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BMPs Are Required at Two Steps of Limb Chondrogenesis: Formation of Prechondrogenic Condensations and Their Differentiation into Chondrocytes

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Formation of the long bones requires a cartilage template. Cartilage formation (chondrogenesis) proceeds through determination of cells and their aggregation into prechondrogenic condensations, differentiation into chondrocytes, and later maturation. Several studies indicate that members of the bone morphogenetic protein (BMP) family promote cartilage formation, but the exact step(s) in which BMPs are involved during this process remains undefined. To resolve this issue, we have used a retroviral vector to misexpress the BMP antagonist Noggin in the embryonic chick limb. Unlike previous reports, we have characterized the resulting phenotype in depth, analyzing histological and early chondrogenic markers, as well as the patterns of cell death and proliferation. Misexpression of *Noggin* prior to the onset of chondrogenesis leads to the total absence of skeletal elements, as previously reported (J. Capdevila and R. L. Johnson, 1998, *Dev. Biol.* 197, 205–217). Noggin inhibits cartilage formation at two distinct steps. First, we demonstrate that mesenchymal cells do not aggregate into prechondrogenic condensations, and additional results suggest that these cells persist in an undifferentiated state. Second, we show that differentiation of chondroprogenitors into chondrocytes can also be blocked, concurrent with expanded expression of a presumptive joint region marker. In addition, we observed alterations in muscle and tendon morphogenesis, and the potential role of BMPs in these processes will be discussed. Our studies therefore provide *in vivo* evidence that BMPs are necessary for different steps of chondrogenesis: chondroprogenitor determination and/or condensation and subsequent differentiation into chondrocytes.

Key Words: **bone morphogenetic proteins; BMP; cartilage formation; chicken; chondrogenesis; Noggin.**

INTRODUCTION

In the vertebrate limb, bone formation proceeds through ossification of a preexisting cartilage template (reviewed in Erlebacher *et al.*, 1995). The first sign of the multistep process of chondrogenesis (see Fig. 1; Wezeman, 1998) is the aggregation of mesenchymal cells into prechondrogenic condensations. Aside from their aggregated state, these chondroprogenitors are morphologically indistinguishable from the rest of the mesenchyme. However, they start to express markers specific for the chondrogenic lineage (for review, Hall and Miyake, 1995). The prechondrogenic condensed mesenchyme then differentiates into small immature chondrocytes. A change in cell shape, gene expression, and increased deposition of cartilage matrix characterize this event. Concomitant with chondrocyte differentiation, a sheath of flattened fibroblastic-like cells—the perichondrium—forms around the cartilage rudiment. Chondrocyte maturation then proceeds, followed by ossification, starting in the center of the element.

The limb cartilage elements form in a temporal proximal-to-distal sequence, but are initially contiguous. Through the gradual recruitment of cells, the primary condensation of the stylopodial element (humerus/femur) bifurcates across the anteroposterior axis to form the zeugopodial (radius–ulna/tibia–fibula) and then the autopodial (wrist/ankle and digits) elements. The individual elements are generated by the progressive cleavage of this continuous condensation along the proximodistal axis at the presump-

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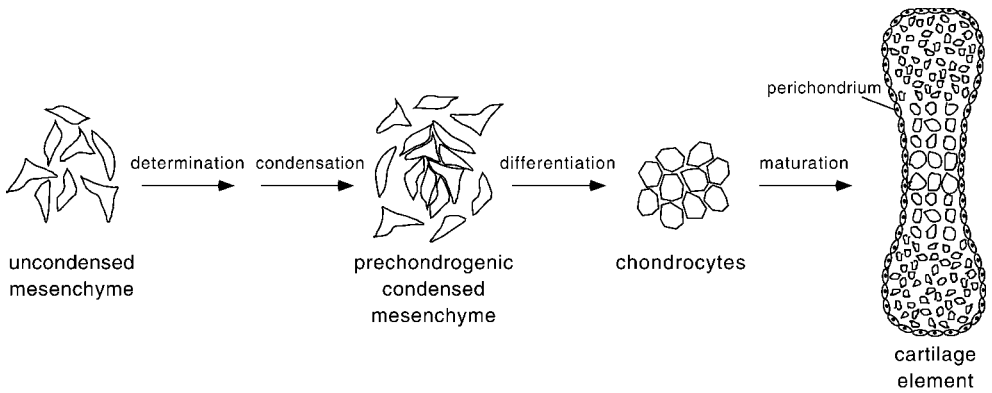


FIG. 1. Schematic illustrating the successive steps of chondrogenesis, see Introduction for details.

tive joint regions (Fell and Canti, 1934), these latter defining the future epiphyseal ends of the skeletal elements. In regions of the condensation where joints will form, condensed chondroprogenitors do not differentiate into chondrocytes and instead adopt a joint fate. This involves expression of new genes and a down-regulation of genes specific to the chondrogenic lineage. The presumptive joint cells then become tightly packed, with a subset undergoing apoptosis as part of their normal program (Haines, 1947). Thus, chondrocytes and joint cells develop in close spatial apposition.

Throughout this paper, we will refer to *determination* as the covert process by which undifferentiated mesenchyme acquires a chondrogenic fate prior to its aggregation into prechondrogenic condensations (Christ *et al.,* 1983) and to *chondrocyte differentiation* as the transition from cells of the prechondrogenic condensations to chondrocytes.

A number of extracellular molecules belonging to the transforming growth factor β (TGFβ) superfamily have been proposed to be key players in chondrogenesis. Among these are members of the bone morphogenetic protein (BMP) family, which can trigger cartilage formation when implanted at ectopic sites in adult animals (Hogan, 1996). In the developing chick limb, *Bmp2, Bmp4,* and *Bmp7* are expressed in the mesenchyme in overlapping patterns, prior to formation of precartilaginous condensations, and in cells adjacent to the condensations during their aggregation. Later, these *Bmp* transcripts are found in the perichondrium. mRNAs of the two types of BMP receptors are present ubiquitously in the mesenchyme throughout limb morphogenesis (*BmpRII* and *BmpRIA*) or, more specifically, in prechondrogenic aggregates, immature chondrocytes, and perichondrium (*BmpRIB*) (see references in Macias *et al.,* 1999). These expression profiles are therefore compatible with BMP signaling at multiple steps of limb chondrogenesis, from determination to maturation.

Little genetic evidence exists to support a role for BMPs in limb chondrogenesis. The phenotype of the *short ear* mouse, a null mutant for *Bmp5,* indicates a function for BMP5 at the condensation step, but not in the limb (King *et al.,* 1994). The existing targeted mutations of other *Bmps* and their receptors are not informative due to either early lethality or lack of a phenotype directly related to cartilage formation (references in Hogan, 1996; Zhang and Bradley, 1996; Dunn *et al.,* 1997; Katagiri *et al.,* 1998). Nonetheless, results from experiments simulating BMP loss of function in the chick embryo show that BMPs act in limb chondrogenesis. Retroviral-mediated misexpression of dominant-negative versions of BMPRs or Noggin, a secreted BMP antagonist [Noggin binds BMP2, BMP4, and to a lesser extent, BMP7, preventing them from interacting with their receptors (Zimmerman *et al.,* 1996)], results in limbs that lack Alcian blue-stained cartilage elements (Kawakami *et al.,* 1996; Zou *et al.,* 1997; Capdevila and Johnson, 1998). These studies did not attempt to define at what step chondrogenesis was affected and therefore the phenotype could be due to either an early or a late defect in cartilage formation.

