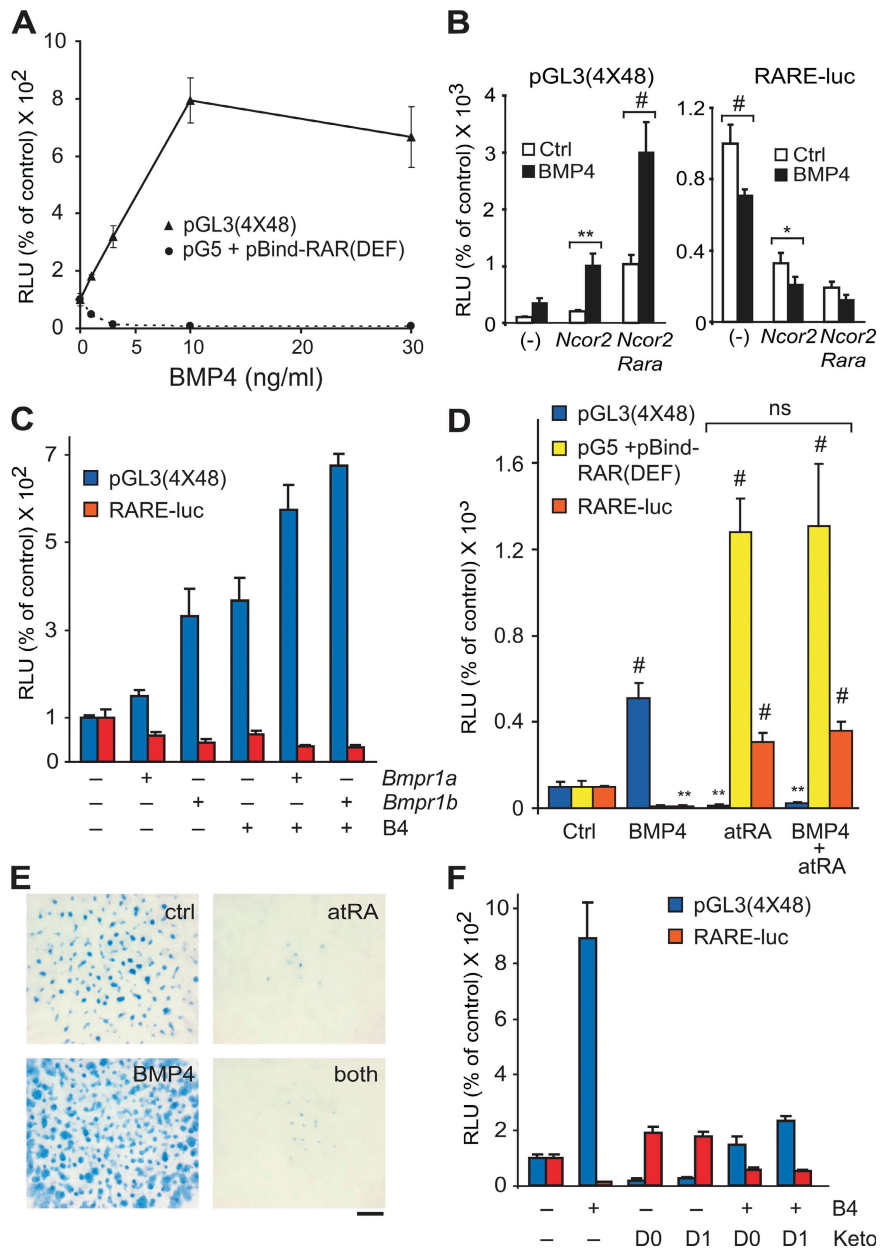


Figure 5. The prochondrogenic activity of BMP4 involves the attenuation of retinoid signaling and is enhanced by RAR-mediated gene repression. (A) Increasing concentrations of BMP4 (added at day 0) lead to a dose-dependent decrease in retinoid signaling (pG5 + Bind-RAR[DEF]) and an increase in SOX9 activity (pGL3[4X48]). (B) The ability of 10 ng/ml BMP4 to stimulate SOX9 activity (pGL3[4X48]) and decrease retinoid signaling (RARE-luc) is enhanced by coexpression of RAR α and/or its nuclear corepressor NCOR2. (C) Expression of the type I BMPRs (*Bmpr1a* or *Bmpr1b*) in the presence and absence of BMP4 also increases SOX9 activity and reduces retinoid signaling. (D and E) The addition of 100 nM atRA effectively neutralizes the stimulatory effect of BMP4 on both SOX9 activity (D) and cartilage formation (E). (F) Inhibition of atRA degradation by ketoconazole only partially interferes with BMP4-induced SOX9 activity. 10 ng/ml BMP4 was added at T = 0 (day 0; D0) and 2 μ M ketoconazole was added at D0 or day 1 (D1). With the exception of B and C, in all experiments, cultures were treated 1 h after transfection; in B and C, cells were treated 24 h after transfection. Error bars represent the SD. **, P < 0.01; #, P < 0.001. Bar, 0.3 mm.

