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ALK2 Functions as a BMP Type I Receptor and Induces Indian Hedgehog in Chondrocytes During Skeletal Development

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ABSTRACT

Growth plate chondrocytes integrate multiple signals during normal development. The type I BMP receptor ALK2 is expressed in cartilage and expression of constitutively active (CA) ALK2 and other activated type I BMP receptors results in maturation-independent expression of Ihh in chondrocytes in vitro and in vivo. The findings suggest that BMP signaling modulates the Ihh/PTHrP signaling pathway that regulates the rate of chondrocyte differentiation.

Introduction: Bone morphogenetic proteins (BMPs) have an important role in vertebrate limb development. The expression of the BMP type I receptors BMPR-IA (ALK3) and BMPR-IB (ALK6) have been more completely characterized in skeletal development than ALK2.
Methods: *ALK2* expression was examined in vitro in isolated chick chondrocytes and osteoblasts and in vivo in the developing chick limb bud. The effect of overexpression of CA ALK2 and the other type I BMP receptors on the expression of genes involved in chondrocyte maturation was determined.
Results: *ALK2* was expressed in isolated chick osteoblasts and chondrocytes and specifically mediated BMP signaling. In the developing chick limb bud, *ALK2* was highly expressed in mesenchymal soft tissues. In skeletal elements, expression was higher in less mature chondrocytes than in chondrocytes undergoing terminal differentiation. CA ALK2 misexpression in vitro enhanced chondrocyte maturation and induced Ihh. Surprisingly, although parathyroid hormone-related peptide (PTHrP) strongly inhibited CA ALK2 mediated chondrocyte differentiation, *Ihh* expression was minimally decreased. CA ALK2 viral infection in stage 19–23 limbs resulted in cartilage expansion with joint fusion. Enhanced periarticular expression of *PTHrP* and delayed maturation of the cartilage elements were observed. In the cartilage element, CA ALK2 misexpression precisely colocalized with the expression with *Ihh*. These findings were most evident in partially infected limbs where normal morphology was maintained. In contrast, *BMP-6* had a normal pattern of differentiation-related expression. CA BMPR-IA and CA BMPR-IB overexpression similarly induced *Ihh* and *PTHrP*.
Conclusions: The findings show that BMP signaling induces *Ihh*. Although the colocalization of the activated type I receptors and Ihh suggests a direct BMP-mediated signaling event, other indirect mechanisms may also be involved. Thus, while BMPs act directly on chondrocytes to induce maturation, this effect is counterbalanced in vivo by induction of the Ihh/PTHrP signaling loop. The findings suggest that BMPs are integrated into the Ihh/PTHrP signaling loop and that a fine balance of BMP signaling is essential for normal chondrocyte maturation and skeletal development.
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Key words: **bone morphogenetic proteins, bone morphogenetic protein receptors, ALK2, cartilage differentiation, limb development**

INTRODUCTION

ENDOCHONDRAL OSSIFICATION IS important for the development of both the axial and appendicular skeleton. This process is initiated with the condensation of mesenchymal cells and development of a cartilaginous template. Chondrocytes subsequently undergo an exquisitely regulated program of sequential proliferation, maturation, hypertrophy, and calcification that culminates in apoptosis. Vascular in-growth occurs into calcified cartilage, which serves as a template and scaffold for primary bone formation. With ongoing ossification, the midportion of the orig-

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inal template becomes bone, with chondrocytes moving toward opposite ends of the long bone. During adolescent growth, the shaft of the bone is mineralized, leaving residual chondrocytes in the metaphyseal regions in structures referred to as growth plates.[1] Endochondral ossification continues in the growth plate until skeletal maturity when hormonal influence cause cessation of growth.

Growth factors play a major role in controlling the events of limb development and endochondral bone formation. Bone morphogenetic proteins (BMPs) are regulators of cell growth, differentiation, apoptosis, cell lineage determination, patterning, and morphogenesis, and are critical for the development of multiple organs and tissues, including the skeleton.[2] In early vertebrate limb development, BMPs control mesoderm cell proliferation, regulate the growth and regression of the apical ectodermal ridge (AER), specify the anteroposterior axis, initiate chondrogenesis, and regulate apoptosis.[3–7] Later, during endochondral ossification, genes for BMPs and BMP receptors are expressed in a distinct spatial and temporal pattern. For example, *BMP-2*, *BMP-4*, *BMP-5*, and *BMP-7* are expressed in the perichondrium surrounding the cartilage elements,[8] while *BMP-6* is expressed in prehypertrophic chondrocytes.[9]

Members of the transforming growth factor-β (TGF-β) superfamily, including TGF-βs, BMPs, activins, inhibins, nodals, and other related factors, bind to two types of transmembrane serine/threonine kinase receptors.[10,11] The type II receptor is a constitutively active kinase, while the type I receptor is inactive until ligand binding results in association with the type II receptor, leading to phosphorylation at the glycerin-serine rich (GS) region. Once activated, type I receptors phosphorylate receptor-associated Smad proteins, which are released from the receptor-complex and associate with Smad4. The activated Smad complexes translocate to the nucleus and induce the expression of target genes.[12–14] Seven type I receptors, also called ALKs (activin receptor-like kinases), have been identified in vertebrates. BMP type IA receptor (BMPR-IA or ALK3) and BMPR-IB (ALK6) are structurally similar to each other and exclusively bind BMPs.[15] ALK2 binds activins and TGF-βs in vitro,[16,17] but recent data suggest that it physiologically functions as type I receptor for BMP signaling. ALK2 mimics the mesoderm ventralizing activity of BMPs but not the effect of activin or TGF-β in *Xenopus* embryo explants.[18] ALK2 phosphorylates the BMP receptor-specific Smads 1, 5, and 8 and not the TGF-β receptor-associated Smads 2 and 3.[19] During vertebrate limb development, *BMPR-IA* is expressed at low levels throughout the limb mesenchyme at early stages and is later found in chondrocytes undergoing hypertrophy, while the expression of *BMPR-IB* prefigures cartilage formation.[20] Although roles of BMPR-IA and -IB in skeletal development have been investigated,[20–25] less is known about the expression and function of ALK2 receptor in skeletogenesis.

Here we show that *ALK2* is expressed in skeletal tissues. Using a constitutively active form of ALK2 that does not require either ligand or type II receptor for signaling, we show that ALK2 functions as a BMP type I receptor in cultured chondrocytes. Moreover, we show that ALK2 induces *Ihh* in cell culture and in vivo, suggesting that the BMP signaling is upstream of *Ihh* and is integrated into the Ihh/PTHrP signaling pathway.

MATERIALS AND METHODS

Growth factors and DNA constructs

PTHrP was purchased from Bachem (Torrance, CA, USA) and TGF-β1 was purchased from Calbiochem (San Diego, CA, USA). BMP-2 was kindly provided by Genetics Institute (Cambridge, MA, USA).