BMP gain-of-function experiments in the chick alter chondrogenesis at an undefined step or in a manner that suggests a late function for BMPs in chondrogenesis. Addition of BMP to *in vitro* limb mesenchyme cultures seems to enhance chondrogenesis after the condensation step (Roark and Greer, 1994). Infection of embryos with retroviruses encoding *Bmp2, Bmp4,* or activated type I *BmpRs* delays chondrocyte maturation and results, by an unclear mechanism, in fusion and hyperplasia of the limb cartilage elements (Duprez *et al.,* 1996; Zou *et al.,* 1997). Application of BMP-soaked beads to undifferentiated mesenchyme causes cell death and expansion and disrupted joint morphogenesis of preexisting cartilage rudiments (Gañan *et al.,* 1996; Macias *et al.,* 1997).

The loss of Noggin function should be equivalent to a derepression of the activity of multiple BMPs. In the limb, *Noggin* is expressed in prechondrogenic condensations and in chondrocytes (Brunet *et al.,* 1998; Capdevila and Johnson, 1998; Merino *et al.,* 1998). In *Noggin* knockout mice, the limb skeletal elements are broader and joint formation

is not initiated. Since the initial condensation phase and proliferation in the epiphyses appear to occur normally, it has been suggested that the cartilage expansion is due to increased recruitment of cells at later stages (Brunet *et al.*, 1998).

Thus, it remains equivocal whether the chondrogenic effects of BMPs target perichondrial activity or chondroprogenitor determination, condensation, and differentiation and whether these effects stem, for instance, from the recruitment of cells or increased proliferation of any of these populations. In sum, the precise role of BMP in limb chondrogenesis is far from settled.

Here we have misexpressed *Noggin* to define BMP requirements during limb chondrogenesis. The affinity of Noggin for multiple BMPs has allowed us to avoid complications related to a potential redundancy in BMPs function. *Noggin* misexpression leads to the complete absence of skeletal elements. We show that this phenotype results from the inhibition of two early steps of chondrogenesis, formation of the prechondrogenic condensations and chondrocyte differentiation, and we explore the mechanism of BMP regulation of these events. Our *in vivo* studies provide definitive proof that BMPs are necessary for prechondrogenic condensations to form and define a novel role for BMPs in the transition of condensed chondroprogenitors into chondrocytes.

MATERIALS AND METHODS

Retroviral Infection

Noggin-RCAS(A) virus (Pizette and Niswander, 1999; titer ~3×10^8 pfu/ml; Morgan and Fekete, 1996) was injected at Hamburger–Hamilton stage 14 (Hamburger and Hamilton, 1992) into the presumptive limb fields or at stage 17 and 20 into the limb buds. For molecular studies other than Alcian blue staining, embryos were injected at stage 14 and fixed no later than stage 27 (E5), since these injections caused lethality between E6 and E7. This protocol leads to infection throughout the limb by stage 24, including the centralmost regions, as determined by section RNA *in situ* hybridization with an RCAS *pol* probe which detects viral transcripts.

RNA in Situ Hybridization and Probes

RNA *in situ* hybridization with digoxigenin-labeled probes was performed as referenced in Zou *et al.* (1997). Antisense probes were generated as described: *BmpRIB* (Zou *et al.*, 1997), *Gdf-5* (Francis-West *et al.*, 1999), *Sox9* (Kent *et al.*, 1996), *Col-II* (Nah *et al.*, 1988), *Tgfβ2* (Merino *et al.*, 1998), *Cek-8* (Sajjadi and Pasquale, 1993), *Pax3* (Goulding *et al.*, 1993), and RCAS *pol* (Crowe *et al.*, 1998). We often find in whole-mount RNA *in situ* hybridization that gene expression in superficial and deep cells can be distinguished by purple and turquoise color, respectively. The differential staining may arise from differences in pH between superficial and deep tissue during the color detection step. In our protocol, the limbs are exposed to NTMT buffer for 20 min prior to addition of the color substrate.

Histology

White Leghorn fertilized chicken eggs (SPAFAS) were incubated at 39°C for the appropriate time. Embryos were fixed in 4% paraformaldehyde (PFA) for whole-mount RNA *in situ* hybridization, 4% PFA–5.5% EDTA decalcifying solution for paraffin section *in situ* and TUNEL, or Carnoy solution for peanut agglutinin (PNA) staining and BrdU detection on paraffin sections. All sections were cut at 8 μm. For whole-mount immunohistochemistry, embryos were fixed by dehydration through an ascending methanol series. BrdU incorporation and detection, whole-mount Nile blue staining, and Alcian blue staining were executed as in Zou *et al.* (1997), TUNEL as in Chen *et al.* (1994), PNA staining as in Stringa and Tuan (1996), and MF20 antibody (Bader *et al.*, 1982) whole-mount staining as in Yokouchi *et al.* (1995).

Micromass Cultures

High-density micromass cultures were prepared from stage 22 to 24 limb buds as described (Zou *et al.*, 1997). The initial incubation of plated cells in media marks day 0; 24 h later marks day 1. For cultures made from RCAS-*Noggin*-infected limbs, embryos were injected in all four limb fields at stage 12 to compensate for the delay between viral infection and transcription, allowing *Noggin* expression by the time of the harvest. Comparable results were obtained with micromass cultures prepared from limb buds infected with RCAS-*Noggin* virus or from uninfected cells incubated with an exogenous source of Noggin. Conditioned medium from control and *Noggin* stably transfected CHO cells (kind gift from R. Harland) was produced as in Lamb *et al.* (1993). In all cases, culture medium was replaced daily. The concentration of Noggin-conditioned medium required to inhibit chondrogenesis was evaluated by serial dilution: a 1:10 dilution into standard medium (as defined in Zou *et al.*, 1997) did not entirely block the formation of cartilaginous nodules, whereas a 1:7 dilution was fully effective. Further assays were conducted using a 1:5 dilution (~60 ng/ml Noggin protein), unless otherwise stated. We also determined the concentration of Noggin-conditioned medium required to antagonize the ectopic chondrogenesis stimulated by exogenous BMP protein. BMP2 at 50 ng/ml (Genetics Institute) was added to day 1 cultures in accordance with studies by Roark and Greer (1994). Incubation in a 1:4 or 1:3 dilution of Noggin-conditioned medium inhibited both the endogenous and the ectopic cartilage formation induced by 50 ng/ml BMP2, whereas 1:7 or 1:5 dilutions of Noggin-conditioned medium only gave partial inhibition. All experiments were done in triplicate and repeated thrice.

