

Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage

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The bone morphogenetic proteins (BMPs), TGF β superfamily members, play diverse roles in embryogenesis, but how the BMPs exert their action is unclear and how different BMP receptors (BMPRs) contribute to this process is not known. Here we demonstrate that the two type I BMPRs, BMPR-IA and BMPR-IB, regulate distinct processes during chick limb development. *BmpR-IB* expression in the embryonic limb prefigures the future cartilage primordium, and its activity is necessary for the initial steps of chondrogenesis. During later chondrogenesis, *BmpR-IA* is specifically expressed in prehypertrophic chondrocytes. BMPR-IA regulates chondrocyte differentiation, serving as a downstream mediator of Indian Hedgehog (IHH) function in both a local signaling loop and a longer-range relay system to PTHrP. BMPR-IB also regulates apoptosis: Expression of activated BMPR-IB results in increased cell death, and we showed previously that dominant-negative BMPR-IB inhibits apoptosis. Our studies indicate that in TGF β signaling systems, different type I receptor isoforms are dedicated to specific functions during embryogenesis.

[Key Words: Bone morphogenetic proteins; BMP receptor; cartilage differentiation; cell death; chondrogenesis; limb development]

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Several families of signaling molecules have been identified that act at multiple points in development to mediate cell-cell communication. The secreted bone morphogenetic proteins (BMPs) have been proposed to control many aspects of vertebrate development (for review, see Hogan 1996). With respect to the development of the vertebrate limb, BMPs have been implicated in the control of pattern formation, interdigital cell death, and bone morphogenesis (see references in Hogan 1996). The question of how BMP signaling mediates these disparate functions remains open.

BMPs can induce ectopic endochondral bone when introduced at intramuscular sites in adult rats (Wozney et al. 1988). Prior to the formation of cartilage elements, *Bmp2*, *Bmp4*, and *Bmp7* are expressed in spatially and temporally dynamic patterns (Francis et al. 1994; Francis-West et al. 1995). Within the developing limb cartilage elements, *Bmp2*, *Bmp4*, and *Bmp7* are expressed in the perichondrium (Lyons et al. 1990; Jones et al. 1991; Wozney et al. 1993; Macias et al. 1997), and *Bmp6* is

expressed in prehypertrophic and hypertrophic chondrocytes (Lyons et al. 1989; Wozney et al. 1993; Vortkamp et al. 1996). *Gdf5*, a BMP-related gene, is expressed in the developing joints and perichondrium (Chang et al. 1994; Storm et al. 1994). However, little is known about the cellular and molecular mechanisms underlying normal or ectopic bone morphogenesis by BMPs.

During endochondral bone development, a cartilage template forms when mesenchymal cells condense and differentiate into chondrocytes. These chondrocytes then undergo a program of proliferation, maturation, hypertrophy, calcification, and cell death (see Fig. 4I below; for review, see Erlebacher et al. 1995). Later, osteoblasts replace the cartilage matrix with bone matrix. This ossification initiates in the center of the cartilage shaft and then expands toward the ends of the cartilage element. Around the cartilage element is a sheath of cells, the perichondrium, which contributes to cartilage growth and regulates the rate of cartilage differentiation.

Because of the relative lack of knowledge of *Bmp* receptor (*BmpR*) localization in the developing cartilage (Yamaguchi et al. 1991; Dewulf et al. 1995; Ishidou et al. 1995; Kawakami et al. 1996), it is not known how BMPs regulate chondrocyte formation and differentiation. However, progress has been made in identifying other

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molecules involved in this process. Recently, *Indian hedgehog* (*Ihh*) has been proposed to regulate the rate of cartilage differentiation (Vortkamp et al. 1996). *Ihh* is expressed in prehypertrophic chondrocytes and its misexpression results in a delay in the maturation to hypertrophic chondrocytes, presumably via the parathyroid hormone-related protein (PTHrP) pathway. Misexpression of *Ihh* induces PTHrP, and PTHrP-defective mice are resistant to the effects of Hedgehog protein (Lanske et al. 1996; Vortkamp et al. 1996). PTHrP (-/-) or PTH/PTHrP receptor (-/-) mutant mice exhibit accelerated differentiation of chondrocytes (Karaplis et al. 1994; Lanske et al. 1996). Conversely, ectopic expression of PTHrP delays chondrocyte differentiation (Weir et al. 1996). The IHH signal has been proposed to act on the perichondrium adjacent to the prehypertrophic zone where the Hedgehog receptor *patched* (*ptc*) is expressed, and then directly or indirectly on the more distant periarticular region to induce PTHrP expression (see Fig. 4J, below). It is not clear whether IHH acts as a long-range signal or whether signaling is mediated by a relay system that ultimately regulates the PTH/PTHrP pathway.

Signaling by BMP involves two types of transmembrane serine/threonine kinases called the type I and type II receptors (for review, see Massagué 1996). Ligand binding to both receptors results in the phosphorylation of the type I receptor by the type II receptor. The type I receptor then transduces the signal by phosphorylating intracellular targets, including members of the Smad family (Hoodless et al. 1996; Liu et al. 1996). Constitutively active mutant forms of the TGF β type I receptor signal in the absence of ligand or type II receptor (Wieser et al. 1995). In vertebrates, two type I BMPRs, BMPR-IA (ALK3, BRK1) and BMPR-IB (ALK6, BRK-II, RPK-1) have been identified that, in combination with the type II receptors BMPR-II or ACTR-II, bind BMP2, BMP4, and BMP7 (Koenig et al. 1994; ten Dijke et al. 1994b; Liu et al. 1995; Nohno et al. 1995; Rosenzweig et al. 1995). Additionally, BMPs can interact with ACTR-I, a type I receptor that is shared with the TGF β -related factor activin (see references in Massagué 1996).

Signaling through type I receptors for different TGF β -related ligands has been shown to result in distinct outcomes (Massagué 1996, and references therein). However, separate roles for different type I receptors within a specific subclass have not been observed previously. For the two type I BMPRs, it is not known whether they have specific roles. In vitro cell culture studies have not identified differences in the signaling function of BMPR-IA and BMPR-IB, despite significant differences in the primary structure of the receptor kinase domains (Liu et al. 1995; Rosenzweig et al. 1995; Hoodless et al. 1996; Kretzschmar et al. 1997). In *Drosophila*, two type I receptors, Thickveins (Tkv) and Saxophone (Sax), both mediate the signaling activity of the BMP2/BMP4 homolog, Decapentaplegic (Dpp) (for review, see Massagué and Weis-Garcia 1996). However, studies to date indicate these receptors are functionally indistinguishable (Brummel et al. 1994; Singer et al. 1997). In contrast, by examining their role in the development of the chicken limb,

we provide the first demonstration that the two type I BMPRs control different developmental responses.

Here we examine the expression pattern of *BmpR-IA* and *BmpR-IB* in the developing limb. We then created point mutations in the two vertebrate type I BMPRs to block or constitutively activate BMP signaling pathways. We demonstrate that BMPR-IA and BMPR-IB play distinct roles in multiple aspects of BMP signaling in the developing limb. BMPR-IB is necessary for the early steps of mesenchyme condensation and cartilage formation. BMPR-IB also regulates programmed cell death. In contrast, BMPR-IA is essential for proper regulation of the later chondrocyte differentiation program. Moreover, our studies indicate the convergence of three signaling pathways in endochondral bone morphogenesis: IHH acts upstream of BMPs, which signal to BMPR-IA in two regions of the cartilage element, locally to influence the prehypertrophic chondrocytes and at a distance to regulate PTHrP production, which feeds back on less mature chondrocytes to regulate the rate of chondrocyte differentiation.