The expression plasmid encoding the CA rat ALK2 receptor (CA rALK2-pcDNA3.1) was a gift from Dr Wylie Vale.[26] The 2.2-kb *Bam*HI fragment containing the coding sequence for rat CA ALK2 was subcloned into a cla12 shuttle vector and then inserted into the replication competent avian sarcoma retrovirus RCASBP(A).[27] The CA human ALK5 cDNA was obtained from Dr Joan Massague[28] and subcloned into the mammalian expression vector pcDNA3.1 (CA hALK5-pcDNA3.1). The full-length chick ALK2 cDNA clone (chALK2-pcDNA3.1) was supplied by Dr Joey Barnett,[29] and a 1.2-kb *Bgl*II-*Xba*I fragment of the chick ALK2 cDNA was subcloned into the KS$^-$ vector (chALK2-KS$^-$). The authenticity of all the constructs was verified by automated DNA sequencing. The TGF-β responsive p3TP-Lux reporter and BMP responsive Xvent2-Luc reporter were gifts from Dr Joan Massague,[30] and the chick Col-X promoter was obtained from Dr Pheobe Leboy.[31] RCAS viruses that encode CA human BMPR-IA or CA chicken BMPR-IB were from Dr Lee Niswander.[20]

Cell cultures

Chick upper sternal chondrocytes were isolated from the cephalic portion of sterna from 13-day chick embryos.[32,33] Cells were resuspended in DMEM with 10% NuSerum IV (Collaborative Biochemical, Bedford, MA, USA) and plated at 2.5 sterna per 100-mm dish. Five to seven days later, chondrocytes were harvested, counted, and replated in 6-well plates for transient transfection experiments or 60-mm dishes for Northern analysis. Chick osteoblasts were isolated from embryonic day 18 chick calvaria,[34] and adolescent growth plate chondrocytes were isolated from 3- to 5-week-old chicks.[35]

RNA isolation and analysis

Total RNA was purified using RNeasy kit (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's directions. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of chick *ALK2, BMPR-IA, BMPR-IB,* and *GAPDH* expression were performed with the Access RT-PCR System (Promega, Madison, WI, USA). RNA (0.5 μg) was used as the template for the RT reaction. The RT reaction was performed at 48°C for 45 minutes, followed by PCR (94°C for 30 s, 58°C for 1 minute, 68°C for 1 minute, 30 cycles). The chick *ALK2* primers were 5′-GATCAGCAAAGGCCAAACAT-3′ (forward) and 5′-AGGAGCTGCCTCTGTAA-3′ (reverse), resulting in a 296-bp product (nt 1444–1739 in U38622). Primers used to amplify BMPR-IA, BMPR-IB, and GAPDH are identical to those described previously.[36]

Northern analysis was performed with denaturing formaldehyde/agarose gels, and RNA was transferred to GeneScreen Plus membranes (NEN, Boston, MA, USA).[33] The membranes were hybridized with a ^{32}P-labeled chick type X collagen or Ihh probe in QuickHyb solution (Stratagene) at 68°C, washed at 60°C in 0.1× SSC and 0.1% SDS, and exposed to Kodak XAR film.[37]

Transient transfection and luciferase assay

Embryonic upper sternal chondrocytes, cultured at 30–40% confluence in 6-well plates, were transfected on day 2 after plating using the transfection reagent Superfect (Qiagen). Cotransfection was performed with 0.5 μg firefly luciferase reporter, 0.5 μg receptor expression plasmid, and 5 ng renilla uniformis luciferase. Twelve hours after transfection, chondrocytes were incubated for 6 h in serum-free media (containing DMEM, hyaluronidase 4 U/ml, and penicillin/streptomycin), followed by addition of 3 ng/ml TGF-β1 or 50 ng/ml BMP-2 as indicated. Eighteen hours after treatment with growth factors, chondrocytes were harvested and assayed for luciferase activities using Dual Luciferase Assay System (Promega), with renilla luciferase values as control for transfection efficiency.[37] Data are represented as the mean of triplicate samples, and error bars represent SEM.

Expression of CA ALK2 in cultured chondrocytes

Chick embryonic fibroblasts (CEFs) grown in DMEM containing 10% FBS and 0.2% fetal chick serum were transfected with the retrovirus RCASBP(A) empty vector or RCASBP(A) containing CA rat ALK2 using Superfect reagent (Qiagen). After transfection, cells were maintained and passed three times in 10-cm dishes. On confluence in the final cultures, fresh DMEM with 10% NuSerum was added to the cultures and collected every 24 h. To infect the chondrocyte cultures with RCAS viruses, cells were treated for the first 48 h in secondary culture with a mixture of 50% fresh DMEM with 10% NuSerum and 50% medium harvested from the RCAS-infected CEF cultures. After 2 days, chondrocytes were treated with fresh medium with or without BMP-2 (50 ng/ml) and/or PTHrP (10^{-7} M) for 7 days. The media, BMP-2, and PTHrP were replenished at 48-h intervals.[37,38]

In vivo expression of CA ALK2 receptor

An established method using infected chick embryo fibroblasts (CEFs) was used to infect the developing limb bud with RCAS viruses.[39] Briefly CEFs were infected with RCASBP(A) virus encoding the CA rat ALK2 receptor. Cells were passed three times, and then pelleted, stained with 0.01% nile blue sulfate, and approximately 1000 cells (0.1 μl) were injected into the forelimb buds of Hamburger-Hamilton (HH) stage 19–23 chick embryos.[40] Chick embryos were harvested at the appropriate stages after implantation and fixed in 4% paraformaldehyde (PFA), followed by dehydration and embedding into paraffin for in situ hybridization and histological analysis.

For Alcian blue-Alizarin red double staining, embryos were fixed in 95% ethanol.[41] Pathogen-free eggs were purchased from SPAFAS (Norwich, CT, USA).

More than 100 embryos were injected with RCAS virus expressing CA ALK2, with approximately 80 embryos processed for morphological analysis using Alcian blue-Alizarin red double staining. More than 20 embryos were analyzed for gene expression by in situ hybridization. Consistent morphological findings and patterns of gene expression were observed in all the embryos. Confirmatory experiments were performed with embryos injected with purified virus expressing CA rat ALK2 receptor and similar morphological and molecular findings were observed (data not shown).

For negative controls, 10 embryos were injected with CEFs alone, 10 were injected with CEFs infected with empty RCAS viruses, and 10 others were injected with purified empty RCAS viruses. No morphological or molecular abnormalities were observed in any of these embryos.

Additional experiments were performed using viruses to overexpress CA BMPR-IA and CA IB. In these experiments, a total of 60 embryos were injected. Thirty injections were performed using each of the constructs (CA BMPR-IA and CA BMPR-IB), with 15 injections using infected CEFs and 15 injections using purified virus. For each set of 15 injections, 10 were processed for Alcian blue-Alizarin red staining, and 5 were processed for in situ hybridization. Similar morphological findings and patterns of gene expression were observed in each of the embryos.

Some experiments were designed to cause partial or less extensive infection of the chick limb. In these experiments, fewer infected CEFs were injected.

In situ hybridization

In situ hybridization was performed on tissue sections using ^{35}S-UTP labeled antisense RNA probes.[42,43] Briefly, after rehydration, the tissues were refixed in 4% PFA, subjected to 20 μg/ml proteinase K treatment at room temperature for 5 minutes, and fixed again in 4% PFA. Hybridization was performed at 55°C in 50% formamide, and the post-hybridization washes were carried out at a final stringency of 50% formamide/1× SSC at 55°C. Probes were kindly provided by Dr Pheobe Leboy (*colIX* and *colX*)[44] and Dr Cliff Tabin (*Ihh*, *PTHrP,* and *BMP-6*).[9] The expression plasmid for CA rat ALK2 (CA rALK2-pcDNA3.1) was digested with *Xba*I and transcribed with SP6 RNA polymerase to generate the rat ALK2 antisense probe. The plasmid which contains the partial chick ALK2 cDNA sequence (chALK2-KS$^-$) was digested with *Sma*I and transcribed with T7 polymerase to generate antisense probe for chick ALK2 or digested with *Xba*I and transcribed with T3 polymerase to generate the sense probe.