RESULTS

Noggin Inhibits Chondrogenesis in Micromass Culture

To begin to define the effects of Noggin on cartilage formation, we have used the micromass culture system. High-density micromass cultures derived from primary limb mesenchyme cells spontaneously differentiate *in vitro* to form cartilage nodules in a way that closely resembles *in vivo* chondrogenesis. The effect of Noggin on chondrogenesis in micromass cultures was first monitored using Alcian blue, which reacts with sulfated proteoglycans deposited in the cartilage matrix. In control cultures, mesenchymal condensations became Alcian blue positive in the central region, where cell density is the highest, by day 3 (Fig. 2A). Over the next few days, the cartilaginous nodules increased

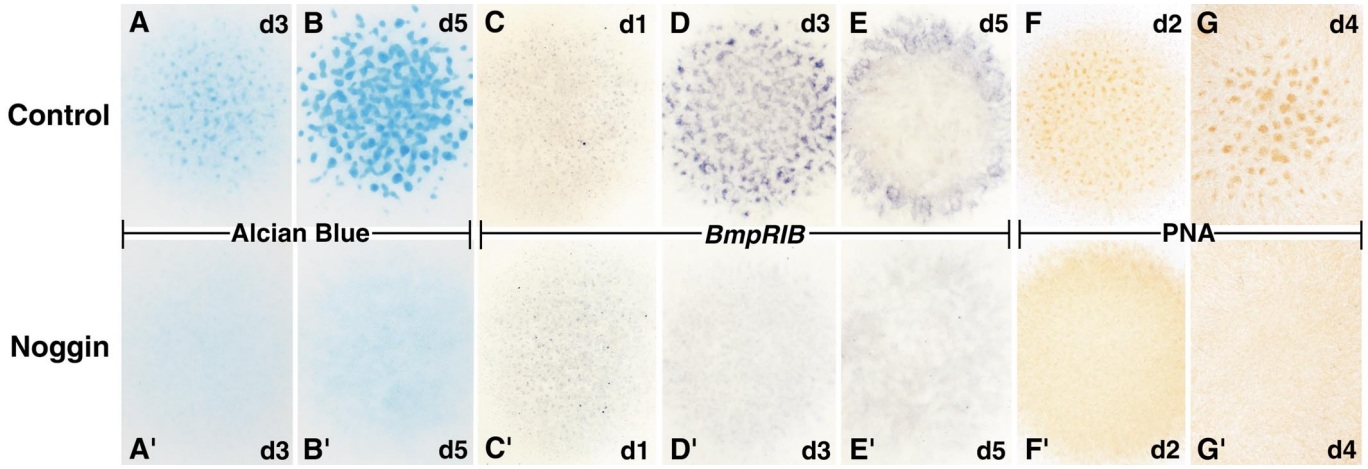


FIG. 2. Noggin blocks chondrogenesis in limb mesenchyme micromass cultures. Control micromasses were cultured in conditioned medium from untransfected CHO cells (A, B, F, G) or standard medium (C–E). (A′, B′, F′, G′) micromasses were cultured in conditioned medium from *Noggin*-transfected CHO cells. (C′–E′) Micromasses were prepared from embryos injected 36 h earlier with RCAS-*Noggin* virus and cultured in standard medium. Alcian blue detects sulfated proteoglycans deposited in cartilage matrix; *BmpRIB* is expressed in, and peanut agglutinin (PNA) selectively binds, prechondrogenic condensations. None of these markers are detected in presence of Noggin except for *BmpRIB* mRNA at day 1 (d1).

in size, stained more strongly, and appeared at the periphery of the culture (Fig. 2B). Addition of a 1:5 dilution of Noggin-conditioned medium (for details, see Materials and Methods) to day 0 cultures fully prevented the formation of Alcian blue-positive nodules over all time points examined (daily intervals up to day 8, Figs. 2A′ and 2B′ and data not shown). In a second experiment, control cultures incubated with 50 ng/ml purified recombinant BMP2 protein (starting on day 1) showed enhanced formation of Alcian blue-stained nodules. A 1:4 dilution of Noggin-conditioned medium abrogated the effects of exogenous BMP2 as well as intrinsic chondrogenesis (not shown). Thus, Noggin-conditioned medium efficiently represses the activity of chondrogenic factors endogenous to the cultures and that of exogenously supplied BMP.

We then tested whether Noggin could affect the expression of early chondrogenic markers. *In vivo*, *BmpRIB* is expressed in prechondrogenic condensed mesenchyme and in immature chondrocytes (Kawakami *et al.*, 1996; Zou *et al.*, 1997). The galactose-specific lectin PNA binds condensed mesenchyme, including precartilaginous condensations (Aulthouse and Solursh, 1987). *BmpRIB* mRNA was already visible throughout control day 1 cultures in a dot-like pattern. The identity of the cell type(s) marked at this time is unclear. From day 2 onward, the staining gained in intensity and was detected in larger foci of cells in the center of the micromass, resembling the distribution of the future cartilage nodules. By day 4, *BmpRIB* expression was also detected at the edges of the culture, and was eventually down-regulated in its center (Figs. 2C–2E). PNA localization was spatially very similar to that of *BmpRIB* transcripts except that PNA staining lagged behind by 1 day (Figs. 2F and 2G). In Noggin-treated cultures, although *BmpRIB* expression at day 1 was similar to that seen in control cultures, it was subsequently neither up-regulated nor found in larger foci. PNA labeling was never observed in Noggin-treated cultures (Figs. 2C′–2G′, PNA binding and *BmpRIB* expression examined up to day 5 and 6, respectively). These results indicate that Noggin abolishes the expression of prechondrogenic condensation markers.

Noggin Misexpression in Vivo Disrupts Chondrogenesis

Noggin misexpression in the limb before overt cartilage formation recapitulated the effects of Noggin protein in micromass cultures. RCAS-*Noggin* virus infection of fore- or hindlimb fields at stages 14, 17, or 20 produced very short limbs completely lacking Alcian blue-stained cartilage (Fig. 3A, $n = 29/30$, assayed up to stage 31). Viral injections restricted to the anterior or posterior region of stage 17 limb buds resulted in loss of anterior or posterior skeletal elements, respectively. However, the remaining digits retained their proper identity, as scored by segmentation pattern ($n = 17/18$, data not shown and Capdevila and Johnson, 1998). Thus, the Noggin-induced phenotype likely is the result of impaired chondrogenesis but not of inhibition of earlier patterning events.

Noggin Prevents Chondrogenesis at the Determination or Condensation Step

We next addressed whether Noggin could affect the expression of prechondrogenic markers *in vivo*. We exam-