action of BMP4 will be potentiated by the presence of NCOR2 and/or RAR α . Indeed, heterologous expression of *Ncor2* or *Ncor2* and *Rara* together increases SOX9 activity by ~2- and 10-fold, respectively. Treatment with BMP4 alone leads to a 3.6-fold increase in reporter gene activity, whereas treatment with BMP4 combined with expression of *Ncor2* or *Ncor2* and *Rara* increases reporter activity by 10- and 30-fold, respectively (Fig. 5 B). Importantly, SOX9 activity correlates inversely with the extent of the decrease in RARE reporter activity (Fig. 5 B). Consistent with our observations, overexpression of either of the BMP receptor (*Bmpr*) type I receptors, *a* or *b*, stimulates SOX9 activity, and this is associated with a

reduction in RARE-luc activity (Fig. 5 C). These effects are potentiated by BMP4 addition. Together, these observations suggest that down-regulation of retinoid signaling in primary limb mesenchymal cells favors expression of a chondrogenic cell fate. Furthermore, addition of 100 nM atRA completely abrogates the prochondrogenic effects of BMP4 on the aforementioned reporter genes and on cartilage nodule formation (Fig. 5, C and D). Interestingly, treatment with ketoconazole on either day 0 or 1 of culture also inhibits the prochondrogenic action of BMP4 (Fig. 5 F), again indicating that even small changes in endogenous retinoid signaling affect expression of the chondrogenic phenotype.

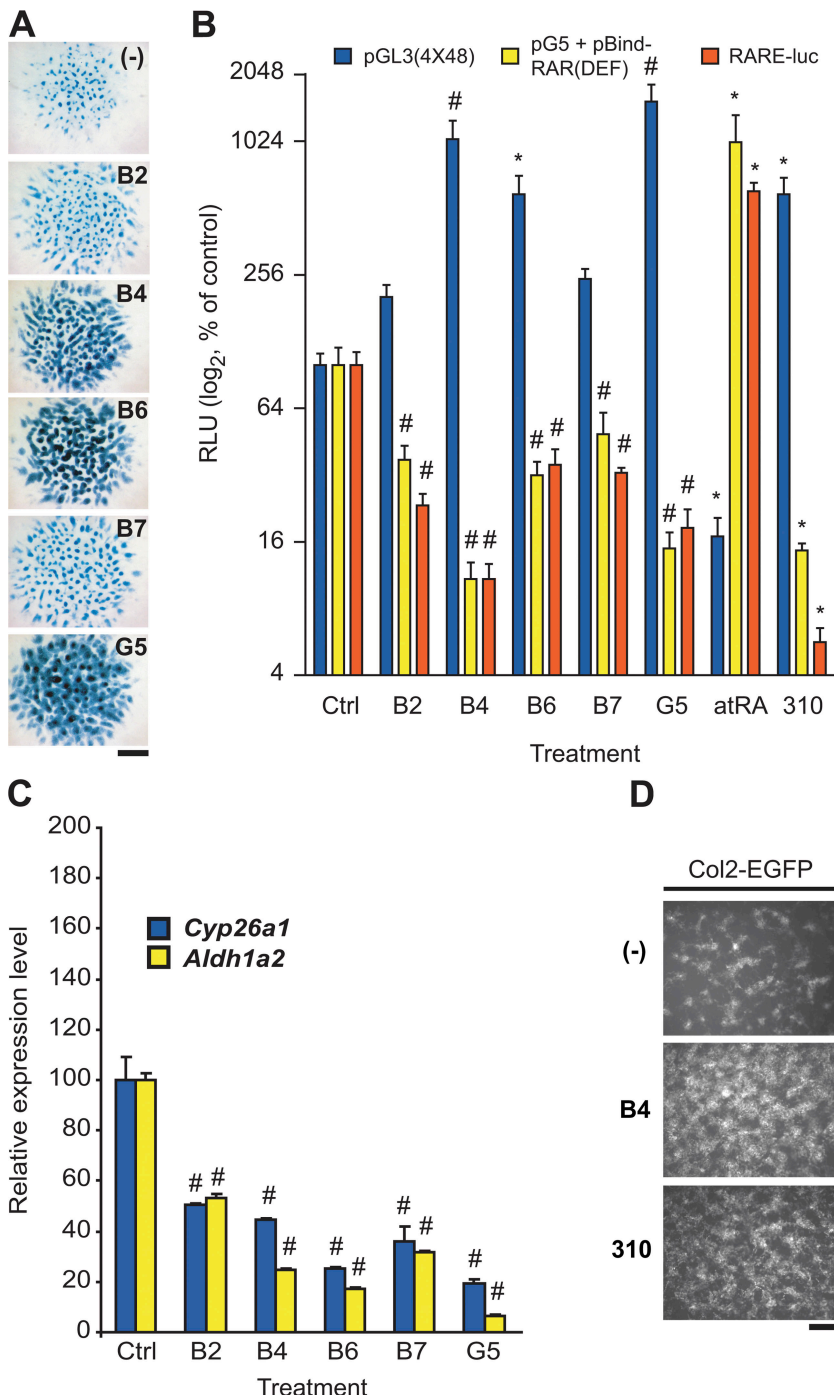


Figure 6. BMPs and GDFs with prochondrogenic activity abrogate retinoid signaling. (A) The BMPs and GDFs increase cartilage formation to varying extents, as determined by staining of 4-d WL cultures with Alcian blue. (B) WL cultures transfected with reporter genes responsive to SOX9 activity [pGL3[4X48]] or retinoid signaling status [RARE-luc; pG5 + pBIND-RAR(DEF)], exhibit increased and decreased activity, respectively, to BMP2 (B2), 4 (B4), 6 (B6), and 7 (B7) and GDF5 (G5). All proteins were added at 10 ng/ml, with the exception of GDF5, which was added at 50 ng/ml; atRA and 310 were supplemented to a final concentration of 100 nM. (C) *Aldh1a2* and *Cyp26a1* expression is inversely proportional to SOX9 activity as determined by qPCR. (D) Antagonism of retinoid signaling phenocopies the effects of BMP4. WL cultures derived from Col2-transgenic mice were treated with vehicle, 20 ng/ml BMP4, or 100 nM of a pan-RAR antagonist (310). Note the more extensive network of EGFP-positive cells in the treated cultures. Images were collected 48 h after culture initiation. Error bars represent the SD. *, $P < 0.05$; #, $P < 0.001$. Bars: (A) 1 mm; (D) 0.25 mm.