RESULTS

ALK2 is expressed in cartilage and functions as a BMP type I receptor in cultured chondrocytes

While the ALK2 receptor is known to have a broad pattern of expression in a variety of tissues,[26,45] its expression in developing skeletal tissues has not been characterized. *ALK2* expression was identified in isolated embryonic upper sternal chondrocytes (USC) and juvenile growth plate chondrocytes (GPC) using RT-PCR with chick-specific

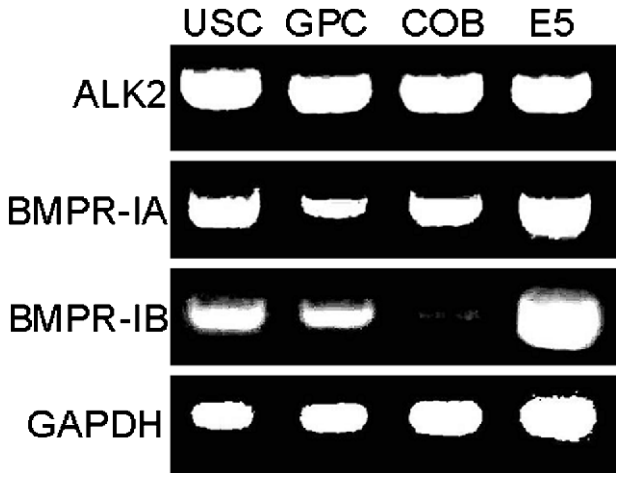


FIG. 1. *ALK2* is expressed in chondrocytes and osteoblasts. *ALK2, BMPR-IA, BMPR-IB,* and *GAPDH* expression was examined in chick embryonic upper sternal chondrocytes (USC), juvenile growth plate chondrocytes (GPC), and calvarial osteoblasts (COB) by RT-PCR. Total RNA from day 5 chick embryo (E5) was used as positive control. PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed.

primers (Fig. 1). *BMPR-IA* and *BMPR-IB* expression was also observed in both sternal and growth plate chondrocytes. In contrast, chick embryonic calvarial osteoblasts (COB) expressed *ALK2* and *BMPR-IA* but had minimal expression of *BMPR-IB*. These findings show that skeletal tissues simultaneously express multiple type I receptors for TGF-β family members and suggest that ALK2 may be a regulator of skeletal development.

ALK2 expression was characterized in vivo at different stages of chick limb development by in situ hybridization (Fig. 2). At embryonic day 5 (E5, HH stage 26), when formation of the cartilage anlage is initiated, *ALK2* was highly expressed throughout the entire limb bud mesenchyme (Fig. 2A). With formation of cartilage (E7 and E9), *ALK2* continued to be highly expressed throughout the mesenchyme. Within cartilage, *ALK2* was expressed at relatively higher levels at the ends of the skeletal elements, which contain less mature chondrocytes, compared with more mature chondrocytes that are found in the central regions of the skeletal elements (Figs. 2B and 2C). By day 11 (E11), *ALK2* was also strongly expressed in the central portion of the skeletal elements where mineralized bone is found (Fig. 2D). High levels of expression were also observed in the perichondrium and periosteum throughout limb development. These findings further suggest a role for ALK2 during formation and growth of the limb.

To examine whether ALK2 functions as a BMP receptor, cultured embryonic upper sternal chondrocytes were transiently transfected with BMP and TGF-β/activin reporter constructs (Fig. 3). BMP-2 induced transactivation of the BMP responsive promoters, Xvent2-Luc[46] and ABC-640-Luc, which are derived from the chick type X collagen promoter.[31] Transient transfection of the CA ALK2 receptor mimicked the effects of BMP-2 and resulted in approximately 3- and 150-fold increases in Xvent-2-Luc and ABC-640-Luc activities, respectively (Figs. 3A and 3B). In contrast, TGF-β signaling, either through addition of recombinant TGF-β1 to the cultures or through cotransfection with the constitutively active TGF-β type I receptor (CA ALK5), inhibited the basal activity of both the Xvent-2 and ABC-640 promoters, consistent with prior data showing antagonism between different members of the TGF-β signaling family.[47] Similarly, while both TGF-β1 and CA ALK5 induced transactivation of the TGF-β responsive reporter, p3TP-Luc,[37] BMP-2 and CA ALK2 reduced basal luciferase activity (Fig. 3C). These findings are consistent with ALK2 as a mediator of BMP signaling.

To determine if ALK2 regulates chondrocyte phenotype in a manner consistent with BMP signaling, we infected chondrocyte cultures with the avian replication competent retroviral vector RCASBP(A) expressing CA rat ALK2. Infection efficiency was >80%, as determined by expression of viral gag protein using immunocytochemistry (data not shown). BMP-2 treatment for 7 days promoted chondrocyte maturation, as shown by induction of *colX* and *Ihh*. Infection with CA ALK2 viruses similarly induced *colX* and *Ihh* (Fig. 4). Interestingly, while PTHrP markedly suppressed the induction of *colX* by BMP-2 and CA ALK2, there was much less suppression of *Ihh*, particularly in the CA ALK2–expressing cultures. These findings further confirm a role for ALK2 as a mediator of BMP signaling events in chondrocytes and suggest that BMP signaling upregulates *Ihh* despite the (1) presence of PTHrP and (2) inhibition of chondrocyte maturation.

CA ALK2 enhances chondrogenesis, delays endochondral bone formation, and disrupts joint formation

To investigate the effect of overexpression of CA ALK2 on chondrocyte differentiation in vivo, chick embryonic fibroblasts (CEFs) infected with the rat CA ALK2 virus were implanted into chick forelimb buds at HH stage 19–23, a time point preceding condensation of the humerus, ulna, and radius.[40] CA ALK2 viral infection for 7 days results in expansion of the cartilage anlage compared with uninfected contralateral control limbs (Figs. 5A–5C). Additionally, there was a relative increase in the size of the cartilage element compared with surrounding connective tissue, suggesting that ALK2 signaling can alter cell fate and induce the undifferentiated mesenchyme into cartilage. The overall length of the infected limb was minimally changed. Finally, the Alcian blue-Alizarin red whole-mount staining suggested a delay in chondrocyte maturation. In the control limbs, Alizarin red staining was present in the midportion of the skeletal elements, consistent with mineralization of the bone. In contrast, there was absence or delay of mineralization in the infected limbs (Figs. 5A–5C).