Results

Generation of BMPR mutations

The studies described here have investigated the role of BMP signaling during three developmental processes. Two of these roles are related to development of the cartilage elements: the early process of its formation and the later process of chondrocyte differentiation. In the third process, BMP signaling regulates apoptosis during limb development.

Mutant forms of the BMPRs were generated. A single amino acid substitution (Q to D) within the GS activation domain was made to generate constitutively active forms (Wieser et al. 1995). The caBMPRs have elevated kinase activity and can signal in the absence of ligand or type II receptor (Hoodless et al. 1996; Kretzschmar et al. 1997; data not shown). Dominant negative mutant forms were generated by a single amino acid substitution (K to R) within the adenosine triphosphate binding site, which reduced kinase activity dramatically (Zou and Niswander 1996; data not shown). Each of the mutant receptor constructs was cloned into an avian replication-competent retroviral vector (RCAS; Hughes et al. 1987), and high titer retroviral stocks were generated and used to infect either the developing chick limb in ovo or limb mesenchyme cultures.

BmpR-IB expression prefigures the cartilage primordia

In the early step of cartilage formation, undifferentiated mesenchymal cells condense and thereafter assume a chondrocytic lineage. As BMPs are thought to be involved in chondrogenesis, we isolated cDNAs encoding chick full-length *BmpR-IA* and *BmpR-IB* and examined their mRNA expression patterns during this process. *BmpR-IB* is strongly expressed in precartilaginous condensation zones in the limb beginning at Hamburger-

Hamilton stage 24 (Fig. 1A; data not shown; Hamburger and Hamilton 1992). *BmpR-IB* expression precedes the early cartilage marker Collagen type II (*Col-II*; Fig. 1B). Following initial chondrocyte differentiation, when *Col-II* is strongly expressed, *BmpR-IB* expression decreases in the core region of the cartilage element but persists in the peripheral, less mature regions (Fig. 1, cf. A and B). From stage 27 to 32, *BmpR-IB* is strongly expressed in the developing digits, particularly in the distal phalangeal region where condensation is still actively occurring (Fig. 1C). A similar expression pattern at this later period has also been reported in chick and mouse limbs (Yamaguchi et al. 1991; Dewulf et al. 1995; Ishidou et al. 1995; Kawakami et al. 1996). Thus, *BmpR-IB* is expressed in all of the cartilage condensations of the limb and is also expressed in other regions of endochondral

bone formation including the vertebrae (data not shown). In contrast, *BmpR-IA* mRNA was detected at low levels throughout the limb bud mesenchyme (from stage 18 to stage 29), with highest levels of expression in the distal progress zone mesenchyme (Fig. 1D; data not shown), where cell fate specification is thought to occur.

BMPR-IB activity promotes cartilage formation

Infection of a stage 20 fore- or hindlimb bud with caBMPR-IB, as well as caBMPR-IA, led to a dramatic expansion of the cartilaginous elements ($n > 100$). The humerus, radius, and ulna, as well as the femur, tibia, and fibula, were often fused, suggesting a disruption of joint formation (Fig. 1E,F). In the most dramatic cases, little or no muscle or other soft tissues was observed in the in-

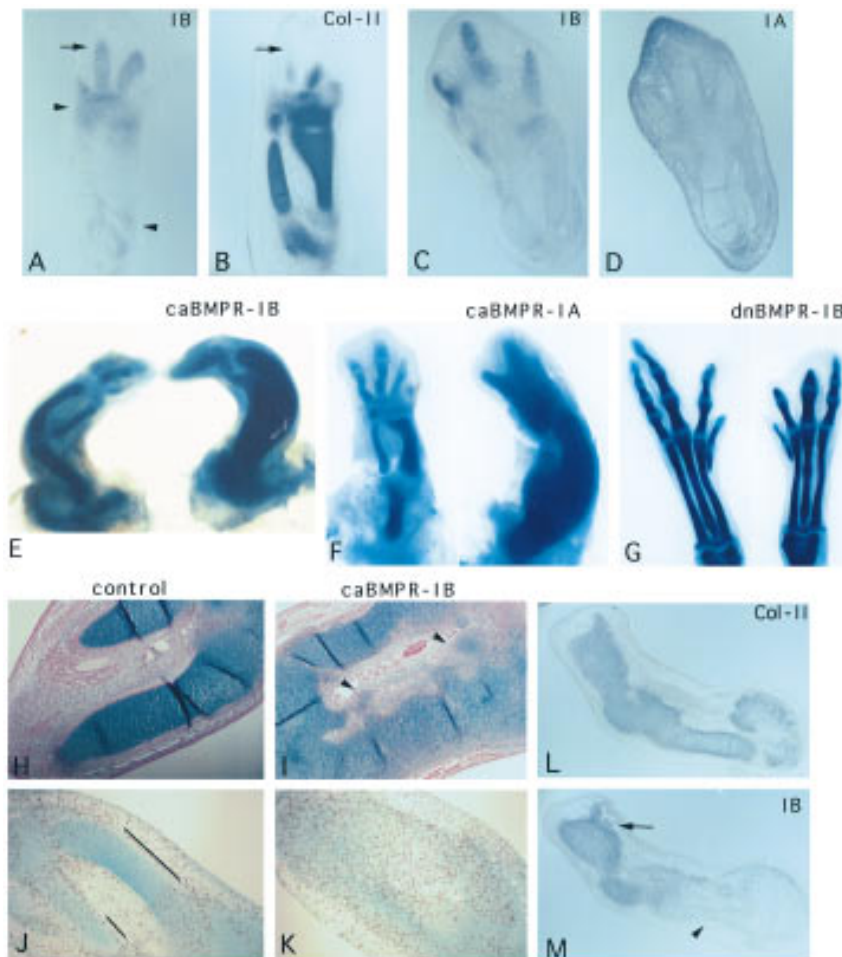


Figure 1. BMPR-IB expression prefigures the future cartilage and its activity is necessary and sufficient for in vivo chondrogenesis. (A–D) Section RNA in situ hybridization with digoxigenin-labeled probes. Stage 27 hindlimb (A) and stage 30 forelimb (C) show that *BmpR-IB* RNA (purple stain) was strongly expressed in precartilaginous condensation zones, at these stages most strongly in the phalangeal region (arrow). Expression of *BmpR-IB* preceded that of *Col-II* (B), an early marker of chondrocytes. *BmpR-IB* expression decreased in the core region of the cartilage element where *Col-II* was strongly expressed, but persisted in regions of less mature chondrocytes (arrowheads). (D) *BmpR-IA* RNA was detected at low levels throughout the limb bud mesenchyme, with highest levels in the distal mesenchyme. (E–G) Day 10 embryos stained with alcian blue to reveal cartilage. (E) Infection of stage 20 forelimb with caBMPR-IB virus or (F) stage 14 presumptive hind-limb field with caBMPR-IA resulted in expanded and fused cartilage elements (right), compared with the contralateral control limb (left). (G) Infection of a stage 21 hindlimb bud with dnBMPR-IB resulted in a loss of distal phalanges (right). Metatarsals were shorter and thinner than in the contralateral limb (left). Regression of the interdigital webbing was inhibited (see also Zou and Niswander 1996). (H,I) Histological section of stage 30 limbs stained with alcian blue. Uninfected left wing has a smooth perichondrium (H). In the contralateral right wing (I) infected at stage 16 with ~3 times less caBMPR-IB virus than in (E,F), the perichondrium was indistinct and swirls of ectopic mesenchymal condensations were observed in the region of soft tissue (arrowheads).