Several of the BMPs and GDFs exhibit chondrogenic stimulatory activity; thus, we were interested in determining whether these factors also influence retinoid signaling. As illustrated in Fig. 6 A, the addition of BMP2, -4, -6, -7, or GDF5 stimulates chondrogenesis to varying extents. All of these factors are also expressed in the developing limb at various times during the formation of skeletal elements. Consistent with their effects on cartilage nodule formation, these ligands also exhibit a similar activity in their ability to increase SOX9 reporter gene expression (Fig. 6 B). Under these conditions, each factor also attenuates retinoid signaling, with BMP4 and GDF5 exhibiting the greatest negative effect (Fig. 6 B). Indeed, quantitative PCR further demonstrates that each factor decreases expression of both *Aldh1a2* and *Cyp26a1* (Fig. 6 C). The promoter of *Cyp26a1* is retinoid-responsive (Loudig et al., 2000) and, thus, its decreased expression under these conditions provides further support that the presence of the aforementioned BMP/GDF abrogates retinoid signaling.

As indicated earlier, the addition of BMP4 leads to widespread cartilage formation both in vitro and in vivo. Enhanced cartilage formation after BMP4 treatment can also be observed within WL cultures derived from Col2-EGFP transgenic mice where BMP4 expression leads to widespread transgene expression. If BMPs operate, at least in part, through attenuation of retinoid signaling, then it would be expected that inhibition of retinoid signaling would phenocopy the action of BMPs. This is indeed the case; treatment of transgenic-derived WL cultures with either a pan-RAR antagonist or the ALDH1 inhibitor DEAB, albeit to a lesser extent, also leads to widespread transgene expression in a pattern reminiscent of BMP4 (Fig. 6 D). Collectively, these experiments suggest that the prochondrogenic activity of many of the BMPs and GDFs resides in their ability to abrogate expression of *Aldh1a2*, consequently resulting in the reduced activation of the retinoid signaling pathway.

RA availability is regulated by BMP signaling in vitro and in vivo

In the forelimb, the autopod cartilages, as determined by *Col2a1* expression, first become evident around early E11, with the hindlimb following ~ 0.5 d later (Fig. 7 A). At the beginning of autopod development, *Col2a1* expression in precartilaginous digital rays is quite weak, but quickly intensifies, such that by late E12 all the digits exhibit strong *Col2a1* expression. In contrast, *Aldh1a2* exhibits a dynamic pattern of expression that quickly changes from a band of expression through the early autopod to a fragmented appearance a few hours later (Fig. 7 A). Bifurcation of *Aldh1a2* expression in the autopod occurs over a relatively short period of time (~ 9 h) and is closely associated with digit formation. As previously reported, *Aldh1a2* expression appears between the developing zeugopodal elements, within the regions flanking the digit cartilages, and within the interdigital region (IDR; Niederreither et al., 1997).

To assess the ability of BMP4 to regulate *Aldh1a2* expression in limb buds, Affi-Gel blue beads soaked in either vehicle or 20 ng/ μ l BMP4 were implanted into the IDR region of late