Joint formation was disrupted in the CA ALK2 infected limbs, with failure of segmentation and joint formation observed. Effects were apparent within 72 h after infection (Fig. 5D). In this example, there is normal formation of the humeral-radial component of the elbow joint but a failure of segmentation of the humerus and ulna. The most dramatic effects were noted in limbs with extensive expansion of the

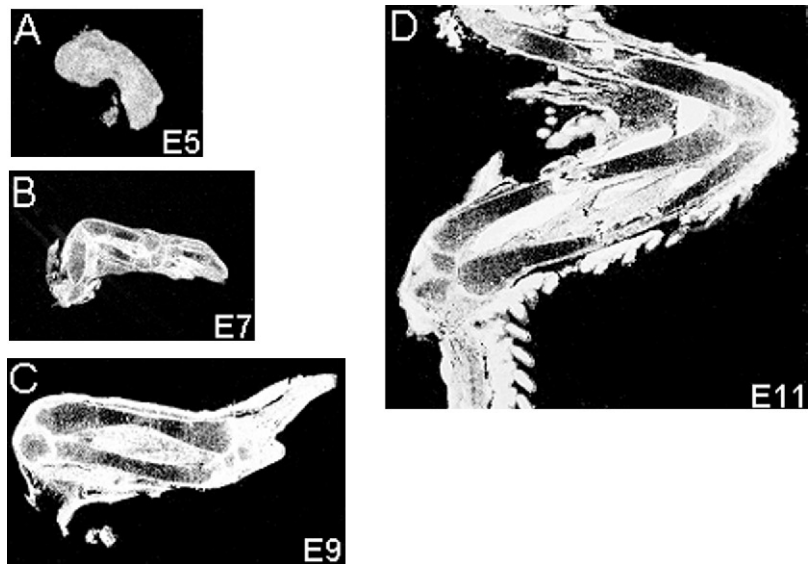


FIG. 2. Expression of *ALK2* in the developing cartilage. Five-micrometer sections from embryonic chick forelimbs were hybridized with a radiolabeled antisense probe for chick *ALK2*. (A) At embryonic day 5 (E5), *ALK2* was diffusely expressed throughout the limb bud mesenchyme. (B–D) With skeletal development, *ALK2* transcripts were detected at relatively higher level in resting and proliferating chondrocytes, and lower level in differentiated chondrocytes, as shown at embryonic days 7, 9, and 11 (E7, E9, and E11).

cartilage elements, where there was complete absence of formation of the elbow joint. While the radius and ulna underwent segmentation, both were fused to the humerus (Figs. 5A–5C).

To make sure that none of the morphological abnormalities were caused by nonspecific effects of the CEFs implantation or viral infection, control experiments were performed in embryos injected with CEFs alone (Fig. 5E, $n = 10$) and in embryos injected with CEFs infected with control empty RCAS virus (Fig. 5F, $n = 10$). None of these embryos had morphological abnormalities on Alcian blue-Alizarin red staining.

CA ALK2 delays chondrocyte differentiation in vivo in the chick limb bud

Because the CA ALK2 was derived from the rat cDNA, in situ hybridization with a rat-specific probe was used to localize the region of viral infection in the chick limb. As shown in Figs. 6C and 6D, the uninfected limb had no hybridization signal, while the infected limb showed infection throughout the expanded cartilage anlage. Thus, the extent of transgene expression can be determined using the rat ALK2 probe. Misexpression of the CA ALK2 receptor in vivo in the chick limb bud resulted in delayed chondrocyte differentiation. Type IX collagen (*colIX*), which is expressed by chondrocytes at all stages of maturation, confirmed the viability and matrix production of the CA ALK2–infected chondrocytes (Figs. 6E and 6F). *ColX*, a marker of hypertrophic chondrocytes, was absent in the cartilage shaft, but was expressed along the perichondrium. Thus, chondrocyte maturation was both delayed and occurred in an abnormal pattern with altered polarity (Figs. 6G and 6H), similar to that observed in PTHrP transgenic mice and in chick limbs with misexpression of Ihh.[9,48] While *Ihh* expression was restricted to the well-defined prehypertrophic regions in the contralateral limb, diffuse expression was apparent throughout the infected cartilage element (Figs. 6I and 6J). Thus, enhanced ALK2 signaling is associated with a dramatic induction of *Ihh*. We also found that misexpression of the CA ALK2 receptor induced *PTHrP* expression in the periarticular/perichondrial region (Figs. 6K and 6L, see arrows). In contrast, *BMP-6* expression localized to prehypertrophic chondrocytes and along the perichondrium in both control and infected limbs (Figs. 6M and 6N). Because misexpression of CA ALK2 is associated with increased periarticular expression of *PTHrP*, the observed delay in maturation in the infected limbs may be because of activation of the Ihh/PTHrP feedback loop. This is a well-characterized negative loop of the rate of chondrocyte differentiation,[9,48] but other mechanisms could be involved as well. Furthermore, the findings indicate that CA ALK2 infection results in constitutive *Ihh* expression with a pattern that is independent of other maturational markers, including *BMP-6* and *colX*.

Gene expression was also examined in embryos injected with CEFs alone ($n = 5$) and embryos injected with CEFs infected with control empty RCAS virus ($n = 5$). No alteration in gene expression was observed in these limbs, showing that the observations are caused by the expression of CA ALK2 (data not shown).

Ihh *expression colocalizes with activated BMP signaling in chondrocytes*

Because the prior experiments represented long-term viral infection (7 days), the induction of *Ihh* expression was examined at 48 h after the ALK2 infection (Fig. 7). This is the earliest time point selected because protein expression is typically delayed between 16 and 24 h after viral infection.[8] *Rat ALK2* expression was apparent by 48 h in the infected limbs, with absent expression in the control limbs. While *Ihh* was expressed in a limited portion of the cartilage element in the control limb, it was dramatically induced in the cartilage element in the infected limb. Moreover, adjacent tissue sections demonstrated that *Ihh* expression colocalized with *ALK2* expression; areas of cartilage that were not infected had absent *Ihh* expression. Thus, it appears

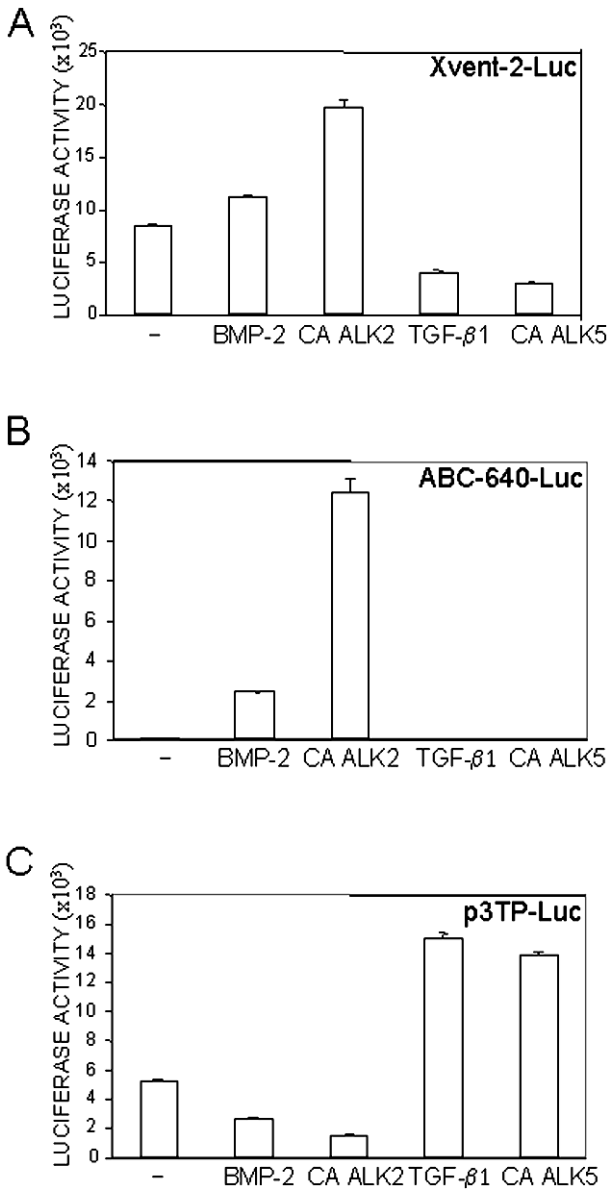


FIG. 3. Signaling specificity of the ALK2 receptor in chondrocytes. Isolated embryonic upper sternal chondrocytes were cotransfected with various luciferase reporters and either control plasmid or plasmid expressing constitutively active ALK2 or ALK5. Control media, or media containing BMP-2 (50 ng/ml) or TGF-β1 (3 ng/ml), was added to the cultures 18 h after transfection, and luciferase activity was measured 18 h later. Constitutively active ALK2 receptor activated the transcription of (A) Xvent-2 and (B) Col-X promoters, which are responsive to BMP signals, but not the (C) p3TP-Luc reporter, which is activated by the TGF-β/activin pathway.