(J,K) BrdU labeling studies. Very few cells incorporated BrdU within the core regions of the cartilage elements of a stage 30 uninfected left wing (J; highlighted by lines), whereas significantly more cells incorporated BrdU throughout the cartilage of the caBMPR-IB-infected contralateral wing (K). High levels of BrdU incorporation in the cartilage were also observed following caBMPR-IA infection (not shown). (L,M) caBMPR-IA results in expansion of the chondrogenic region as distinguished by *Col-II* expression (L) and induces the ectopic expression of endogenous *BmpR-IB* (M; arrow). *BmpR-IB* expression is down-regulated in more mature chondrocytes (arrowheads), similar to normal chondrogenesis.

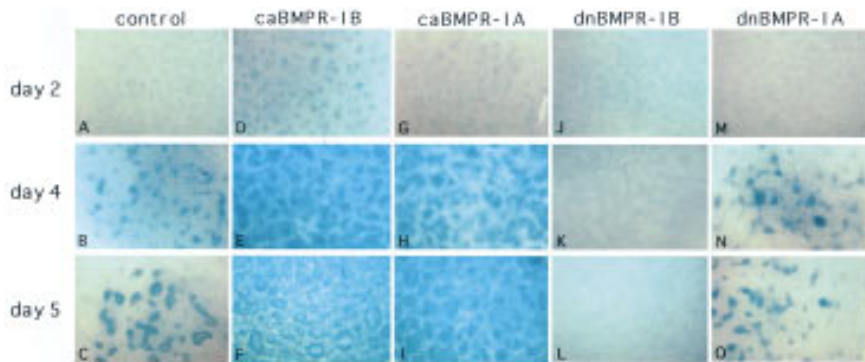


Figure 2. BMPR-IB activity is necessary for *in vitro* cartilage formation. High-density micromass cultures derived from stage 22–24 limb buds stained with alcian blue to reveal cartilage nodules after culture for 2 days (*top row*); 4 days (*middle row*), or 5 days (*bottom row*) following (A–C) no viral infection, or infection with (D–F) caBMPR-IB; (G–I) caBMPR-IA; (J–L) dnBMPR-IB; (M–O) dnBMPR-IA virus.

fectured limb. The length of most skeletal elements was comparable to those of the contralateral control limb. The number of digits was often reduced by one (similar reduction was observed following caBMPR-IA infection; see Fig. 1F). Because of the aberrant morphology and lack of molecular probes that distinguish specific digits, we cannot distinguish between a patterning defect or an alteration in the distribution of mesenchymal cells into the expanded condensations such that fewer mesenchymal cells may be left to contribute to subsequent condensations.

To analyze the effect of caBMPR-IB on early cartilage condensation, the limbs were examined histologically following infection with a lesser amount of virus for more subtle effects. Cartilage condensation still occurred over a much broader region than in the contralateral limb (Fig. 1, cf. H and I). Whereas the normal perichondrium formed a smooth sheath of spindle-shaped cells around the cartilage (Fig. 1H), the perichondrium of the infected elements was severely disrupted histologically and as evidenced by the lack of expression of perichondrial molecular markers, for example, PTH/PTHrP receptor and Gli (data not shown). Ectopic mesenchymal condensations were observed and the cells were classified as chondrocytes by alcian blue staining (Fig. 1I) and *Col-II* RNA expression (not shown). Thus, misexpression of activated BMPR-IB in the undifferentiated limb mesenchyme results in an expansion of the chondrogenic region.

This expansion may result from an increase in the rate of chondrocyte proliferation and/or recruitment of muscle/soft tissue into a chondrocytic lineage. To study the former possibility, bromodeoxyuridine (BrdU) incorporation analyses were performed ($n = 3$ each for caBMPR-IB and for caBMPR-IA). In control limbs (Fig. 1J), very few cells incorporated BrdU in the core region of the cartilage elements, but higher levels of proliferation were noted in the flanking chondrogenic regions, the perichondrium, and soft tissues. In caBMPR-IB infected limbs (Fig. 1K), significantly more cells incorporated BrdU in the core region of the cartilage, such that the number of BrdU-labeled cells was similar along the entire cartilage shaft. Similar results were noted for caBMPR-IA infected limbs. Therefore, increased chondrocyte proliferation can account at least in part for the dramatic expansion of the chondrogenic region.

We further confirmed that BMPR-IB-mediated activity promotes the early steps of chondrogenesis using limb mesenchyme micromass cultures. Uninfected micromass cultures differentiate after 4 days of culture, giving rise to multiple cartilage nodules (Fig. 2A–C). Micromass cultures infected with caBmpR-IB RCAS virus gave rise to cartilage nodules after 2 days, almost two days earlier than control cultures (Fig. 2, cf. D with A and B). There was also a marked increase in the number and size of cartilage nodules (Fig. 2E,F), similar to that observed in micromass cultures treated with BMP protein (Duprez et al. 1996b; S. Pizette and L. Niswander, unpubl.) and with caBMPR-IA virus (see Fig. 2G–I).

BMPR-IB is necessary for cartilage formation

As *BmpR-IB* is strongly expressed in the precartilaginous mesenchymal condensations and ectopic expression of caBMPR-IB elicits extensive mesenchymal condensation, we sought to determine whether BMPR-IB is essential for cartilage formation. Ectopic expression of dominant-negative BMPR-IB (dnBMPR-IB) *in vivo* disrupted cartilage formation and resulted in the loss of the distal phalanges ($n > 30$; Zou and Niswander 1996; Fig. 1G). In addition, the metatarsals were usually shorter and thinner than normal. In more severe cases in the hindlimb, the fibula was missing and the pelvis was disrupted (data not shown).

In limb micromass cultures, dnBMPR-IB completely blocked cartilage formation (Fig. 2J–L). Interestingly, infection of the micromass culture with dnBMPR-IA had no effect on nodule formation (Fig. 2M–O; Kawakami et al. 1996). Although both caBMPR-IB and caBMPR-IA can promote chondrogenesis, BMPR-IB appears to be a more direct effector of chondrogenesis. Examination of endogenous *BmpR-IB* transcripts after infection with caBMPR-IA both *in vitro* and *in vivo* revealed that *BmpR-IB* mRNA was induced by caBMPR-IA but not vice versa (Fig. 1M; data not shown). This suggests that there may exist a hierarchy of receptor action such that BMPR-IB is the mediator of chondrogenesis and the promotion activity of caBMPR-IA on chondrogenesis is a result of induction of endogenous *BmpR-IB* (H. Zou et al., in prep.). Taken together, our results indicate that BMPR-IB transduces BMP signals necessary for the early steps of cartilage formation.

BmpR-IA is expressed in prehypertrophic chondrocytes overlapping the Ihh domain

During endochondral bone formation and later in the growth plate, chondrocytes differentiate progressively, passing sequentially through proliferative, hypertrophic, and degenerative stages (see Fig. 4I, below). Several *Bmps* are expressed in the perichondrium, including *Bmp2*, *Bmp4*, and *Bmp7* (shown in Fig. 3A–C; Kingsley 1994), and *Bmp6* is expressed in prehypertrophic and hypertrophic cells (Kingsley 1994; Vortkamp et al. 1996). However, it is not known which chondrocytes receive and respond to BMP signaling, or what role this signaling plays in the regulation of chondrocyte function and differentiation.