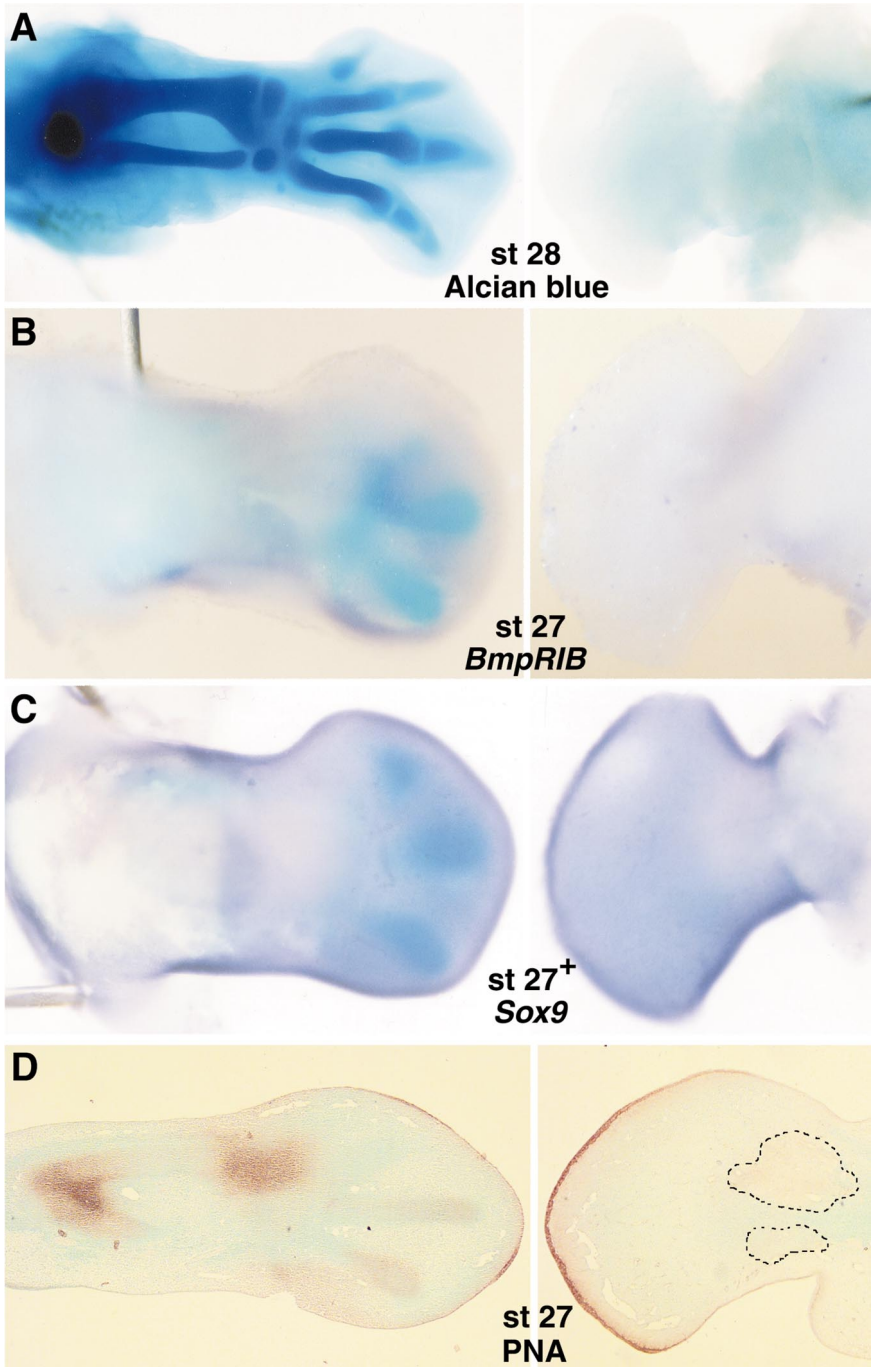


FIG. 3. *In vivo* cartilage formation is dramatically inhibited following *Noggin* misexpression. Control uninfected and RCAS-*Noggin*-infected hindlimbs on left and right, respectively. Limb primordia were injected at stage 14 (B–D) or stage 17 (A) and embryos fixed at stages 27–28. (A–C) Alcian blue staining is absent from RCAS-*Noggin*-infected limbs and *BmpRIB* and *Sox9* expression is greatly reduced [superficial *Sox9* expression (purple staining) is unaffected]. (D) In representative sections, the lack of PNA labeling and faint methyl green counterstaining in the infected autopod indicate that chondrogenesis is blocked at, or before, the condensation step. A faint PNA signal is, however, present in the infected zeugopod (dotted outline). Strong methyl green highlights areas of increased cell density but does not specifically label prechondrogenic condensations. Whole-mount embryos shown in dorsal view, anterior to the top; control and infected samples are at the same magnification.