likely that ALK2 signaling leads directly to the induction of *Ihh.*

To further confirm the spatial relationship of *Ihh* expression and *ALK2* infection, we targeted infection to the posterior limb bud and injected fewer CEFs, therefore restricting infection to the developing ulna. This approach has the additional advantage of minimally altering limb morphology. The extent of infection was confirmed using the *rat*

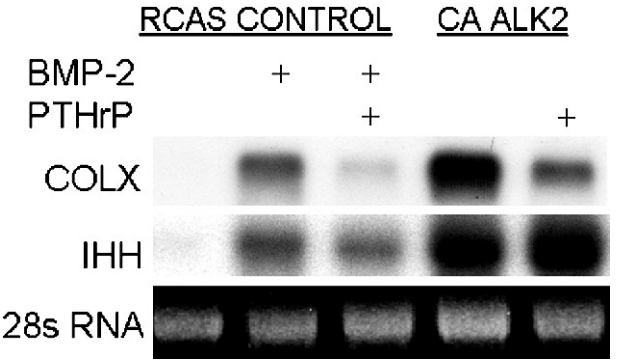


FIG. 4. ALK2 induces *colX* and *Ihh* in cultured chondrocytes. Embryonic upper sternal chondrocytes were infected with either control RCAS virus without insert (RCAS control) or with a RCAS virus overexpressing the CA ALK2 receptor. The cultures were treated with BMP-2 (50 ng/ml) or PTHrP (10^{-7}M) continuously for 7 days, as described in the Materials and Methods section. Total RNA was harvested, and *colX* and *Ihh* expression was measured by Northern blot. The ethidium bromide–stained 18s RNA was used as a loading control.

ALK2 riboprobe, and was limited to a portion of the cartilage in the ulna. In the uninfected radius, a normal pattern of endogenous *Ihh* expression was observed. However, in the infected ulna, areas of misexpression of *Ihh* occurred and colocalized to areas of *ALK2* transgene expression. Interestingly, adjacent areas of uninfected cartilage did not express *Ihh* (Fig. 8, see arrows). In contrast, *CA ALK2* expression did not result in *Ihh* expression in the soft tissues. The highly specific colocalization of *CA ALK2* expression and *Ihh* induction indicates that the CA ALK2 receptor acts upstream to induce *Ihh* expression within the cartilage elements. The effect could be either direct or indirect, although the spatial colocalization suggests a dependence on activated BMP signaling in the *Ihh*-expressing cells.

To examine whether induction of *Ihh* is unique to the ALK2 receptor, CEFs infected with CA BMPR-IA or -IB viruses were also implanted into HH stage 19–23 embryonic chick limbs. In all the limbs examined, CA BMPR-IA infection induced *Ihh* expression ($n = 5$, Fig. 9), as did the CA BMPR-IB infection ($n = 5$, data not shown). Furthermore, the pattern of *CA BMPR-IA* and *Ihh* expression was highly colocalized in partially infected limbs, similar to the findings described with *ALK2* misexpression. When we injected concentrated BMPR-IA or -IB viruses instead of infected CEF cells, similar results were obtained ($n = 5$ for IA infection, $n = 5$ for IB infection, data not shown). These findings are consistent with our previous results showing that both CA BMPR-IA and -IB receptors induce *Ihh* in cultured chondrocytes.[22] Thus, the induction of *Ihh* is a general response to BMP signaling in chondrocytes, rather than the effect of a specific BMP receptor.

DISCUSSION

BMP signaling has been shown to be essential for the development of mesenchymal tissues and subsequent formation of the skeleton. Mice deficient in the BMP type II receptor lack formation of mesenchyme,[49] and there is

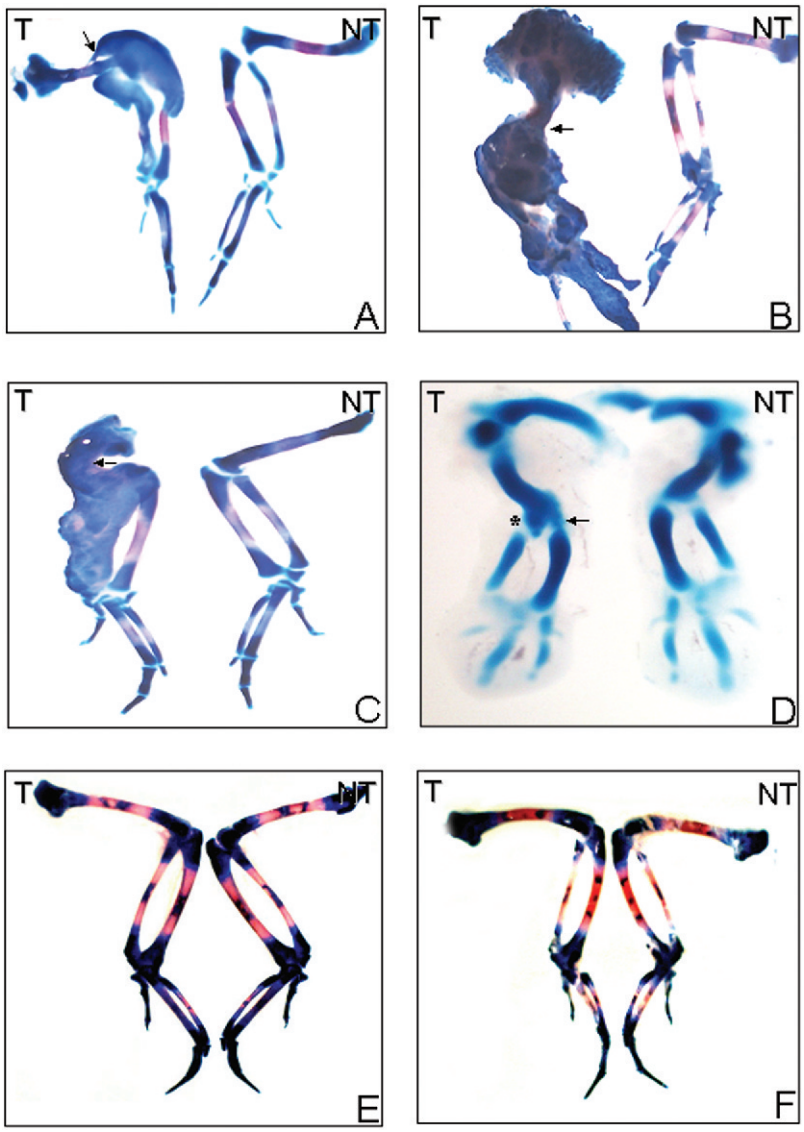
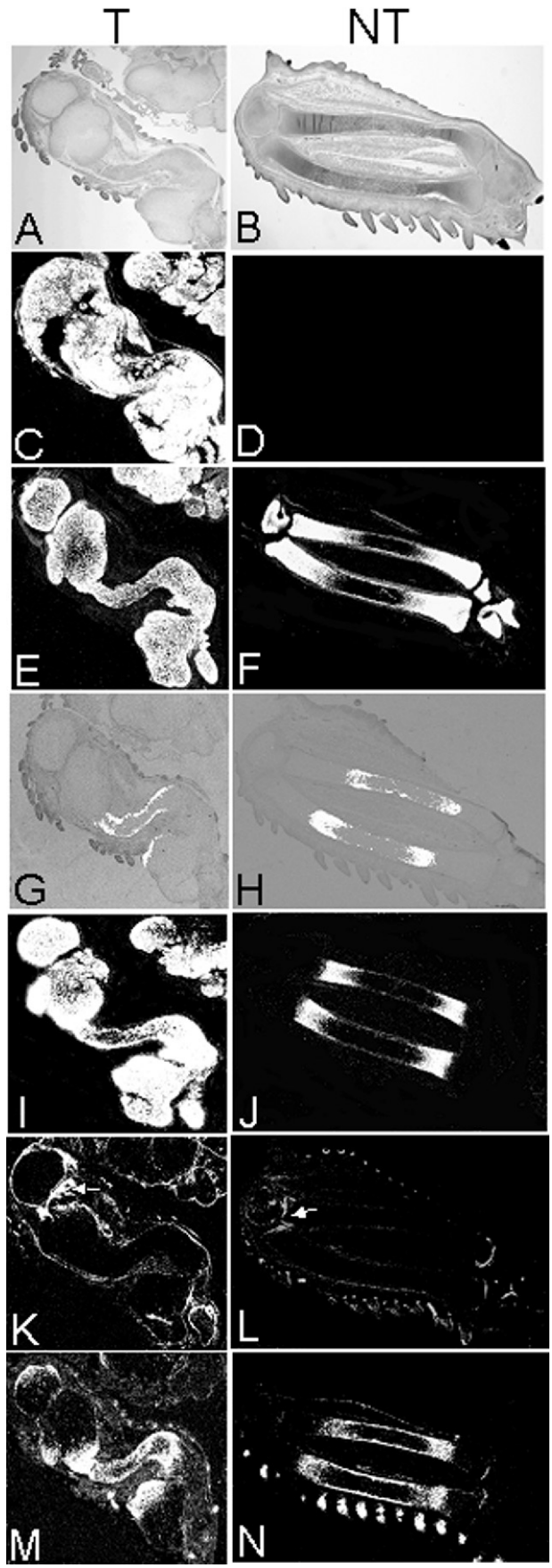