We first examined the expression patterns of *BmpR-IA* and *BmpR-IB* in the limb cartilage elements. *BmpR-IB* appears to be expressed at a low level in the cartilage elements after their initial formation (Fig. 1A,C; data not shown). *BmpR-IA*, in contrast to its earlier ubiquitous expression, became restricted and was highly expressed in prehypertrophic chondrocytes, as defined histologically and by overlapping expression with the *Ihh* domain (Fig. 3D,E). The onset of expression of *Ihh* in prehypertrophic chondrocytes precedes that of *BmpR-IA* by ~1 day. As chondrocyte differentiation proceeded, *BmpR-IA* expression faded in the central hypertrophic region [as defined by expression of collagen type-X (*Col-X*)] but continued to be expressed in prehypertrophic chondrocytes that now flank the hypertrophic region (data not shown), again similar to that of *Ihh*. This pattern of overlapping *BmpR-IA* and *Ihh* expression continued in the fetal growth plate (Fig. 3F,G). *BmpR-IA* was also expressed in the joint region similar to that of PTHrP (Fig. 3H,I; Vortkamp et al. 1996), and in the inner layer of the

perichondrium (Fig. 3F). The highly localized expression pattern of *BmpR-IA* within prehypertrophic chondrocytes suggests that these cells are the direct target of BMP signals secreted from the adjacent perichondrium. The prehypertrophic chondrocytes themselves, as well as hypertrophic chondrocytes, which express *Bmp6*, may also signal to BMPR-IA. However, the affinity of BMP6 for BMPR-IA is less well characterized. In addition, BMPs in the perichondrium may signal to the periarticular joint region, which expresses *BmpR-IA*.

caBMPR-IA delays chondrocyte differentiation

To investigate the role of BMPR-IA-mediated signaling during chondrogenesis, *caBMPR-IA* infected wings were examined histologically. At embryonic day 9, the mid-section of the contralateral uninfected radius and ulna had undergone hypertrophic cartilage differentiation (Fig. 4A). In the majority of infected limbs, however, the cartilage elements lacked hypertrophic cartilage, and the chondrocytes appeared immature (Fig. 4B).

This apparent delay in differentiation was investigated further using molecular markers. In Figure 4C–F, infection was targeted to the posterior distal region of a stage 21 limb (note expansion, fusion, and developmental delay of the posterior digits). *Col-II*, a general marker of all chondrocytes except hypertrophic chondrocytes, was expressed throughout the infected posterior region, indicating the chondrocytes were viable and produced cartilage matrix (Fig. 4C). In contrast, *Col-X* could not be detected in the infected region, even though it was expressed in hypertrophic cartilage in uninfected regions and in the contralateral limb (Fig. 4D). *Ihh* was expressed in uninfected prehypertrophic chondrocytes but was not de-

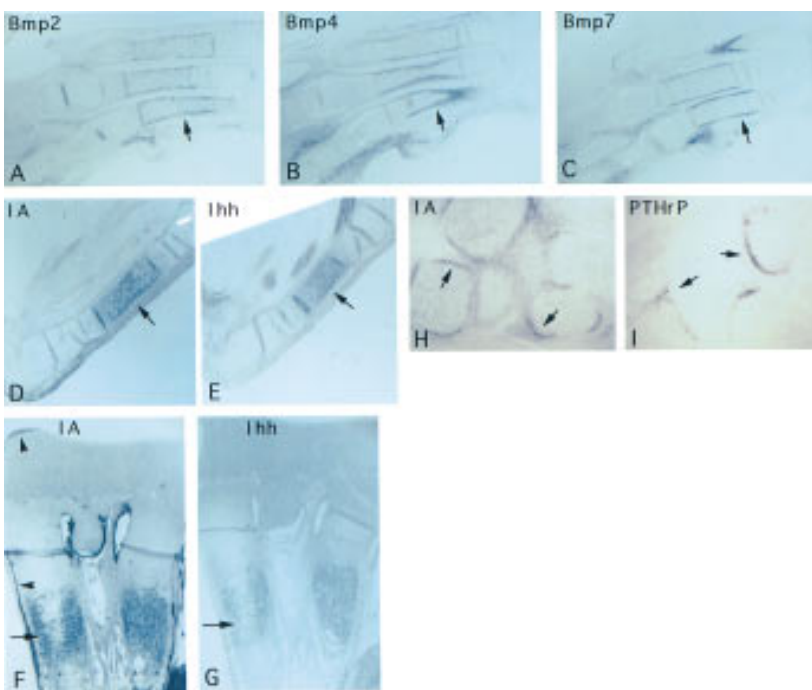


Figure 3. Expression patterns of *Bmp*, *BmpR-IA*, and *Ihh* during cartilage differentiation. (A–C) Serial sections of a stage 31 metatarsal region processed for RNA in situ hybridization with (A) *Bmp2*, (B) *Bmp4*, and (C) *Bmp7*. All three *Bmps* are expressed in the perichondrium that surrounds the cartilage elements. Serial sections through a stage 32 ulna (D,E) or through the growth plate of an embryonic day 15 distal tibia (F,G) hybridized with (D,F) *BmpR-IA* or (E,G) *Ihh*. *BmpR-IA* and *Ihh* are largely coexpressed in the prehypertrophic chondrocytes (arrows). *BmpR-IA* RNA is also detected in the periarticular region, as well as in the inner layer of the perichondrium (arrowheads in F). (H,I) Sections through the periarticular region of a stage 34 carpal region hybridized with (H) *BmpR-IA* and (I) PTHrP. Both *BmpR-IA* and PTHrP are expressed in other joints (not shown). Arrows in (A–I) highlight the expression domains.

tected in the infected region (Fig. 4F). *ptc* and *Gli*, presumed molecular markers of Hedgehog signaling, were also not detected in the infected regions (data not shown). PTH/PTHrP receptor expression normally overlaps slightly with the *Ihh* domain, extending into a region of less mature chondrocytes and in the perichondrium. In the infected elements, PTH/PTHrP receptor was expressed at low levels and not highly localized (Fig. 4E). Therefore, our histological and molecular analysis

indicates that the delay in differentiation resulting from misexpression of caBMPR-IA is at a step prior to the formation of prehypertrophic chondrocytes. Interestingly, the ectopic caBMPR-IA phenotype is similar to that observed following ectopic expression of *Ihh* or *Bmp* in the limb (Duprez et al. 1996a; Vortkamp et al. 1996).

In the studies of Vortkamp et al. (1996), misexpression of *Ihh*-induced PTHrP in the periarticular joint region. We found that misexpression of caBMPR-IA also induced