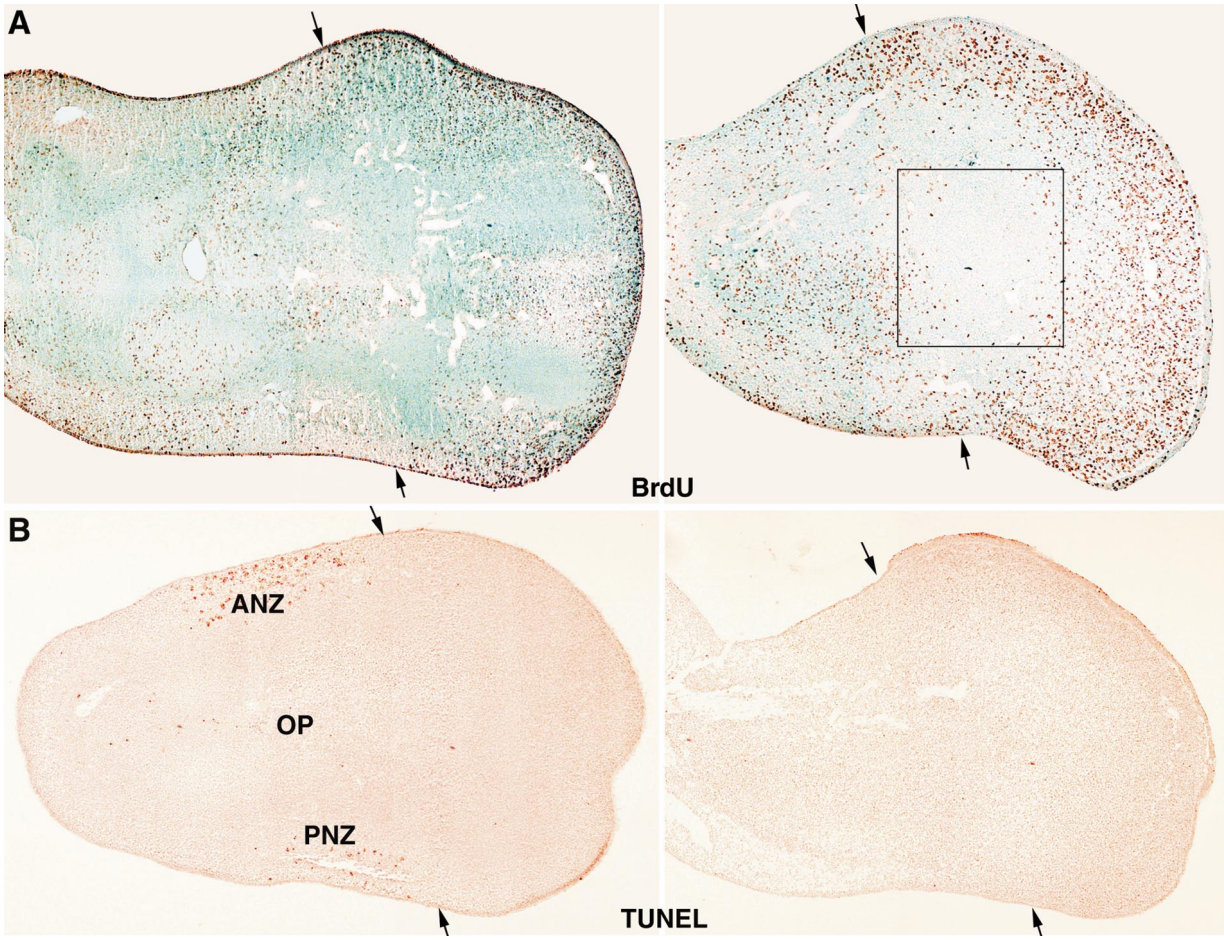


FIG. 4. Analysis of cell proliferation and cell death in stage 27 limbs. RCAS-*Noggin* virus was injected into the limb primordia at stage 14 and infected and uninfected limbs were fixed at stage 27, sectioned, and processed for BrdU incorporation (A, hindlimbs) or TUNEL (B, forelimbs). Representative sections of control and RCAS-*Noggin*-infected limbs are on left and right, respectively. Arrows indicate the approximate zeugopod/autopod boundary. Anterior is up, distal to right. (A) In control, brown BrdU-labeled cells are detected throughout the tissue except in condensed mesenchyme, which stains intensely with methyl green. In infected limbs, BrdU is incorporated into cells in the distal but not proximal (square) part of the autopod, where light methyl green staining indicates a lack of mesenchyme condensation. (B) In control limbs, TUNEL-positive cells are present in the anterior and posterior necrotic zones (ANZ, PNZ) and opaque patch (OP). No apoptosis is observed in the autopod and zeugopod of RCAS-*Noggin*-infected limbs (every section was examined).

ined PNA binding on sections and the expression of *BmpRIB* and the *Sry*-related *Sox9* transcription factor by whole-mount *in situ* hybridization. *Sox9* is transcribed in precartilaginous condensations and chondrocytes (Ng *et al.*, 1997), similar to *BmpRIB*, and activates the transcription of type II Collagen (*Col-II*), a major component of the cartilage matrix (Bell *et al.*, 1997). In stage 27 control autopods, *BmpRIB* and *Sox9* were expressed in, and PNA bound to, the wrist/ankle prechondrogenic condensations and the digital rays. In stage 27 limbs infected at stage 14 with RCAS-*Noggin*, *BmpRIB* and *Sox9* expression were extremely reduced or below detection in prechondrogenic regions (Figs. 3B and 3C, $n = 10/10$ for both probes), suggesting that condensation of the autopodial elements did not occur in infected limbs. This was further corroborated by the lack of PNA staining in the autopod of infected embryos ($n = 10/10$, Fig. 3D). In contrast, a PNA signal was present within the zeugopod region of infected limbs. This result will be discussed later.

If all the markers studied above are direct downstream targets of the BMP pathway (Harada *et al.*, 1998; Healy *et al.*, 1999), the absence of their expression would not necessarily imply an absence of the cell types which would synthesize these molecules. The lack of cellular aggregation in the autopod was nevertheless confirmed histologically by semithin plastic sections (not shown) and the faint methyl green counterstain which normally highlights regions of high cell density (Figs. 3D and 4A). Hence, our data

provide evidence that *Noggin* misexpression compromises chondrogenesis at the condensation step or upstream of this event, at the determination step.

Absence of Autopodial Condensations in RCAS-Noggin-Infected Limbs Is Not Due to Cell Death

To determine why prechondrogenic condensations do not form in the autopod of RCAS-*Noggin*-infected limbs, we examined the patterns of cell death and proliferation. To assay cell death, we performed whole-mount Nile blue staining and TUNEL on sections from stage 24 to 27 limbs. No apoptosis was detected at stage 27 in the autopod (and zeugopod) of infected limbs ($n = 7/7$, Fig. 4B and Pizette and Niswander, 1999) when condensations should be forming. Apoptosis was also not detected at earlier stages (stages 24 and 25) in the distal mesenchyme underlying the apical ectodermal ridge (AER), which should contain the progenitors of the autopodial elements (Figs. 6A and 6B and data not shown). Note, however, that there is ectopic cell death in the zeugopod region at these stages and this will be addressed later. We then addressed proliferation by assessing BrdU incorporation on sections of stage 27 limbs, concentrating in particular on the core mesenchyme where the chondrogenic areas should be found. Compared to controls, infected limbs demonstrated extensive mesenchyme proliferation in the distal part of the autopod beneath the AER, most likely a consequence of enhanced AER function following *Noggin* misexpression (see Pizette and Niswander, 1999). Interestingly, cells within the proximal part of the autopod were largely BrdU-negative, not aggregated, and very sparsely distributed (Fig. 4A, boxed area; $n = 6/6$). Control autopods also exhibited regions of low mitotic index, but these were restricted to the areas of condensations (Fig. 4A).

These results show that *Noggin* misexpression does not trigger death of the cells normally destined to form the prechondrogenic condensations of the autopod. These cells therefore persist, although their proliferation rate appears altered. These data do not indicate the state of differentiation of these cells, which may never have been, or may no longer be, chondroprogenitors. It is also possible that they have acquired another cell fate.

Alternate Cell Fate for Autopodial Chondroprogenitors in RCAS-Noggin-Infected Limbs?

Mesenchymal progenitors have a developmental plasticity, such that they can give rise to chondroblasts, osteoblasts, adipocytes, myoblasts, or connective tissue (Christ *et al.*, 1983; Tajbakhsh *et al.*, 1996; Wang *et al.*, 1993). To evaluate whether cells failing to receive a BMP signal are diverted to another cell lineage, we examined the expression of available markers of limb mesenchyme subpopulations, namely tendon, muscle (despite the fact that their limb precursors originate in the somites), and joint.

Individual tendons arise from three dorsoventral pairs of primordia distributed along the proximodistal axis of the limb. Since our interest is in the fate of cells that should have formed the precartilaginous condensations of the autopod, we focused on the distal tendon primordia. These were visualized by their expression of *TGFβ2* and the *Eph*-related receptor *Cek-8* (Merino *et al.*, 1998; Patel *et al.*, 1996). At stage 27, RCAS-*Noggin*-infected limbs displayed relatively normal levels of *TGFβ2* and *Cek-8* RNAs. However, the distal tendon primordia of these limbs were highly disorganized. They showed no sign of segregation into the tendon blastemas that normally associate with the digital rays at this stage and instead spanned the length of the anteroposterior axis ($n = 15/15$ and $10/10$, Fig. 5A and data not shown, respectively).