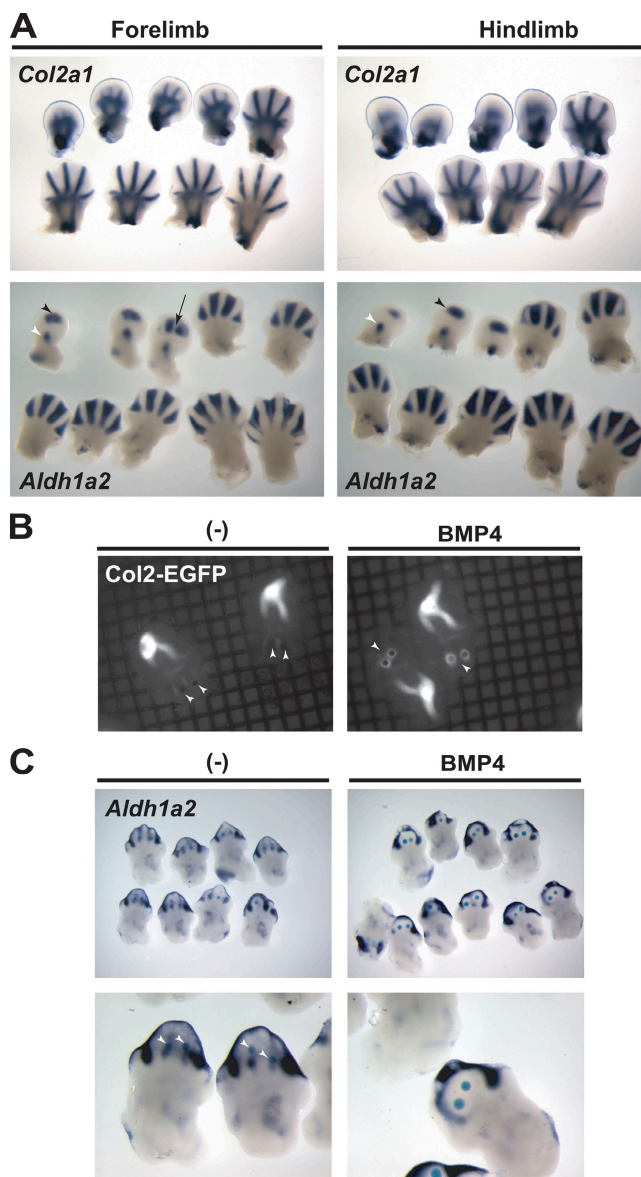


Figure 7. *Aldh1a2* and *Col2a1* exhibit dynamic, but distinct, expression patterns during zeugopod and autopod formation, and BMP4 down-regulates *Aldh1a2* expression in the developing autopod. (A) At the beginning of E11, *Col2a1* expression is evident in the developing zeugopod, with very weak expression evident in the autopod of the forelimb and hindlimb. During E11 in the forelimb, *Col2a1* becomes abundantly expressed in the autopod, and by late E11, *Col2a1* appears strongly with the hindlimb autopod (upper top right). *Aldh1a2* is expressed between the developing zeugopods (white arrowheads) and in a band throughout the presumptive autopod (black arrowheads). During a 6–8 h period in the forelimb and hindlimb (top series in both images), *Aldh1a2* expression in the autopod becomes fragmented (arrow) eventually localizing to regions that flank the developing digits. (B) Introduction of beads soaked in vehicle (-) had no apparent effect on EGFP expression (white arrowheads) in limb buds derived from Col2-EGFP forelimbs, whereas beads soaked with 20 ng/ μ l BMP4 stimulated EGFP expression (white arrowheads). (C) BMP containing Affi-Gel blue beads (blue) down-regulates *Aldh1a2* in the same limb buds as measured by whole-mount in situ hybridization. Beads soaked in either vehicle or BMP4 were implanted into the IDR of \sim E11.75 forelimb buds, and limbs were visualized and fixed after 24 h of culture. In the control limbs, all beads (white arrowheads) were associated with various amounts of *Aldh1a2* expression and no ectopic EGFP expression (B and C). Ectopic EGFP expression was observed around all BMP4-containing beads, and this was accompanied by decreased *Aldh1a2* expression (B and C).

E11 limb buds, followed by 24 h of organ culture. Implantation of BMP4-soaked beads into limb organ cultures stimulates ectopic Col2-EGFP expression (Fig. 7 B) and substantially modifies *Aldh1a2* expression (Fig. 7 C). Consistent with the ability of BMP4 to stimulate ectopic cartilage in the early IDR as reported by others, BMP-soaked beads stimulated EGFP expression around the bead of all implanted limb buds ($n = 10$). EGFP expression was not detected around control beads ($n = 8$). *Aldh1a2* was expressed to varying levels around control beads. In contrast, *Aldh1a2* expression was excluded from those regions around the BMP4-soaked beads (Fig. 7 C). Together, these results demonstrate that BMP4 down-regulates the expression of *Aldh1a2* in limb buds.

During early appendicular skeletal development, the BMPs, GDFs, and their receptors exhibit a complex pattern of expression, appearing at various times and locations within the cartilaginous anlagen. In this regard, activated BMP signaling has been observed in the developing cartilages, and an absence of the *Bmpr1a* and *-1b* severely compromises precartilaginous condensation formation and chondroblast differentiation (Yoon et al., 2005). Activation of either of these two receptors leads to a stage-dependent increase in cartilage formation (Zou et al., 1997). In contrast, activated retinoid signaling inhibits chondrogenesis, whereas decreased retinoid signaling, either through antagonism of the receptors or in compound *Rar* knockouts, promotes chondrogenesis in vitro and in vivo (Zou et al., 1997; Weston et al., 2003a,b). Together, these observations would suggest that *Aldh1a2* and liganded *Rars* should be excluded from developing cartilages. To examine the relationship of *Aldh1a2*-expressing cells and developing cartilages, double in situ hybridization was used in vitro and in vivo in WL cultures and E11.25–12.5 embryos, respectively. *Aldh1a2* and *Col2a1* are expressed in spatially distinct regions (Fig. 8, A and B). More specifically, *Aldh1a2*-expressing cells are excluded from *Col2a1*-expressing regions and from the apical ectodermal ridge and subjacent mesenchyme. During autopod development, small rays of *Col2a1*-expressing cells can be observed to bisect the band of *Aldh1a2* expression (Fig. 8 A). Consistent with these observations, *Aldh1a2*-expressing cells are excluded from *Col2a1*-expressing regions in WL cultures. Furthermore, the distribution of *Aldh1a2*-positive cells would be expected to cause localized activation of the retinoid signaling pathway. This is, indeed, the case because establishment of WL cultures from a transgenic mouse harboring an RA-responsive reporter gene (RARE-hsp68-lacZ; Rossant et al., 1991) demonstrates foci of transgene-expressing cells, which are distinct from the developing cartilages (Fig. 8 C). Transgene expression is markedly increased and decreased in response to 50 or 100 nM of exogenous atRA or the addition of 100 nM of 310, respectively (unpublished data). In both culture systems, BMP4 leads to expansion of *Col2a1*-expressing cells and increased cartilage formation, as well as substantial reduction in *Aldh1a2* expression and RARE activity (Fig. 8 B, C). In aggregate, these findings suggest that during autopod development BMP signaling regulates expression of the chondrogenic phenotype by controlling RA availability.