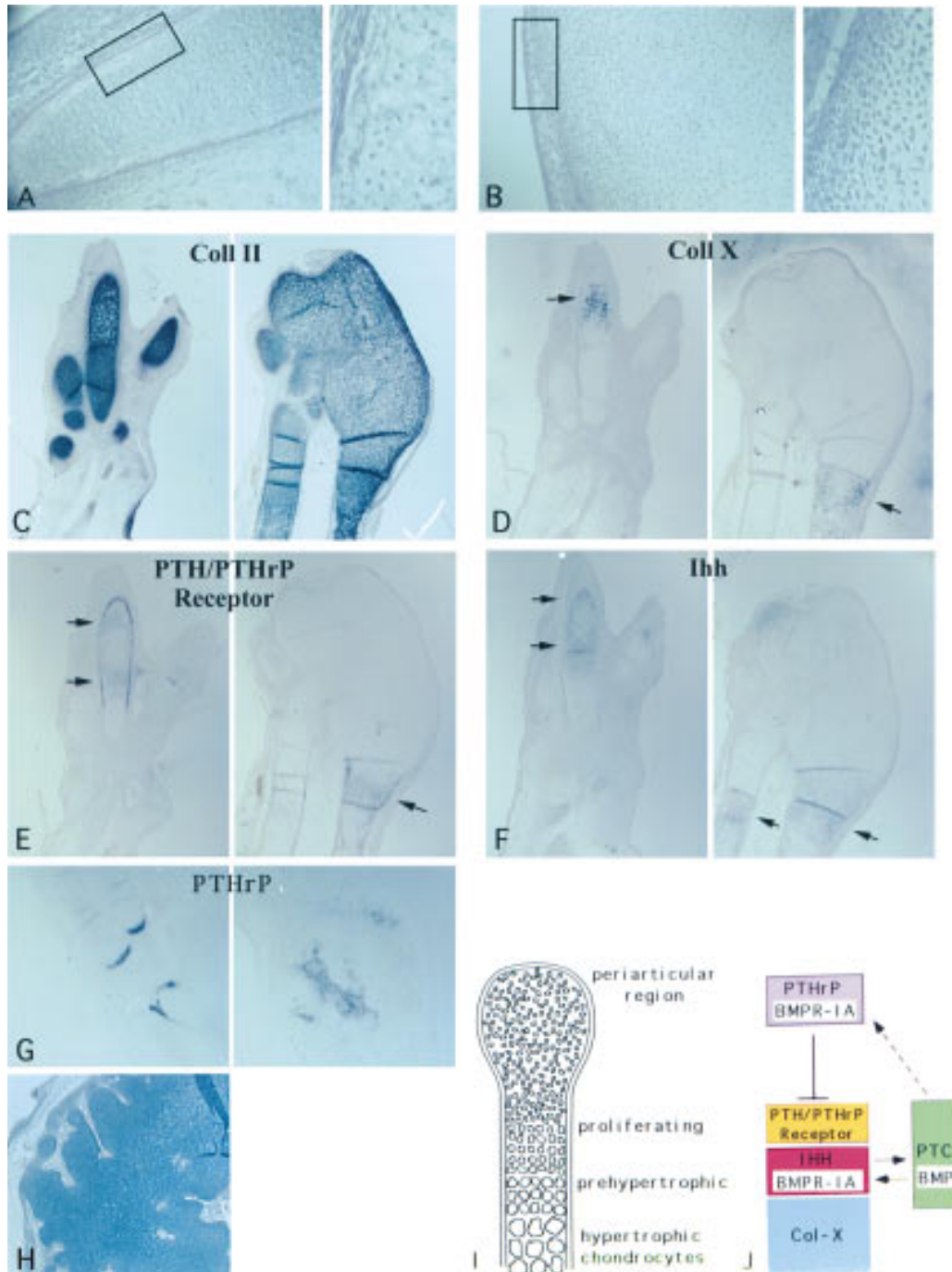


Figure 4. (See facing page for legend.)

PTHrP expression in a similar manner (Fig. 4G). However, as indicated above, ectopic caBMPR-IA does not induce *Ihh* expression.

caBMPR-IB does not delay chondrocyte differentiation

We also examined the consequences of misexpression of caBMPR-IB on chondrocyte differentiation. Although there was extensive cartilage formation following ectopic caBMPR-IB (shown in Figs. 1 and 5B), there was no apparent delay in chondrocyte differentiation as shown by *Col-X*, *Ihh*, and PTH/PTHrP receptor expression. Interestingly, the proximodistal (i.e., longitudinal) differentiation pattern within each infected cartilage element was affected. *Col-X* and PTH/PTHrP receptor are normally expressed in discrete proximodistal regions of each cartilage element in a complementary pattern, with *Col-X* expression in the center and PTH/PTHrP receptor expression flanking (Fig. 5C,E). In the infected limb where phalanges were fused and joints missing, these mRNAs were expressed along the entire proximodistal axis, although they still displayed a complementary pattern (Fig. 5D,F).

Irregular differentiation and ossification following caBMPR-IA misexpression

By day 13 of incubation, in caBMPR-IA infected limbs, we noted a disarray in the pattern of ossification. During endochondral development, blood vessel invasion and ossification normally start in the midsection of the cartilage element. Strikingly, however, in the caBMPR-IA-infected limbs, this occurred at multiple sites scattered

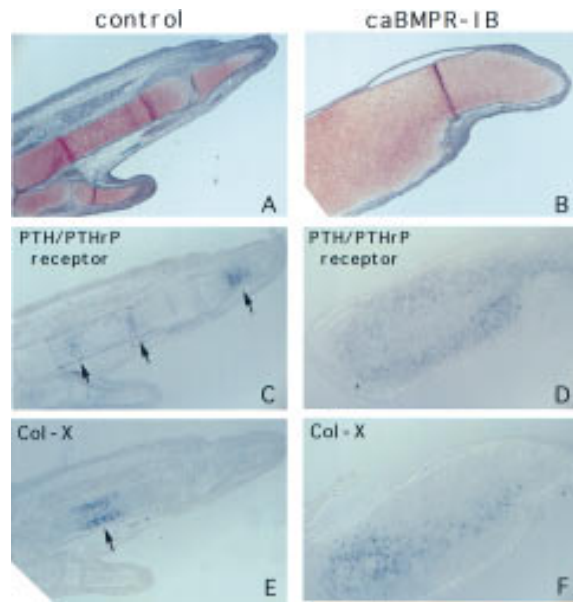


Figure 5. caBMPR-IB does not delay chondrocyte differentiation. Serial sections through the digit region of a day 8.5 uninfected limb (A,C,E) and limb infected with caBMPR-IA at stage 20 (B,D,F). (A,B) Weigert-Safranin stain revealed extensive cartilage formation, fusion of the phalanges, and lack of joint formation in the infected limb. (C-F) RNA section in situ hybridization with PTH/PTHrP receptor (C,D) or *Col-X* (E,F). In the infected fused phalanges (D,F), PTHrP receptor and *Col-X* mRNAs displayed complementary expression patterns but were detected along the entire proximodistal axis; in contrast to the discreet proximodistal localization of these RNAs in the contralateral limb (C,E; arrows).

Figure 4. caBMPR-IA delays chondrocyte differentiation and induces PTHrP expression. Histological sections of day 9 embryonic wings (A) uninfected and (B) caBMPR-IA-infected at stage 14. The infected cartilage element lacked hypertrophic chondrocytes (HC) that were clearly present in the contralateral control. The boxed areas are enlarged on right. (C-F) Serial sections of day 8 uninfected and caBMPR-IA-infected wings (left and right, respectively). Infection was targeted to posterior distal limb mesenchyme at stage 21 resulting in expansion and fusion of the posterior digits. RNA in situ hybridization revealed that (C) *Col-II* was expressed in both the infected and uninfected limbs, indicating the cells were viable; (D) *Col-X* was expressed in HC in the uninfected limb and in the proximal part of the infected limb (arrows); however, it could not be detected in the infected digit region; (E) *Ihh* was expressed in prehypertrophic chondrocytes (preHC) in the uninfected limb and in the proximal part of the infected limb (arrows), but could not be detected in the infected digit region; (F) PTHrP Receptor was expressed in the uninfected chondrocytes (arrows) overlapping the expression domain of *Ihh* (F) and in the perichondrium; it was also readily detected in the proximal part of the infected limb (arrow), but was not highly localized in the infected digit region. (G) RNA section in situ hybridization of stage 30 tarsal region of uninfected and caBMPR-IA-infected hindlimb (left and right, respectively). PTHrP expression was induced in a broader region of the periarticular cartilage by misexpression of caBMPR-IA. (H) Histological section through an embryonic day 10.3 joint region of a limb infected with caBMPR-IA at stage 14. Ectopic vascularization was scattered throughout the cartilage element, including the joint area (shown here), which normally is not invaded by blood vessels. (I) Schematic drawing of the cartilage differentiation program. Chondrocytes undergo a program of proliferation, maturation from preHC to HC, and, eventually (not shown), calcification and cell death. (J) Model of regulation of chondrocyte differentiation through BMPR-IA-mediated signaling. We propose that there exists a local signal relay loop (depicted by two arrows). Proliferating chondrocytes (expressing PTH/PTHrP Receptor; yellow) exit the cell cycle and begin their maturation from preHC (expressing *Ihh* and *BmpR-IA*; red) to HC (expressing *Col-X*; blue). IHH, produced by preHC, signals to the adjacent perichondrial cells (which express *ptc*; green; Vortkamp et al. 1996). The perichondrium responds to IHH by expressing *Bmps* (green) and the BMP proteins signal back to BMPR-IA in the preHC. We also suggest that BMP from the perichondrium is an important relay signal in the proposed regulatory loop between IHH and PTHrP. Perichondrial BMPs could act on (dotted arrow) BMPR-IA in the periarticular region (purple) to regulate PTHrP production. PTHrP then signals to chondrocytes expressing PTH/PTHrP receptor (yellow) to regulate the rate of differentiation. Thus, we suggest that *BmpR-IA* expression in both the preHC and periarticular chondrocytes is important in regulating the progression of chondrocytes through the differentiation pathway. Model adapted from Vortkamp et al. (1996).