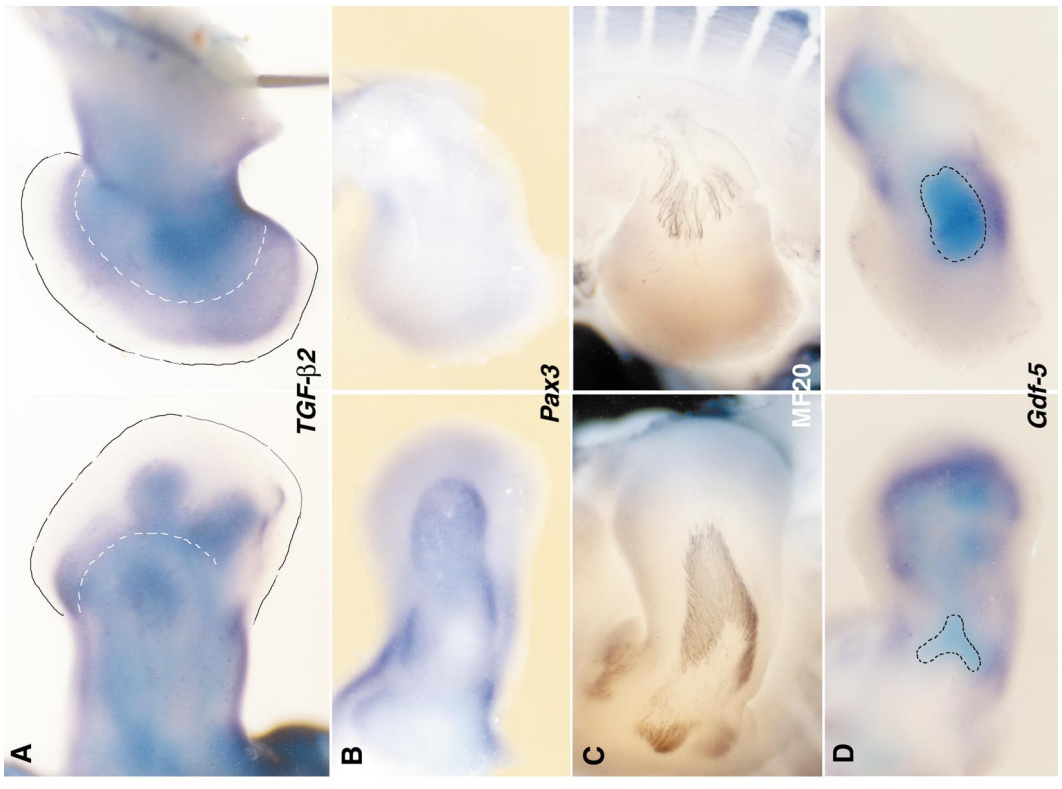
Pax3 expression correlates with the proliferative potential of presumptive muscle cells; it marks these cells during their migration into and within the limb and later outlines the edges of the differentiating muscle masses (Amthor *et al.*, 1998; Williams and Ordhal, 1994). At stage 22, both control and injected wing buds exhibited a similar distribution of *Pax3* transcripts, indicating that *Noggin* misexpression did not interfere with the initial myogenic cell migration ($n = 6/6$, data not shown). However, at stage 27, *Pax3* expression in infected limbs was drastically reduced along the entire proximodistal axis ($n = 10/11$, Fig. 5B). Differentiation into muscle fibers was monitored at stage 27 with the MF20 antibody (Bader *et al.*, 1982), establishing that in infected limbs the overall number of myotubes was greatly reduced and that some of them were improperly oriented ($n = 9/10$, Fig. 5C).

Gdf-5 (growth and differentiation factor-5), a TGF*β* superfamily member, marks the prospective joints of the appendicular skeleton (Storm and Kingsley, 1996). In stage 27 control autopods, *Gdf-5* was expressed in the wrist/ankle joint-forming region, as well as the interdigital mesenchyme. In infected autopods, *Gdf-5* mRNA was undetectable ($n = 11/12$, Fig. 5D). Unexpectedly, we consistently noted ectopic *Gdf-5* expression in a more proximal area of the infected limbs, where the zeugopod elements would normally form. This will be addressed in the next section.

In summary, instead of an expansion in populations adopting the tested cell fates, we observed either no noticeable change or a decrease in the extent of expression of tendon, muscle, and joint markers in infected limbs. These data do not support the idea that *Noggin* misexpression causes the cells that would normally become the autopod chondroprogenitors to be directed to another fate. We therefore tentatively conclude that the progenitors of the autopodial skeleton persist as undifferentiated mesenchymal cells.

Noggin Blocks Chondrocyte Differentiation

Stage 14 viral infections lead to widespread Noggin misexpression in the limb prior to normal condensation of autopodial and zeugopodial skeletal elements and result in



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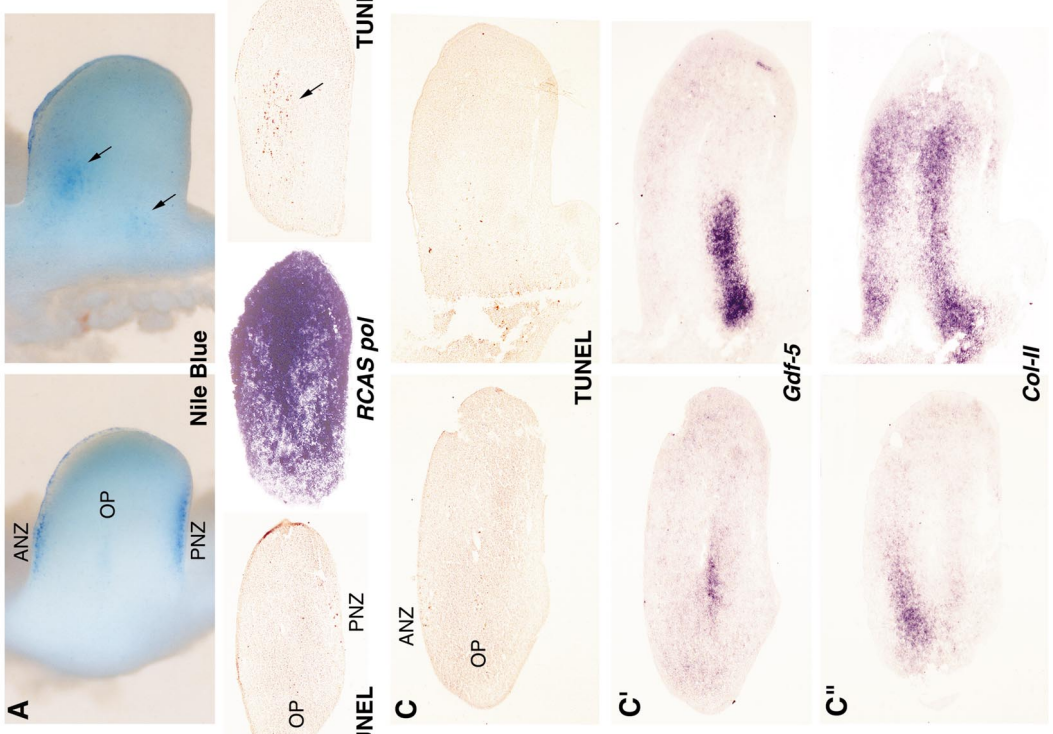

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total loss of Alcian blue-stained cartilage (Fig. 3A). However, unlike in the autopod, three pieces of evidence indicated that prechondrogenic condensations formed in the zeugopod of infected limbs. First, cells of the precartilaginous condensations normally give rise to both chondrocytes and joint cells, and *Gdf-5* marks the presumptive joint cells in the condensation. Since *Gdf-5* was expressed in the center of the infected zeugopod at stage 27 (Fig. 5D), this result suggests that prechondrogenic cells aggregated in this region. Second, PNA staining (Fig. 3D) indicated again that condensation took place in the infected zeugopod. Third, histological examination of infected limbs at stage 27 confirmed the presence of condensed mesenchyme in the zeugopod (concentrated methyl green staining), but also demonstrated a complete absence of chondrocytes (Figs. 3D and 4A). These results imply that BMPs are required for the differentiation of chondroprogenitors into chondrocytes. BMPs may act, for instance, as survival factors for condensed chondroprogenitors or as repressors of the joint fate (see Brunet *et al.*, 1998; Merino *et al.*, 1999a). Both hypotheses will be addressed below.