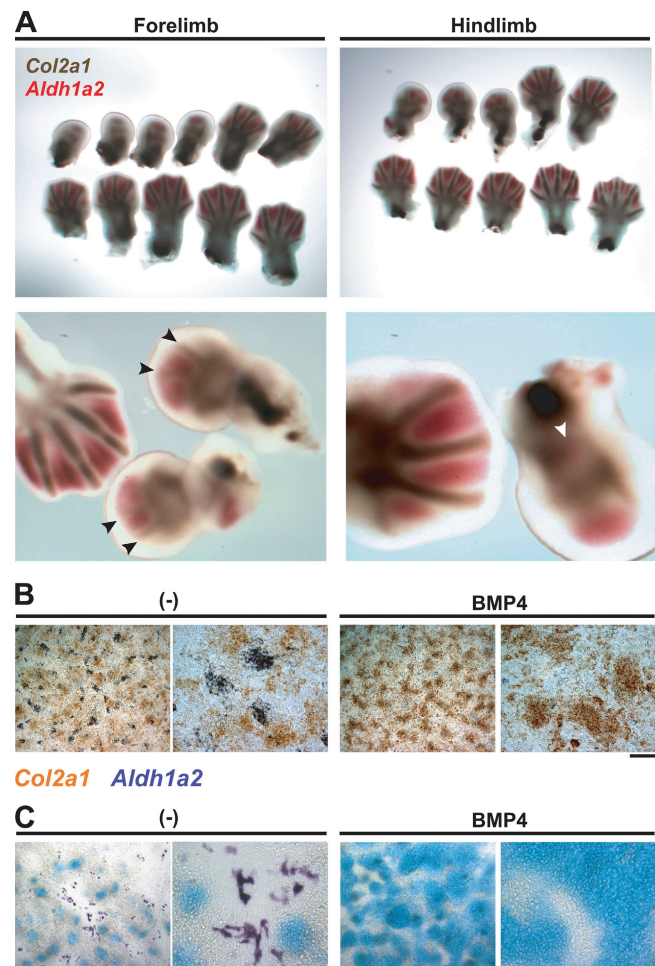


Figure 8. ***Aldh1a2*-expressing cells are distinct from *Col2a1*-expressing cells in vitro and in vivo.** (A) *Aldh1a2*-expressing cells (red staining) are excluded from *Col2a1*-positive (brown staining) regions as determined by double whole-mount in situ hybridization. During autopod development, *Aldh1a2* expression is progressively extinguished in those regions that begin to express *Col2a1*. In the bottom image, *Aldh1a2* expression is subdivided by the developing digits (black arrowheads) and *Aldh1a2* expression is expressed between the developing zeugopod elements (white arrowhead). (B) Similar to what was observed in vivo, double in situ hybridization of *Aldh1a2* (purple) and *Col2a1* (orange) of day 1 WL cultures reveals that *Aldh1a2*-expressing cells are excluded from *Col2a1*-expressing regions. Addition of BMP4, however, leads to extensive *Col2a1* expression and loss of *Aldh1a2* expression. (C) Similarly, WL cultures established from RARE-lacZ reporter mice exhibit foci of transgene-expressing cells (purple-stained cells) after 3 d of culture, which are distinct from Alcian blue-stained regions. Further, addition of 20 ng/ml BMP4 abolishes expression of the transgene. Bars: (B and C, left) 0.15 mm; (B and C, right) 0.04 mm.

Discussion

Manipulation of either the BMP or the retinoid signaling pathway severely compromises skeletal ontogenesis. Although the mechanism of action of BMPs in chondrogenesis is poorly defined, previous studies have implied a potential connection between the BMP and RA pathways (Weston et al., 2000). Using an unbiased genome-wide expression profiling strategy, we have defined the precise nature of this relationship and demonstrate, both in cell and organ culture, that BMP signaling controls RA availability by regulating *Aldh1a2* expression. Furthermore,

unliganded *Rars* and RAR-mediated repression support expression of the chondrogenic phenotype. Thus, BMP signaling promotes chondrogenesis by enhancing RAR-mediated repression, which itself is regulated via attenuation of RA availability.

Function of BMP signaling in chondrogenesis: involvement of the retinoid signaling pathway

BMP2, -4 and -7, along with GDF5, bind and activate the BMPR type I *a* and *b* receptors. Several hallmarks of activated BMP/GDF signaling are observed in the developing skeleton, including the phosphorylation of Smads (1, 5, or 8; Brunet et al., 1998; Yoon et al., 2005). *Noggin* is also often up-regulated in response to the activation of BMPRI (Canalis et al., 2003); this, too, was observed in this study (Fig. 2). An essential requirement for BMPRI-mediated signaling in endochondral ossification has been elegantly demonstrated by Yoon et al. (2005) through generation of compound null mutants of *Bmpr1a* and *-1b*. In this study, mutant animals were found to exhibit severe deficiencies in cartilage formation. More specifically, these animals present with poorly defined precartilaginous condensations that weakly express *Sox9*. Similarly, excess *Noggin* in vitro or in vivo leads to reduced cartilage formation, and decreased *Sox9* expression and activity (Pizette and Niswander, 2000; Weston et al., 2000; unpublished data). Reduced activity of BMPs or GDFs through targeted or spontaneous mutations also negatively impacts the formation of precartilaginous condensations and subsequent cartilage formation. In contrast, expression of wild-type (Fig. 5 C) or constitutively active versions of the BMPR type I receptors promotes chondrogenesis (Zou et al., 1997; unpublished data). Furthermore, ectopic BMP signaling in vivo and in vitro expands cartilage formation, likely through recruitment of cells not destined to become chondrocytes (Duprez et al., 1996a,b). Thus, BMP signaling appears to act at multiple stages within the chondrogenic program, including the establishment, maintenance, and differentiation of chondroprogenitors. The ability of BMPs to stimulate chondroblast differentiation is influenced by retinoid signaling (Figs. 4, 5, and 6), suggesting that at least a subset of these activities involves BMP-mediated attenuation of the retinoid signaling pathway.