FIG. 5. Skeletal abnormalities in CA ALK2 infected limbs. HH stage 19–23 (E3.5) chick forelimbs were implanted with CEFs infected with CA ALK2 viruses, and embryos were harvested at (A–C) day 10.5 and (D) day 6.5, stained with Alcian blue-Alizarin red to reveal nonmineralized cartilage and mineralized bone. (A–C) Misexpression of ALK2 enhanced chondrogenesis and resulted in expanded cartilage elements compared with contralateral control. ALK2 infection also delayed endochondral bone formation, as evidenced by absent Alizarin red staining in the infected skeletal elements, showing delayed formation of mineralized bone. Fusion of the elbow joint was present (see arrows). (D) Stage 21 forelimb was infected with CA ALK2 viruses, and limbs was harvested 3 days later and stained. A more subtle elbow joint abnormality was apparent at this early stage of infection with fusion of the humerus and ulna present (see arrow), but with formation of the radial-humeral articulation (*). To make sure that the morphological changes are caused by ALK2 infection and not simply the result of either the implantation of CEFs or a nonspecific effect caused by RCAS infection, limbs were injected with either (E) CEFs alone or with (F) CEFs infected with control RCAS virus. T, injected limb; NT, contralateral control limb of the same embryo.

evidence that ALK2 participates in the process of mesoderm formation.[45,50] In cardiac explants harvested from stage 13–18 chick embryos, antisera to chick ALK2 inhibited mesenchyme formation up to 50%.[29] During limb development, BMP signaling has been shown to stimulate the differentiation of mesenchyme to cartilage.[6] This effect has been observed in vitro, where BMPs and their activated receptors have been shown to induce chondrogenesis from mesenchymal cell lines,[51] primary cultures of mouse and chick embryonic limb bud mesenchymal cells,[52] and organ cultures of mouse embryonic limb rudiments.[23,53] Zou et al.[20] have previously established a similar effect in vivo from observations that the constitutively active BMPR-IA and -IB receptors resulted in enhanced chondrogenesis. The current findings suggest a role for ALK2 as a BMP receptor involved in limb development, because high levels are expressed in mesenchymal tissues, and overexpression of CA ALK2 in the limb bud enhances chondrogenesis similar to the other type I BMP receptors.

Previously, *ALK2* expression has been observed in cartilage from mouse embryonic limb bud explants.[23] We similarly observed *ALK2* expression in cartilage and found the highest levels in resting and proliferating chondrocytes. *ALK2* expression was also observed in cultures of embryonic chick sternal chondrocytes and calvarial osteoblasts. In chick sternal chondrocyte cultures, CA ALK2 stimulated BMP signaling events and enhanced *colX* expression, similar to the effect of recombinant BMP-2. Thus, in addition to potentially modulating chondrogenesis, ALK2 seems to also have importance during later events, such as endochondral bone formation. However, because the ALK2 knockout mouse dies during early embryogenesis before formation of skeletal elements,[45,50] tissue targeted gene deletion will be required to further investigate the individual contribution of ALK2 function in the skeleton.

Misexpression of ALK2 in vivo altered the normal pattern of chondrocyte maturation. The paradigm explaining the rate of chondrocyte differentiation during endochondral



ossification in the developing limb focuses on the Ihh/PTHrP signaling pathway.[9,54] Genetic studies show that underexpression of *PTHrP* results in an accelerated rate of chondrocyte maturation, whereas overexpression results in delayed maturation, and in both cases, severe growth abnormalities ensue.[48,55,56] The source of PTHrP during development is the periarticular/ perichondrium, and thus this molecule acts as a morphogen on the growth plate. *PTHrP* expression is induced by Ihh, which is expressed by chondrocytes in the transitional zone of the growth plate that have undergone commitment to terminal differentiation. Elevated levels of PTHrP, which block differentiation, result in a decrease in the number of cells expressing *Ihh*. This in turn, results in a subsequent decline in *PTHrP* expression. The decrease in PTHrP permits cells to escape and complete maturation with re-expression of *Ihh* and completion of a cycle of signaling.