To explore whether condensed chondroprogenitors that would give rise to zeugopodial chondrocytes die, we performed whole-mount Nile blue staining. At stage 24, when condensations of the zeugopodial elements are normally laid down, 89% of RCAS-*Noggin*-infected limbs ($n = 18$) lacked the zones of normal cell death present in the zeugopod of control limbs, but 45% (mostly forelimbs) also exhibited ectopic staining in regions other than the progress zone (Fig. 6A). The ectopic cell death was transient, observed at stage 25 in only 33% of the infected limbs [$n = 6$ (all forelimbs)/18, not shown] and not at all at stage 27 (Pizette and Niswander, 1999). Therefore, BMP signaling could be needed for survival of condensing cartilage precursors.

To determine if this transient ectopic cell death correlated with regions of prechondrogenic condensation, alternate sections of stage 24 limbs were assayed for TUNEL and expression of *Col-II* (which faintly marks precartilaginous condensations; Nah *et al.*, 1988), *Gdf-5* (to look for aberrant conversion of cells to a joint fate), and RCAS *pol* (for viral transcripts). Forelimb and hindlimb analyses gave different results, probably due to the relative developmental delay of chondrogenesis in forelimbs. The Noggin-induced phenotype in the wings presumably represents an earlier stage of the phenotype seen in the legs, and these results are presented separately. All infected forelimbs ($n = 3$) had a significant number of dying cells located in the zeugopod, outside of the normal apoptotic domains. No apoptosis, however, was detected in the distal mesenchyme (Fig. 6B). None of these zeugopods showed *Gdf-5* expression at this stage (at stage 27 *Gdf-5* is strongly misexpressed in the zeugopod of infected legs *and* wings, Fig. 5D), and *Col-II* stained faintly and diffusely (not shown). These expression patterns resembled that of control forelimbs (not shown). Although these results cannot clearly identify the dying cells, we can conclude that the *Noggin*-induced cell death is not a consequence of the ectopic *Gdf-5* expression.

The infected hindlimb zeugopods ($n = 2$) had fewer TUNEL-labeled cells than infected wings, but these were again abnormally localized. Since whole-mount preparations show that the ectopic cell death is transient, this may indicate that the wave of cell death in the legs is nearing completion at this stage. In control legs, two faint areas of *Col-II* staining prefigured the tibia and fibula, and *Gdf-5* was expressed in a narrow region between the proximal ends of these domains, which appeared to correlate with dying cells of the opaque patch (Figs. 6C–6C″). In contrast, both infected legs showed ectopic and high levels of *Col-II*

FIG. 5. Expression of tendon, muscle, and joint markers in RCAS-*Noggin*-infected limbs. Control (left) and infected (right) limbs shown in dorsal view, anterior at top, same magnification for all. (A) Hindlimbs and (B–D) forelimbs. *Noggin* virus was injected at stage 14 and the embryos were fixed at stage 27 and processed for (A, B, D) whole-mount RNA *in situ* hybridization or (C) whole-mount detection of muscle fibers using the MF20 antibody. (A) In the control autopod, the tendon primordia visualized by the marker *Tgfβ2* have segregated (purple staining distal to the white dotted line). However, in the infected autopod, *Tgfβ2* expression distal to the white line is uniform, indicating that the distal tendon primordia do not segregate properly. (B) *Pax3* RNA is detected in the muscle precursors in the control limb, but is barely detectable in infected limbs. (C) Muscle fibers in infected limbs are disorganized and reduced in number. (D) In control, *Gdf-5* expression prefigures the elbow (dotted outline) and wrist joints and outlines the digital rays and interdigit. In infected limbs, *Gdf-5* is not expressed in the autopod, but is ectopically expressed at high levels in the zeugopod (dotted outline). In (A and D), superficial and deep mesenchymal expression is detected as purple and turquoise staining, respectively (see Materials and Methods).

FIG. 6. Analysis of cell death and prechondrogenic condensation markers in stage 24 limbs. Control uninfected (left) and RCAS-*Noggin*-infected (right and center in B) limbs, anterior at top and distal to right, dorsal views in (A). Control and infected samples are at the same magnification. *Noggin* virus was injected at stage 14 and the embryos were fixed at stage 24. Cell death was detected by whole-mount staining with Nile blue (A) or on sections with TUNEL (B and C; brown nuclei). (A and B) In control forelimbs, the ANZ, PNZ, and OP are visible, whereas they are not in infected forelimbs, and instead there is widespread ectopic cell death in the zeugopod region (highlighted by arrows). Note that there is no apoptosis in the distal mesenchyme of infected limbs, even though there is widespread viral expression (RCAS *pol* probe on alternate section). (C, C′, C″) Alternate sections of hindlimbs. Controls display developmentally programmed cell deaths (PNZ is not present in this section) and expression of *Gdf-5* in between the zeugopod condensations which are marked by *Col-II*. Infected hindlimbs have fewer TUNEL-positive cells than infected forelimbs, but these are again ectopic and the normal apoptotic zones are absent. Infected hindlimbs show up-regulated *Col-II* expression distributed throughout the mesenchyme, abnormally overlapping with *Gdf-5* expression which is also up-regulated and expanded (C–C″ and not shown).

mRNA throughout the mesenchyme and increased and expanded *Gdf-5* expression in the center of the zeugopod, overlapping that of *Col-II*. The few apoptotic cells present in these samples were excluded from the *Gdf-5* domain, but likely colocalized with *Col-II* transcripts (Figs. 6C–6C″ and data not shown). The significance of the deregulated *Col-II* expression and its relationship to the state of mesenchyme condensation in the infected hindlimb zeugopods is unclear. Thus, it remains uncertain if dying cells are condensed chondroprogenitors. However, the ectopic *Gdf-5* expression and its overlap with *Col-II* expression suggest that cells of the precartilaginous condensations may be abnormally directed into a joint fate.

DISCUSSION

Bmps and *BmpRs* are expressed in the limb mesenchyme prior to aggregation of prechondrogenic condensations and then in or around condensations. Later, their transcripts are found in the chondrocytes and/or perichondrium. BMPs are thus present at the right times and places to direct several stages of cartilage formation. Here, we have misexpressed *Noggin* both *in vitro* and *in vivo* to carefully examine the role(s) of BMPs during early limb chondrogenesis. Our results define two steps at which BMPs act (see Fig. 1). Inhibition of BMP signaling prevents the appearance of prechondrogenic condensations and also blocks their differentiation into chondrocytes. Therefore, BMPs are involved in the initial steps of chondrogenesis.

Specificity of the Noggin-Mediated Antagonism

Activin and TGFβ2 have been proposed to be regulators of early steps of chondrogenesis in the chick autopod, based on their expression pattern and ability to stimulate *de novo* chondrogenesis. Their effects are suggested to be mediated through induction of *BmpRIB* expression, the expression of which then allows BMPs to signal and regulate later steps of chondrogenesis (Merino *et al.*, 1998, 1999b). GDF-5 may also participate in the initial stages of cartilage formation and is expressed in both the zeugopod and the autopod (Francis-West *et al.*, 1999). Noggin binds BMP2, BMP4, and BMP7, preventing them from interacting with their receptors, but does not bind activin or TGFβ (Zimmerman *et al.*, 1996). However, Noggin also binds GDF-5, blocking its activity (Merino *et al.*, 1999a). Hence, BMP signaling aside, GDF-5 signaling could have been antagonized by *Noggin* misexpression. Could the loss of GDF-5 function account for the described phenotypes?