randomly throughout the cartilage shaft, sometimes even including the joint regions (Fig. 4H). This ectopic vascularization and irregular ossification is similar to that following misexpression of *Ihh* in the chick limb, and of PTHrP in transgenic mice (Vortkamp et al. 1996; Weir et al. 1996).

Near the ectopic ossification sites, we also observed an apparent reversal of polarity in the pattern of chondrocyte differentiation: Histologically chondrocytes became hypertrophic at the circumference of the long bone rather than the middle. Chondrocytes in the middle were separated by large amounts of matrix that was not stained by cartilage and bone-specific dyes (Alcian blue or Safranin O), suggesting that those cells were not viable. At later embryonic stages, irregular areas of cartilage persisted in the bone marrow cavity. The bones were abnormal in shape and lacked normal growth plates. The reversal in chondrocyte differentiation pattern is similar to that observed in PTHrP transgenic mice (Weir et al. 1996). Consistent with this, misexpression of caBMPR-IA in the limb induced expression of PTHrP (described above). Thus, this phenotype is likely to occur through the PTH/PTHrP pathway.

BMPR-IB is involved in programmed cell death in the embryonic limb

We showed previously that blocking BMP signaling by dnBMPR-IB in the embryonic limb results in a reduction in apoptosis leading to soft tissue syndactyly (webbing) (Zou and Niswander 1996). Here we have used caBMPR-

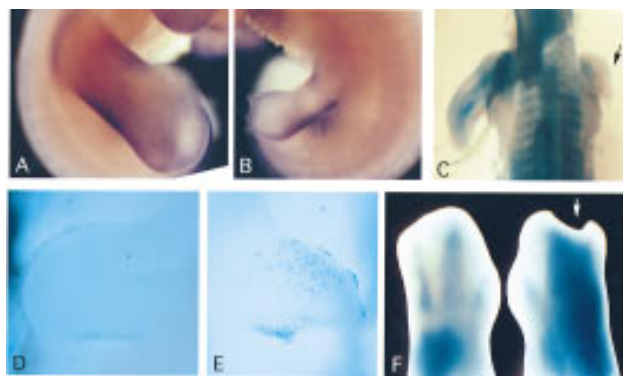


Figure 6. caBMPR-IB regulates cell death in the embryonic limb. (A) Day 7 control uninfected hindlimb. (B) caBMPR-IB infection of the contralateral limb at stage 15 resulted in a very thin limb that also displayed a degenerating AER and distal shortening. (C) In the most dramatic cases, caBMPR-IB infection resulted in extreme truncation of the limb (arrow). (D, E) Nile blue staining to detect nonviable cells. Forty-eight hours after caBMPR-IB infection, the infected forelimb (E) displayed a marked increase in the number of Nile blue-stained mesenchymal and AER cells compared with the contralateral limb (D). (F) Posteriorly localized caBMPR-IB infection of a stage 22 forelimb resulted in an accelerated regression of interdigital soft tissue at stage 30 (right) compared with the contralateral limb (left). The posterior phalanges were expanded and fused.

IB and found the opposite effect: caBMPR-IB infection of the developing limb led to increased cell death. Early caBMPR-IB infection throughout the limb field (before stage 17) resulted in a very thin limb, with the antero-posterior diameter about half the size of a control limb (Fig. 6A,B). The proximodistal length was also often affected, resulting in distal truncations. The cartilage that did form was expanded to fill the limb, as described above. In the most dramatic cases (~10%), the infected limb was severely truncated or absent (Fig. 6C). Examination 2 days following extensive infection revealed that the apical ectodermal ridge (AER) was disrupted, with some sections apparently degenerating. The distal portion of the limb was often split into several small outgrowths, presumably the consequence of degeneration of the AER and underlying mesenchymal tissue. caBMPR-IB infected limbs stained with a vital dye showed a marked increase in the number of nonviable mesenchymal and AER cells (Fig. 6D,E).

Later and more localized caBMPR-IB infection of stage 22 posterior mesenchyme resulted in accelerated regression of the interdigital soft tissue (Fig. 6F), opposite to the dnBMPR-IB-induced webbing phenotype. Massive cell death was not observed in these later infected limbs. In contrast, caBMPR-IA did not result in increased cell death, nor did dnBMPR-IA give rise to webbing phenotype (infection performed between stage 14 and 24).

Discussion

The type I BMPRs control distinct processes during vertebrate limb development

A major conclusion from this study is that the two type I BMPRs mediate distinct responses during limb development (summarized in Fig. 7). Several lines of evidence support this conclusion. First, the expression pattern of the two type I receptors differ markedly. *BmpR-IB* expression is not detectable in early limb mesenchyme but is detected in early condensations where expression prefigures the cartilage primordia (this paper; Kawakami et al. 1996). *BmpR-IA* is expressed at low levels throughout the limb mesenchyme with highest expression in the distal mesenchyme, and later becomes localized to the prehypertrophic chondrocytes. Second, BMPR-IB activity is necessary and sufficient for cartilage condensation, both in vivo and in vitro, as tested by misexpression of both gain-of-function caBMPR-IB and loss-of-function dnBMPR-IB. BMPR-IA does not appear to be a direct effector of cartilage condensation. Instead, our data suggest that BMPR-IA acts through BMPR-IB to elicit mesenchyme condensation (Fig. 1M; H. Zou et al., in prep.). Third, BMPR-IA is involved in the regulation of chondrocyte differentiation during late stages of cartilage development. *BmpR-IA* is expressed in prehypertrophic chondrocytes, and misexpression of caBMPR-IA results in a delay in chondrocyte differentiation. *BmpR-IB* expression is down-regulated during chondrogenesis and misexpression of caBMPR-IB does not inhibit chondro-

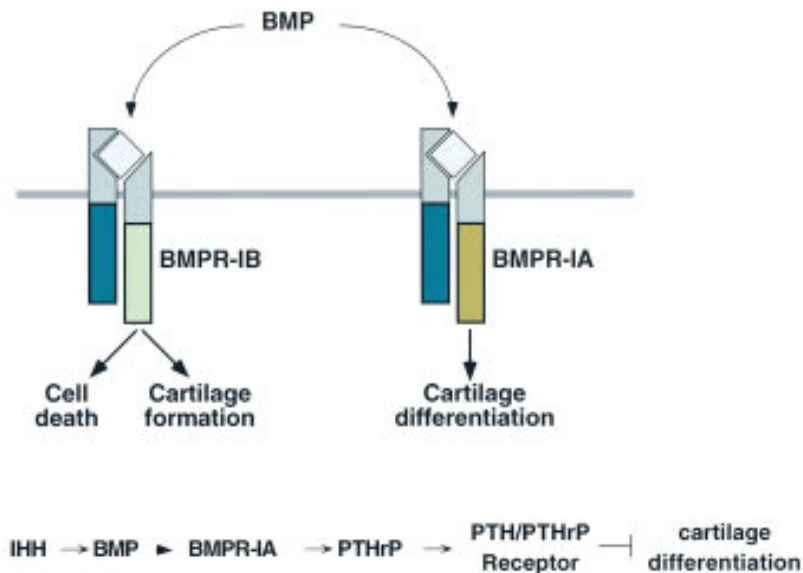


Figure 7. BMPR-specific functions in limb development and molecular pathways elicited by IHH signaling. (*Top*) We propose that BMPR-IB specifically regulates the intracellular pathways that lead to cell death and formation of the initial cartilaginous skeleton in response to BMP, whereas BMPR-IA regulates the rate of chondrocyte differentiation. Schematic adapted from Massagué and Weis-Garcia (1996). (*Bottom*) Proposed molecular pathway downstream of IHH. See Discussion for description. Direct interaction is indicated by an arrowhead. Steps indicated by an arrow may be direct or indirect.