The signals controlling *Ihh* expression in the growth plate are critically important because of their role as a regulator of chondrocyte differentiation. Initially, it was thought that *Ihh* was simply a maturational marker, expressed by late proliferating chondrocytes committed to undergo terminal maturation.[9] However, recent evidence has accumulated to show that *Ihh* regulation is more complex and that BMPs may be involved in the regulation of *Ihh* expression. The BMP antagonist, noggin, is highly expressed in the growth plate and is found primarily in immature chondrocytes. In noggin$^{-/-}$ mice, *Ihh* is diffusely expressed throughout the growth plate.[57] Similarly, delivery of BMP impregnated heparin-acrylic beads to the chick embryo induced high levels of *Ihh* in the cartilaginous skeletal element adjacent to the bead.[58] In vitro findings in our lab have similarly suggested induction of *Ihh* by BMP signaling. Treatment of embryonic sternal chondrocyte cultures with BMP-6 or constitutively active BMPR-IA or -IB receptors induce *Ihh*, and the effect occurred even in the presence of inhibition of

FIG. 6. ALK2 misexpression delays chondrocyte differentiation and induces both *Ihh* and *PTHrP* expression. HH stage 19–23 (E3.5) chick forelimbs were implanted with CEFs infected with CA ALK2 viruses, and embryos were harvested at day 10.5. Safranin O-Fast green–stained sections demonstrated abnormal cartilage formation in (A) the CA ALK2 infected limb compared with (B) the contralateral control limb. Serial sections of CA ALK2 infected limb (T) and contralateral control limb (NT) were hybridized with ^{35}S-labeled RNA probes for (C and D) *rat ALK2*, (E and F) *colIX*, (G and H) *colX*, (I and J) *Ihh*, (K and L) *PTHrP*, and (M and N) *BMP-6*. All figures showed dark field images, except G and H, in which composite images were generated by overlaying the dark field signal with hematoxylin-counterstained tissue. The extent of ALK2 virus infection was determined by hybridization with (C) *rat ALK2* probe, which did not hybridize with (D) uninfected chick tissue. *ColIX* was expressed in both the (E) infected and (F) uninfected limbs, indicating the cells were viable. *ColX* was detected in hypertrophic chondrocytes in the (H) uninfected limb and was expressed in the perichondrium region in the (G) infected limb. *Ihh* expression was in the prehypertrophic chondrocytes in (J) the uninfected limb, while its expression was dramatically induced throughout the (I) infected cartilage element. *PTHrP* was enhanced and induced in a broader region in the periarticular perichondrium of the (K) infected limb compared with (L) control (see arrows). *BMP-6* was expressed in prehypertrophic chondrocytes and along the perichondrium in both (M) infected and (N) control limbs.

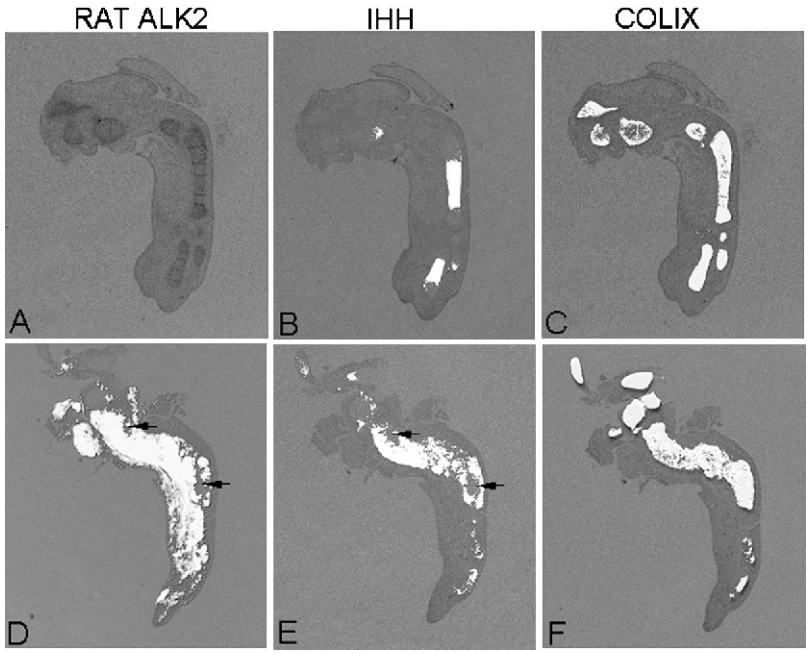


FIG. 7. *Ihh* is induced in the cartilage 48 h after ALK2 infection. HH stage 19–23 (E3.5) chick forelimbs were implanted with CEFs infected with CA ALK2 viruses, and embryos were harvested at day 5.5. In situ hybridization was performed using ^{35}S-labeled RNA probes for (A and D) *rat ALK2*, (B and E) *Ihh*, and (C and F) *colIX* on serial section of (A–C) control limbs and (D–F) ALK2 virus-infected limbs. Shown are composite images generated by overlaying the dark field signal with hematoxylin-counterstained tissue. The extent of infection status was determined by hybridization with (A and D) *rat ALK2* probe. (C and F) *ColIX* probe was used to indicate the site of cartilage formation. (E) *Ihh* induction was apparent in the infected limb and was spatially localized to areas of ALK2 transgene expression, as observed on serial sections. In contrast, in adjacent areas there was no *rat ALK2* transgene expression, *Ihh* expression was absent (see arrows).

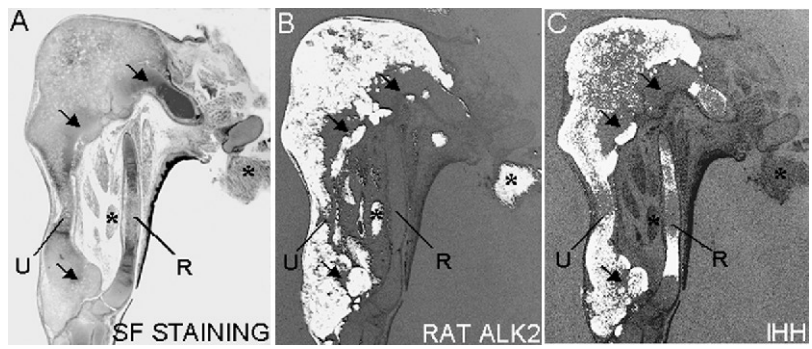


FIG. 8. *Ihh* induction colocalizes with ALK2 misexpression in the cartilage of partially infected forelimbs. HH stage 19–23 (E3.5) chick forelimbs were injected with a reduced number of CA ALK2 infected CEFs. Embryos were harvested at day 10.5, and serial sections were stained with (A) Safranin O-Fast green or in situ hybridization performed using ^{35}S-labeled RNA probes for (B) *rat ALK2* and (C) *Ihh*. The infected ulna stained weakly with Safranin O compared with uninfected radius, and within the ulna, Safranin O staining was stronger in uninfected regions (see arrows) compared with other infected regions of the ulna. (B) The extent of ALK2 infection was determined by in situ hybridization with the *rat ALK2* probe. The radius was not infected, and the ulna was partially infected, as *rat ALK2* signals were not detected in some areas of ulna (see arrows). (C) The uninfected radius had a normal pattern of *Ihh* expression, while in the partially infected ulna, *Ihh* expression colocalized to areas of *ALK2* infection. In adjacent uninfected cartilage, *Ihh* was not expressed (see arrows). *ALK2* infection outside the cartilage region was not associated with *Ihh* expression (see asterisk). U, ulna; R, radius.

differentiation by PTHrP.[22,32] Recently, Minina et al.[59] demonstrated regulation of *Ihh* expression by BMP signaling, with gain of function leading to enhanced *Ihh* expression, while loss of BMP signaling resulted in decreased expression in a mouse embryonic limb culture model.