Cells throughout the early autopod exhibit high chondrogenic potential, and these cells can be directed under a variety of conditions to form cartilage in vitro and in vivo (Lee et al., 1994; Ganan et al., 1996; Rodriguez-Leon et al., 1999). As autopod development ensues, the chondrogenic potential of many of these cells is lost, especially those within the IDRs, i.e., prechondrogenic mesenchyme loses its ability to become cartilage or is inhibited from doing so. In the presence of BMP signals, however, these cells express a chondrogenic fate (Fig. 7 A); this is especially apparent in the *Noggin*-null mice, which present with very broad digits and greatly limited IDR (Brunet et al., 1998). Importantly, during the establishment of these distinct regions, *Aldh1a2* is dynamically expressed within these domains. Before overt chondrogenesis and robust expression of *Col2a1*, *Aldh1a2* appears as a broad band throughout the autopod. As digit formation proceeds, *Aldh1a2* expression is quickly extinguished in cartilage-forming regions, becoming localized

to the nonchondrogenic flanking regions. At this embryonic stage, the developing limb is exquisitely sensitive to changes in retinoid status (Weston et al., 2003b), and the presence of *Aldh1a2* is congruent with a role for retinoid signaling in inhibiting expression of the chondrogenic phenotype in cells flanking the developing digits. Moreover, activation of a RA-responsive reporter in transgenic mice can be observed within the early IDR (Rossant et al., 1991), and RARE-expressing cells are excluded from developing cartilages (Fig. 8 C). In contrast, numerous studies have demonstrated that diminished retinoid signaling promotes chondrogenesis in vitro and in vivo (Weston et al., 2003a,b). In this study, we show that manipulation of endogenous RA production and signaling significantly impacts chondrogenesis (Figs. 4 and 5). In aggregate, these results suggest dual roles for the RARs in regulating chondrogenesis. Unliganded receptors promote expression of the chondroblastic phenotype, whereas liganded receptors inhibit differentiation of prechondrogenic cells. In this regard, local activation of the retinoid signaling pathway in the developing autopod would specify nonchondrogenic regions. Consequently, under conditions where retinoid signaling is diminished in the developing autopod, widened phalanges and/or cartilage formation in the IDR would be expected. Interestingly, *Rara/g* double-null mice present with both of these phenotypes. More specifically, a bulbous phalange abnormality is consistently observed, along with a less frequent appearance of ectopic cartilages in the IDRs (Lohnes et al., 1994). These mice also exhibit ectopic cartilage at several extraskelatal sites (Lohnes et al., 1994; Mendelsohn et al., 1994).

Parallels in axial and appendicular skeletogenesis: overlapping players involving different mechanisms

In the axial skeleton, BMPs play a role in the establishment and maintenance of an autoregulatory loop between the transcriptional repressor NKX3.2 and SOX9, thereby promoting axial chondrogenesis (Zeng et al., 2002). Interestingly, even though BMPs are sufficient and necessary for axial and appendicular chondrogenesis, their regulation of *Sox9* appears to operate through an NKX3.2-independent manner in appendicular skeletogenesis (Zeng et al., 2002). In axial chondrogenesis, expression of a chondrogenic fate in somitic mesoderm appears to involve derepression of *Sox9* by NKX3.2-mediated downregulation of gene X, which is a transcriptional repressor of *Sox9*. Likewise, the retinoid pathway appears to be operating in a similar manner in the appendicular skeleton to regulate *Sox9* and expression of a chondrogenic fate. In this scenario, activation of the retinoid signaling pathway up-regulates the expression of a negative regulator of *Sox9* expression, gene Y, leading to decreased *Sox9* expression and reduced cartilage formation. Conversely, antagonism of RAR-mediated signaling downregulates the expression of Y, leading to derepression of *Sox9* and increased cartilage formation, and, thus, supporting a chondrogenic-permissive condition. Thus, at least in the limb, BMPs function by attenuating the expression of *Aldh1a2*, thereby stimulating *Sox9* expression and activity, and the elaboration of a chondroblastic phenotype. Both programs appear to converge

on negative regulators of *Sox9*, genes X and Y. Further complicating an understanding of BMP action in the limb is that BMPs do not appear to act directly to regulate *Aldh1a2* expression, as protein synthesis is required for this activity. Thus, BMPs possibly act to increase the expression of a negative regulator of *Aldh1a2* expression, or rather that BMPs may act directly through a relatively labile protein. Evidence for this latter scenario comes from the observation that in the absence of protein synthesis (Fig. 3 C), BMP4 addition leads to an initial transient decrease in *Aldh1a2* expression. Collectively, these findings demonstrate that BMP4-mediated establishment and maintenance of *Sox9* expression in limb chondrogenesis involves a cascade of factors with the retinoid signaling pathway figuring prominently in this program. Further elucidation of the factors operating upstream and downstream of the RA signaling pathway will provide additional important insights into the molecular mechanisms underlying the chondrogenic program.