cyte differentiation. Finally, BMPR-IB is involved in BMP-mediated programmed cell death. Use of the dn-BMPR-IB to block BMP signaling results in decreased apoptosis in the interdigital soft tissue, leading to a webbed phenotype, whereas caBMPR-IB causes a marked increase of cell death in soft tissues. In contrast, our dnBMPR-IA and caBMPR-IA point mutations do not affect cell death.

Our studies thus provide the first demonstration that different type I BMPRs can elicit distinct developmental outcomes when expressed in the same cells. In vitro cell culture studies have not separated type I BMPR action, although this may reflect the paucity of biochemical and molecular criteria to evaluate receptor signaling. Two *Drosophila* type I BMPRs, Tkv and Sax, appear to be functionally equivalent, as loss-of function Tkv and Sax clones in the embryo and wing disc produce similar phenotypes, and Tkv can functionally substitute for Sax (Brummel et al. 1994; Nellen et al. 1994; Singer et al. 1997).

The vertebrate BMPR-IA and BMPR-IB have similar but not identical binding affinities in vitro for the BMP ligands that are expressed in the developing limb (Francis et al. 1994; Koenig et al. 1994; ten Dijke et al. 1994b; Francis-West et al. 1995; Liu et al. 1995; Nohno et al. 1995; Rosenzweig et al. 1995). It has been suggested that the temporally and spatially regulated expression of BMPs controls the formation of particular skeletal elements (Kingsley 1994). Further refinement may be regulated by the temporal and spatial expression and activity of the type I and type II BMPRs. Additional specificity may also be controlled by the differential intracellular response to BMPR-mediated signal transduction. Future studies should help distinguish how different combinations of TGF β -family ligands, their receptors, and intracellular targets exquisitely regulate the complex formation of skeletal elements in the proper number, position, shape, and size.

Regulation of chondrocyte differentiation through BMPR-IA mediated signaling

The delay in chondrocyte differentiation caused by misexpression of caBMPR-IA phenocopies misexpression of *Ihh*. Expression of *BmpR-IA* in prehypertrophic chondrocytes overlaps that of *Ihh*. It has been suggested that IHH signals to the perichondrium, which expresses *ptc*, a proposed Hedgehog target that is also involved in reception of the HH signal (Nusse 1996; Vortkamp et al. 1996, and references therein). Studies in *Drosophila* and vertebrates suggest that signaling by the Hh/Ptc pathway may regulate BMP expression (references in Laufer et al. 1994; Blair 1995). Consistent with this, *Bmp2*, *Bmp4*, and *Bmp7* are expressed in the perichondrium (Fig. 3A–C; Kingsley 1994). These ligands bind BMPR-IA, which is expressed in prehypertrophic chondrocytes (Fig. 3D,F). This suggests the existence of a local signal relay loop, as illustrated in Figures 4J and 7, which could provide a means to regulate the rate of chondrocyte differentiation and balance the relative populations of chondrocytes among the different zones. In our model, as proliferating chondrocytes exit the cell cycle and start to hypertrophy, they transiently express high levels of *Ihh*. IHH acts on the adjacent perichondrium, which expresses *ptc*. These cells respond to IHH by expressing BMPs, which then signal back to BMPR-IA in the prehypertrophic chondrocytes. Our misexpression studies indicate that the downregulation of BMPR-IA activity is necessary for the initiation of the hypertrophic differentiation process. Thus, we propose another role for BMP signaling in the limb: the regulation of the rate of chondrocyte differentiation through BMPR-IA. These studies also imply that the perichondrium acts as an intermediate in the regulation of cartilage differentiation. Thus, as maturing chondrocytes produce IHH, they set off a signaling cascade mediated through BMPs to inhibit the differentiation of neighboring cells.

BMP signaling from the perichondrium might also be an important relay signal in the proposed regulatory loop between IHH and PTHrP (see Figs. 4J and 7). Misexpression of caBMPR-IA or BMPs delays chondrocyte differentiation, similar to the misexpression phenotypes of IHH or PTHrP (this paper; Duprez et al. 1996a; Vortkamp et al. 1996; Weir et al. 1996). Moreover, ectopic caBMPR-IA or IHH results in increased PTHrP expression (this paper; Vortkamp et al. 1996). PTHrP is localized to the periarticular region, both normally and in limbs misexpressing caBMPR-IA or IHH, quite distant from the endogenous *Ihh* domain. In the model proposed by Vortkamp et al. (1996), IHH regulates PTHrP expression in the periarticular region, most likely indirectly based on lack of *ptc* expression in the periarticular region. One possible mechanism for this longer-range effect involves relay signals. BMPs expressed in the perichondrium could act on the periarticular joint region, where *BmpR-IA* is expressed, to relay the IHH signal to PTHrP. Thus, it is possible that BMP in the cartilaginous skeleton mediates both local effects on the prehypertrophic chondrocytes, as well as longer-range effects to regulate PTHrP in the periarticular cartilage.

Our work, and that by Vortkamp et al. (1996), suggest that the sequential progression of chondrogenic differentiation may require appropriate activation as well as down-regulation of a network of signaling molecules and their receptors. Our data indicate that BMP signals mediate, and are functionally downstream of, the effects of IHH on chondrocyte differentiation. Future studies should cast additional light on the details of the network of regulatory interactions linking *Ihh*, *ptc*, *Bmp*, *BmpR-IA*, *PTHrP*, and *PTH/PTHrP* receptor to the control of the sequential steps of chondrocyte differentiation.

BMPs and programmed cell death

BMP signaling has been implicated in the regulation of embryonic programmed cell death (Graham et al. 1994; Yokouchi et al. 1996; Zou and Niswander 1996; Macias et al. 1997). Our studies support a distinct role for BMPR-IB in the regulation of BMP-mediated cell death. We have found that early caBMPR-IB infection greatly increases cell death in the developing limb. Later infection results in premature regression of the interdigital soft tissue. Moreover, dnBMPR-IB results in decreased apoptosis in the interdigital region (Zou and Niswander 1996). In contrast, neither our activated nor our dominant-negative forms of BMPR-IA affected cell death. However, Yokouchi et al. (1996) observed a decrease in apoptosis in the limb following overexpression of a different dominant-negative BMPR-IA construct. This construct lacks the entire intracellular portion of the polypeptide, including the kinase and the GS activation domains, whereas our dnBMPR-IB construct creates a single amino acid change in the ATP-binding site. A truncated BMPR-IB construct blocks chondrogenesis in limb mesenchyme cultures (Kawakami et al. 1996), as does our point-mutant dnBMPR-IB (this paper). However, the effect of the truncated dnBMPR-IB construct on cell death has not been reported.