Both in vitro and in vivo findings provide evidence that BMP signaling stimulates expression of *Ihh*. The in vivo studies are particularly convincing because we used a constitutively active rat receptor. Based on sequence differences with the chick, we were able to perform in situ hybridization with a rat-specific probe to precisely determine the extent of infection in the limbs. We found that areas of *Ihh* induction precisely colocalized with *CA ALK2* misexpression. *Ihh* expression was widespread and did not correlate with the expression of other maturational markers, such as *BMP-6* or *colX*. In contrast to the infected chondrocytes, adjacent uninfected chondrocytes did not have ectopic or maturation independent expression of *Ihh*. Although this suggests that activation of BMP signaling by CA BMP receptors is required for *Ihh* expression in cartilage, it does not rule out the additional participation of secreted factors from either infected or uninfected cartilage and adjacent tissues.

The findings are at variance with the results of Zou et al.,[20] who did not observe increased expression of *Ihh* in cartilage after infection with constitutively active BMPR-IA

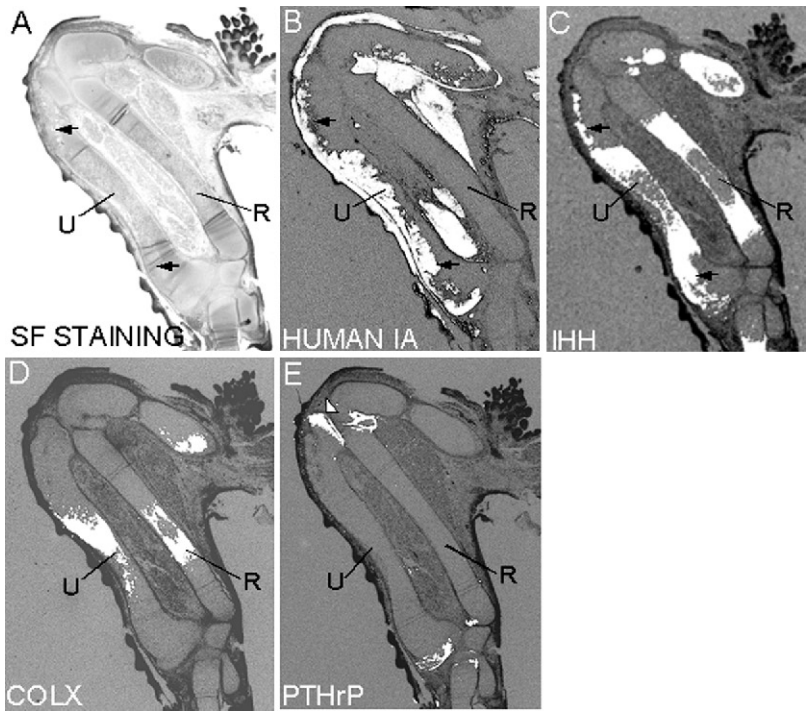


FIG. 9. CA BMPR-IA infection induces *Ihh* expression. Stage 19–23 forelimbs were implanted with CEFs infected with CA BMPR-IA viruses, and the embryos were harvested at day 10.5. (A) Histological analysis of BMPR-IA infected limb by Safranin O-Fast green staining. (B) The extent of infection status was determined by in situ hybridization with a human BMPR-IA probe, which did not hybridize with uninfected chick tissue. (C) Endogenous *Ihh* expression was present in regions flanking the midportion of both the radius and ulna. Additional areas of *Ihh* expression extended into regions of less mature cartilage in the infected ulna (see arrows). These regions of *Ihh* expression colocalized with areas of *CA BMPR-IA* misexpression. (D) *ColX* expression was localized to the midportion of the skeletal element in this partially infected limb. Interestingly, *CA BMPR-IA* misexpression did not induce *Ihh* in infected hypertrophic chondrocytes. (E) *PTHrP* expression was present in the periarticular region and was enhanced in the infected ulna (see arrowhead).

and -IB receptors. One explanation for this difference is the possibility that ALK2 may result in unique signals compared with BMPR-IA and BMPR-IB. In vitro studies have suggested that while BMPR-IA and IB stimulate phosphorylation of Smads 1, 5, and 8, ALK2 may have a more restricted pattern of Smad activation, limited to Smads 1 and 5.[60] Furthermore, activation of other signaling pathways by the BMP receptors, including p38 MAP kinase, has been described.[61] To assess this possibility, we directly examined the effects of BMPR-IA and -IB and compared them with our observations with the constitutively active ALK2 receptor. Surprisingly, we found that BMPR-IA and -IB also induced *Ihh* expression in chondrocytes in vivo. In the case of *BMPR-IA*, which is a human gene, we were also able to determine the extent of infection. We similarly found a high correlation between areas of infection and areas of *Ihh* misexpression. This correlates with in vitro findings in our laboratory that show *Ihh* induction in sternal chondrocytes by the constitutively active BMPR-IA and -IB receptors.[22] Thus, based on these findings, we conclude that all of the BMP receptors are capable of stimulating *Ihh* expression in cartilage. The methods used to detect *Ihh* in the current manuscript are different than those previously used by Zou et al.[20] In the prior manuscript, in situ hybridization was performed with a nonradioactive method employing digoxigenin-labeled probes. In contrast, our studies used ^{35}S-radiolabeled probes, which have been shown to be a more sensitive method of gene detection.[62,63]

Our findings suggest that BMPs may act in a paracrine manner to inhibit differentiation; BMP signaling induces *Ihh* and leads to a suppression of maturation by increasing *PTHrP* through the Ihh/PTHrP signaling loop. Prior work by Zou et al.[20] also found that increased signaling through the BMPR-IA receptor inhibited differentiation. However, because they did not detect increased expression of *Ihh* in the growth plate, they concluded that BMP signaling acted directly on perichondrial cells to stimulate *PTHrP* expression. In contrast, our findings support a model whereby enhanced BMP signaling in the growth plate leads to an autocrine stimulation of *Ihh* expression in these cells and a subsequent induction in *PTHrP*.

In addition to a paracrine effect, where BMPs may inhibit differentiation, there is also strong evidence to suggest that BMPs have direct effects on chondrocytes as stimulators of chondrocyte differentiation. Multiple studies have shown that BMPs and/or CA BMP receptors can directly promote maturation of isolated chondrocytes,[32,64] consistent with the in vitro finding in the current study in which both BMP-2 and CA ALK2 induced the expression of *colX* in chondrocyte cultures. Thus, the discrepant results between in vitro and in vivo studies examining the functional role of BMPs in chondrocyte differentiation may be related to disparate local and paracrine effects of these signaling molecules. The final effect of BMPs on chondrocyte differentiation may be because of the balance of the local and paracrine effects.

This study used a gain of function strategy in the chick limb development model to gain insight into the role of the ALK2 receptor during development. Using this approach, we have been able to establish that *Ihh* is induced by BMP-mediated signaling events. We also found an increase in the expression of *PTHrP* in the periarticular region. The basis of this increased expression is likely because of the overexpression of Ihh in the growth plate, but could be contributed to direct induction of PTHrP in the periarticular region by BMP signaling, as has been previously sug-

gested.[20] However, Minina et al.[59] recently demonstrated that BMP signaling does not act as a secondary signal to induce PTHrP expression in the periarticular cartilage. The mechanism involved in BMP and PTHrP interactions, as well as the manner in which BMP signaling integrates into the Ihh/PTHrP signaling pathway, will require further study. However, determination of these events will provide a more comprehensive understanding of the events involved in skeletal development.

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