Materials and methods

Reagents

atRA, ketoconazole, diethyl aminobenzaldehyde (DEAB), cycloheximide, and actinomycin-D were obtained from Sigma-Aldrich; all compounds were dissolved in 95% ethanol, with the exception of ketoconazole, which was dissolved in deionized water. BMPs and GDFs were purchased from R&D Systems and resuspended according to the manufacturer's instructions. BMPs were added to media at a concentration of 10–20 ng/ml, whereas GDF5 was used at a concentration of 50 ng/ml to reflect its much lower activity (R&D Systems). AGN194310 was provided by R. Chandraratna (Vitae Pharmaceuticals, Irvine, CA).

Plasmid constructs

Reporter plasmids containing SOX9 binding sites (pGL3[4X48]) or trimerized RARE-luc were previously described (Weston et al., 2002). pG5, which is a reporter that contains five GAL4 binding domains, was obtained from Promega. To generate GAL4–RAR(DEF) fusions, the C-terminal region of *Rara* encompassing the ligand-binding domain was subcloned in-frame into pBIND (Promega). For expression in primary cells, full-length versions of murine *Cyp26a1* (provided by M. Snead, University of California, Los Angeles, Los Angeles, CA) and *Rara1* were subcloned into a modified pSG5 (Weston et al., 2002), and the construct containing an N-terminal EGFP fusion to full-length *Ncor2* (provided by M. Privalsky, University of California, Davis, Davis, CA) was as previously described (Goodson et al., 2005). Mammalian expression plasmids containing *BMPRI1a* and *Bmpr1b* were provided by J. Wrana (University of Toronto, Toronto, Canada).

Establishment and transfection of primary mesenchymal cultures

Primary mesenchymal cultures from CD-1 E11.5 mouse limb buds were established as previously described (Weston et al., 2000) with the following modifications. For microarray analyses and other specified experiments, distal limb bud tips (subridge region) were dissected from E11.5 mouse embryos (the morning of the plug was considered E0.5) as described by Gay and Kosher (1984), with the excised region extending 0.3–0.4 mm from the distal apex of the limb to the proximal cut edge. After proteolytic digestion, cells were filtered through a cell strainer (40 μ M; BD Biosciences) to obtain a single cell suspension. Cells were pelleted and resuspended at a density of 2×10^7 /ml and 12–15 10- μ l aliquots of this suspension were plated into a 35-mm tissue culture dish (Nunc) and allowed to adhere for 1 h. After this period, 2 ml of culture medium consisting of 60% F12/40% DME and supplemented with 10% FBS (Qualified; Invitrogen) was added to each well with or without 20 ng/ml BMP4 (R&D Systems); this time was considered T = 0. Cultures were maintained for a period of up to 3 d; to minimize handling, culture media was replaced on alternate days. Cultures from either RARE-hsp68-lacZ (CD-1 background; provided by J. Greer, University of Alberta, Edmonton, CA) or Col2-EGFP mice (CD-1 background; derived from breeding of heterozygous transgenic males with CD-1 females) were established in a similar manner. Alcian blue staining, β -galactosidase staining, and the establishment and transient transfection of cultures derived from WL buds was performed as

described in Weston et al. (2000, 2002). In brief, cultures were transfected at day 0 and extracts for luciferase analysis were collected at day 2 unless indicated otherwise. For experiments involving primary limb mesenchymal cells alone or cells transfected with reporter genes only, factors or compounds were added at the time of media addition (T = 0). For experiments involving cotransfection with expression plasmids, factors and compounds were added 24 h after transfection.

Limb bud organ culture and bead implantation

WL buds were collected in PSA from \sim E11.5 Col2-EGFP embryos. Affi-Gel blue beads (Bio-Rad Laboratories) soaked in either vehicle or BMP4 (20 ng/ μ l) for 2 h were transferred into the IDR of the limb buds. Limb buds were cultured on Nucleopore Track-Etch membranes (Whatman) at the air–liquid interface on top of stainless steel mesh in 12-well tissue culture plates. PSA was aspirated from each well and replaced with BGJb medium (Invitrogen) containing 10% FBS and antibiotics. The level of culture medium should not exceed the height of the membranes. Limbs were incubated under standard tissue culture conditions. After a 24-h incubation, EGFP expression was visualized using a dissection microscope (model MZ12; Leica) with epifluorescence. Limbs were subsequently fixed in 4% PFA before processing for whole-mount in situ hybridization.

Transcriptional profiling with microarrays: experimental design and analysis

RNA was harvested from primary cultures using RNeasy (QIAGEN) according to the manufacturer's instructions. For the zero time point, cells were allowed to attach for 1 h and were subsequently processed for RNA isolation. For other cultures, the media was gently aspirated, and any remaining media was blotted from the well before the addition of the lysis reagent. After isolation, the RNA was precipitated, and resuspended at 2 μ g/ml; RNA quality was examined on a Bioanalyzer 2100 (Agilent), and the expression of *Sox9* and *Col2a1* were measured using real-time PCR.