Overexpression of a truncated or point mutant type I receptor may cause a general dominant-negative effect by sequestering ligands and type II receptors that are shared with other type I receptors. The dominant-negative effect of a point mutant type I receptor might be more specific because this construct could also sequester intracellular proteins such as Smads, which associate with type I receptors (Macias-Silva et al. 1996; Zhang et al. 1996; Kretzschmar et al. 1997). Nonetheless, in a combinatorial system of multifunctional receptors, it is not possible to determine the role of a specific receptor in a particular process through the use of dominant-negative constructs. In contrast, the ability of the activated BMPR-IB, but not the activated BMPR-IA, to elicit cell death provides evidence that the apoptotic pathway is triggered specifically through BMPR-IB-mediated signal transduction.

Our studies show that caBMPR-IB causes extensive cell death when expressed in young limb buds. However, the only obvious effect on cell death following infection of later stage limbs is an accelerated regression of interdigital soft tissue. Interdigital soft tissue seems to be the primary target tissue of BMP-mediated apoptosis, whereas the condensed digital mesenchyme cells appear to be resistant to this apoptotic signal. This difference in response of young versus old, interdigital versus digital cells to BMP-mediated apoptosis could be a result of differences in their developmental history or their state of differentiation.

Conclusion

Our studies reveal a mechanism by which specific intracellular effects can be generated in response to the same or closely related extracellular BMP signals. During embryogenesis, multiple BMPs and BMPRs are often coexpressed or display a close spatial-temporal association. Our work demonstrates that the type I BMPRs provide specificity by triggering distinct phenotypic responses. This study also presents evidence for the integration of the BMP signaling pathway with the IHH/PTHrP pathway implicated in regulation of bone morphogenesis: IHH acts upstream of BMPs, which then work through BMPR-IA in prehypertrophic chondrocytes and in the periarticular region to regulate PTHrP production, thus serving to control chondrocyte differentiation. The mutant forms of the type I BMPRs provide powerful tools to experimentally manipulate BMP signaling in developing tissues that are dependent on BMP such as the kidney, eye, mammary gland, and nervous system, as well as to elucidate the role of BMPs in triggering cell death. In the future, it will be of interest to determine the identity of the intracellular molecules that specifically interpret the signals from the different BMPRs.

Materials and methods

Cloning of chicken type I BMPRs

Full-length chicken *BmpR-IA* and *BmpR-IB* cDNA clones were isolated from a stage 12-15 chick cDNA library (kind gift of D.

Wilkinson, Imperial Cancer Research Fund, London, UK) using human *BmpR-IA* and mouse *BmpR-IB* as probes (kindly provided by K. Miyazono and P. ten Dijke, Ludwig Institute, Uppsala, Sweden) under low-stringency hybridization conditions. DNA sequence was compared with the reported sequences for human and chick *BmpR-IA* (ten Dijke et al. 1993, 1994a; Kawakami et al. 1996) and chick *BmpR-IB* (Sumitomo et al. 1993). The coding regions of the two chick BMPRs were amplified by polymerase chain reaction, subcloned into pBlue-script (Stratagene), and used to generate antisense dioxygenin probes. The 1.6-kb *BmpR-IA* was cloned into *EcoRI-SalI* sites, digested with *EcoRI*, and transcribed with T7 polymerase. The 1.5-kb *BmpR-IB* was cloned into *BamHI-EcoRI*, digested with *BamHI*, and transcribed with T7 polymerase.

Generation of mutant type I BMPRs and in ovo infection

cDNAs containing the coding region of chick *BmpR-IB* and human *BmpR-IA* cloned into pBluescript were subjected to oligonucleotide-mediated site-directed mutagenesis (Clontech) to change Lys-231 to Arg for dnBMPR-IB (similar phenotypes were noted with mouse dnBMPR-IB), Lys-261 to Arg for dnBMPR-IA, Gln-203 to Asp for caBMPR-IB, and Gln-233 to Asp for caBMPR-IA. The mutant constructs were cloned into *Clal2-Nco* shuttle vector and then into the replication-competent avian retroviral vector RCAS(A) (Hughes et al. 1987). Viruses were generated as described (Morgan and Fekete 1996). In all cases, similar results were obtained from multiple viral preparations of a given construct. Concentrated RCAS(A) virus encoding the various mutant type I BMPRs was injected into the presumptive limb field or developing limb bud.

Histological analysis

Limbs from various stages were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Limbs from day 7 or older embryos were decalcified in 5.5% EDTA /4% PFA for up to a week at 4°C, with the solution changed every 24 hr. Limbs were then dehydrated, embedded in paraffin, and sectioned at 8 µm. Sections were stained with hematoxylin and eosin or alcian blue (pH 2.5) and counter-stained with Nuclear Fast Red to localize sulfated proteoglycans, or by Weigert-Safranin staining (Prophet et al. 1994).

RNA in situ hybridization

For serial sections, two consecutive sections were collected on seven alternating slides. Section RNA in situ hybridization with digoxigenin-labeled probes was done according to Neubuser and Balling (Neubuser et al. 1995 and pers. comm.). Briefly, after rehydration, the tissues were refixed in 4% PFA, subjected to 10 µg/ml of proteinase K treatment at room temperature for 5–7 min, and fixed again in 4% PFA. Hybridization was performed at 65°C in 40% formamide, and posthybridization washes were carried out at a final stringency of 20% formamide/0.5× SSC at 60°C. Detection was performed using BM-purple substrate (Boehringer) from 12 hr to 1 week, depending on the probe. Whole-mount RNA in situ hybridization was performed essentially as described (Henrique et al. 1995). Probes were kindly provided by P. Brickell (*Bmp2* and *Bmp4*; Francis et al. 1994), B. Houston (*Bmp7*; Houston et al. 1994), C. Tabin (*Ihh*, *ptc*, *Gli*, *PTHrP receptor*; references in Vortkamp et al. 1996), W. Upholt (Col-II, Hyun-Duck et al. 1988), B. Olsen (collagen type X; Ninomiya et al. 1986), and G. Stewler (PTHrP; Schermer et al. 1991).

BrdU incorporation

Undiluted BrdU labeling reagent (250 µl) (Amersham Life Science) was injected into the amniotic cavity of day 6 chick embryos (controls or caBMPR-IA/-IB infected at stage 19–20 into the limb bud). The embryos were returned to the incubator for 2.5 hr, then sacrificed and placed in Carnoy's Fix. Paraffin sections (8 µm) were collected and processed using Amersham's Cell Proliferation Kit. The DAB color reactions were stopped after 2 min and sections counterstained briefly in 1% methyl green.

High-density micromass cultures

Stage 22–24 limb buds were isolated; the ectoderm was removed by trypsin treatment; and the mesenchyme dissociated as described (Ahrens et al. 1977; Swalla and Solursh 1986). Mesenchyme was resuspended at a density of 2×10^7 cells/ml; 12 µl aliquots were infected with 1 µl concentrated RCAS virus encoding the different BMPR mutants and plated on 35-mm tissue culture dishes. One hour later, Medium 199 (GIBCO) containing 10% fetal calf serum and 2% chick serum was added. Each 35-mm culture dish contained three similarly treated micromasses, and multiple dishes were plated allowing analysis at 24-hr intervals. The cultures were stained with alcian blue (pH 2.5) to visualize chondrogenic nodule formation. These experiments were repeated three times with similar results.

Nile blue detection of cell death

Vital staining by Nile blue sulfate (Sigma N-5632) was performed according to Tone et al. (1983). Briefly, embryos were dissected in cold PBS, transferred to prewarmed Medium 199 containing 0.001% Nile blue, incubated at 37°C for 30–45 min, washed in cold PBS for 5 hr, and photographed.

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