For each time point, a minimum of two biological replicates were analyzed on U74V2 chips A and B. 10 μ g of RNA was labeled and hybridized to the chips using the manufacturer's recommended protocol. Gene expression profiles were subsequently analyzed using MAS 5.0 (Affymetrix) and GeneTraffic UNO bioinformatics programs (Stratagene). All datasets were initially filtered to remove genes called absent by MAS 5.0, and were further filtered as indicated in the text using GeneTraffic. Hierarchical clustering was performed in GeneTraffic using gene clustering and a Pearson correlation distance metric.

Real-time PCR and whole-mount in situ hybridizations

To follow the expression of transcripts for *Aldh1a2*, *Cyp26a1*, *Col2a1*, and *Sox9*, quantitative real-time PCR was performed using the 7900HT Sequence Detection System (Applied Biosystems). Primers and MGB probes (TaqMan) were designed using PrimerDesigner 2.0 (Applied Biosystems). The primer/probe sets used for detection of *Sox9* and *Col2a1* were as described in Weston et al. (2002) and the following primer/probe sets were used for quantifying *Cyp26a1* transcript abundance: forward primer, 5'-CTCCAACCTGCACGATTCCT-3', reverse primer, 5'-CGGCTGAAGCGCTGCAT-3', probe 6FAM-5'-CAGCGAAAGAAGGTG-3'-MGBNFQ. *Aldh1a2* transcripts were detected using the forward primer, 5'-GGTATCCTCCGCAATGCAA-3', reverse primer, 5'-GCGCATTAAGGCATTGTAACA-3', and probe, 6FAM-5'-CTGGGACAGTTTGGATC-3'-MGBNFQ. Primer and probe concentrations were optimized according to the manufacturer's instructions. Total RNA was isolated from primary cultures as described above. Quantification was performed using \sim 25 ng of total RNA and the expression of all genes relative to endogenous rRNA was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems) and the comparative C_T method as described in User Bulletin #2 (Applied Biosystems).

Whole-mount in situ hybridization was performed on primary mesenchymal cultures as previously described (Weston et al., 2000) with the following modifications. After permeabilization using 10 μ g/ml proteinase K in PBS supplemented with 0.05% Triton X-100, cells were postfixed in 4% PFA and 2% glutaraldehyde in PBS. Blocking and antibody incubations were performed in 1% blocking reagent (Roche) in 1 \times maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5). Riboprobes were synthesized in the presence of UTP-digoxigenin with the appropriate RNA polymerase and linearized template DNA, according to manufacturer's instructions (Roche). Riboprobe complementary to *Col2a1* was generated from BamHI-linearized pBluescript containing 1.1 kb of the C-propeptide-encoding region of *Col2*, and transcribed in vitro with T7 RNA polymerase. *Aldh1a2* riboprobe was transcribed from EcoRI-linearized modified pBluescript containing a 400-bp fragment of the *Aldh1a2* coding sequence

using T3 RNA polymerase. Double in situ hybridizations were performed as above, but with the following modifications. Hybridization was performed using both DIG- and fluorescein-labeled RNA probes. Cultures were subsequently incubated overnight in fluorescein-labeled antibody (for detection of *Aldh1a2*). After incubation in NBT/BCIP/10% PVA-staining buffer, cultures were rinsed twice with PBS, and then fixed for 10 h in a 4% PFA/PBS solution. Cultures were again briefly rinsed twice in PBS, and the blocking antibody and staining steps were repeated, using an anti-DIG-AP antibody and INT/BCIP for detection of *Col2a1*.

Single and double whole-mount in situ hybridization on limb buds and cultured limbs was performed as described by Hargrave and Koopman (2000). Double in situ hybridizations were performed as described in the previous paragraph, except that BCIP alone was used for detection of *Col2a1*. To enhance visualization of the light blue (BCIP) and purple staining (NBT/BCIP), all images were adjusted to maximum hue in Photoshop (Adobe).

Microscopy and image acquisition

Images of fixed cultures (in 70% ethanol or Tris-buffered salt solution with 0.1% Triton X-100 [TBTX]) were collected at room temperature using either a dissection microscope (Stemi SV11 Apo, S1.6× objective; Carl Zeiss MicroImaging, Inc.) or an inverted microscope (Axiovert S100; Carl Zeiss MicroImaging, Inc.) fitted with a CP-Achromat 5×, 0.12 NA, CP-Achromat 10×, 0.25 NA, LD Achromat, 0.55 NA, 10× Fluor, 0.5 NA, and an LD Achromat 40×, 0.6 NA. Monochromatic and color images were acquired from a QImaging Retiga Exi (12-bit) and a QImaging Retiga 1300i (12-bit) camera, respectively, using Openlab 4 software (Improvision). Live cell and organ culture images were collected at room temperature in culture media with either an Axiovert S100 microscope or a dissection microscope (MZ FLIII; Plan 1.0× objective; Leica). Photoshop was used to adjust some image levels.

Statistical analysis

All luciferase assays were performed in quadruplicate and repeated using three distinct preparations of primary cells. Real-time PCR analysis was performed in duplicate and repeated a minimum of two times with independent RNA samples. Real-time PCR and luciferase reporter data were analyzed by one-way analysis of variance, followed by a Bonferroni post-test for multiple comparisons using GraphPad Prism, Version 4.0 (GraphPad Software, Inc.). Significance is represented as follows: *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$. One representative experiment is shown for all luciferase and real-time PCR results.

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