In the autopod, *Gdf-5* is expressed in presumptive joint regions and around the well-developed condensations, but not in or around early condensations (Merino *et al.*, 1999a, and our unpublished observations). Accordingly, digit condensations in the *Gdf-5* loss-of-function mouse mutant, *brachypodism*, are formed but are thinner than normal (Storm *et al.*, 1994; Grüneberg and Lee, 1973). These data suggest a restricted role for GDF-5 in recruitment of cells to preexisting cartilaginous condensations of the autopod. We thus propose that the total absence of autopodial prechondrogenic condensations in the Noggin-induced phenotype arises from loss of BMP signaling.

In the zeugopod, *Gdf-5* is expressed in the joint-forming area of the precartilaginous condensations (Fig. 6C′). GDF5 has been implicated in joint formation as well as in proliferation of immature chondrocytes in the adjacent epiphyses, and chondrocyte differentiation is delayed in the autopod of the *brachypodism* mutant (Storm and Kingsley, 1996; Merino *et al.*, 1999a; Francis-West *et al.*, 1999; Storm and Kingsley, 1999). Therefore, the later effects of *Noggin* misexpression on chondrogenesis may occur through loss of GDF and/or BMP signaling and the specific contribution cannot be inferred from other studies. Indeed, GDF-5 and BMPs share the same receptors and may form heterodimers (Erlacher *et al.*, 1998; Chang and Hemmati-Brivanlou, 1999), indicating that alterations of the activity of one of these factors could alter the activity of the others.

Effects of Noggin Misexpression on the Early Steps of Chondrogenesis

Autopod. In the adult, exogenous BMPs clearly induce *de novo* ectopic chondrogenesis, strongly suggesting that *Bmps* are master genes able to trigger the entire series of events needed for normal chondrogenesis. However, the origin and state of differentiation of the cells recruited to form the adult ectopic cartilage have not been determined. In the RCAS-*Noggin*-infected embryonic autopod, condensed chondroprogenitors are absent and there is a defect in proliferation, but no ectopic cell death is detected at the time and location these cells would normally be condensing or specified. Thus, BMPs are not survival signals for autopodial chondroprogenitors, but they may stimulate chondroprogenitor proliferation prior to condensation. Furthermore, other mesenchymal populations such as joint, tendon, and muscle did not appear to be expanded. Therefore, BMPs do not seem to repress the potential of chondroprogenitors to acquire alternate fates. Whether BMPs regulate the initiation of chondrogenesis at the level of chondroprogenitor aggregation or determination cannot be distinguished, due to a lack of preaggregation chondroprogenitor-specific markers.

Activin and TGFβ2 have been proposed to participate in autopodial chondrogenesis by controlling *BmpRIB* expression. Although our results do not exclude a role for these factors in this process, the lack of *BmpRIB* expression in RCAS-*Noggin*-infected autopods clearly indicates a BMP requirement at this step. In addition, unlike BMPs, TGFβs do not promote ectopic cartilage formation in the adult (Roark and Greer, 1994). One discrepancy is that BMPs cannot induce ectopic cartilage in the embryonic interdigital spaces (Gañan *et al.*, 1996), whereas TGFβ can. However, since BMPs promote apoptosis in this region, it is

possible that the apoptotic response supercedes a potential chondrogenic response.

Zeugopod. In the RCAS-*Noggin*-infected zeugopod, chondrocytes are not morphologically discernible but prechondrogenic condensations are, and they are marked by PNA labeling and *Gdf-5* expression. BMP/GDF signaling is therefore required for chondrocyte differentiation.

This signal may maintain the chondrogenic potential of chondroprogenitors, for instance by repressing joint fate, as *Noggin* misexpression results in an expansion of *Gdf-5* expression into areas that abnormally overlap with the cartilage-forming regions. Three hypotheses could be advanced to explain the expansion of the *Gdf-5* domain. First, BMP/GDF could normally directly repress *Gdf-5* transcription, although *Noggin* misexpression does not activate *Gdf-5* expression in the autopod. Second, BMP/GDF signaling may, directly or indirectly, limit proliferation of the joint cell population. *Noggin* misexpression would thus lead to an increase in the number of cells legitimately expressing *Gdf-5*. Third, because precartilaginous condensations give rise to both joint cells and chondrocytes, it is tempting to speculate that the chondroprogenitors have been converted to a joint fate.

In contrast to the autopod, the ectopic cell death present in the infected zeugopods suggests that BMP/GDF may also act as survival signals for chondroprogenitors once they have condensed. As GDF-5 has been proposed to mediate cell death (Storm and Kingsley, 1999), another possibility is that this ectopic cell death is connected to the expansion of *Gdf-5* expression. The fact that an exogenous source of GDF-5 induces apoptosis only weakly (Merino *et al.*, 1999a) and that expansion of *Gdf-5* expression in infected limbs occurs after the wave of cell death implies that Noggin misexpression triggers ectopic cell death independent of GDF-5. In addition, *Noggin* misexpression is likely to inhibit GDF-5 activity.

Differences along the proximodistal axis. Our early viral infections (stage 14) direct *Noggin* misexpression before condensation of both zeugopodial and autopodial elements. The differing results in the autopod versus the zeugopod could be explained as differences in the nature of the proteins involved in chondrogenesis in these two regions, as for instance activin and TGFβ2 appear to play a role in autopodial but not zeugopodial chondrogenesis (Merino *et al.*, 1998, 1999b). Alternatively, there could be a difference in the extent of *Noggin* misexpression in the zeugopod versus the autopod, and a function for BMPs in the aggregation of chondroprogenitors in the zeugopod, or in their differentiation into chondrocytes in the autopod, could exist. To attempt to address this possibility, we have modified the timing of *Noggin* misexpression by introducing *Noggin* virus at earlier and later times (stages 10 and 20, respectively). Our preliminary data (histological analysis and PNA staining; not shown) indicate that injection of *Noggin* virus at the earlier time results in the absence of zeugopodial condensations. Injection at the later stage still prevents the formation of prechondrogenic condensations in the autopod and still blocks chondrocyte differentiation in the zeugopod. The results of the early injections support a requirement for BMPs in the formation of prechondrogenic condensations in the zeugopod. The lack of condensations in the autopod following late injection precludes an analysis of the role of BMPs in chondrocyte differentiation in the autopod.

Effects of Noggin Misexpression on Limb Myogenesis and Tendon Formation

In the autopod, *Noggin* misexpression prevents chondroprogenitors from condensing or from being determined. To test whether these cells had switched to another fate, we examined the expression of markers of different cell types. We did not find any changes consistent with this hypothesis. However, we saw alterations in muscle and tendon morphogenesis.

Muscle. BMPs have been reported to promote proliferation and/or inhibit differentiation of the myogenic progenitors in the somites and the limbs (Amthor *et al.*, 1998, 1999). In this scheme, removal of BMP signals would deplete the pool of proliferative presumptive muscle cells, thus diminishing the total number of myofibers.

In RCAS-*Noggin*-infected wing buds, the early phase of *Pax3* expression is normal, indicating that muscle progenitors have migrated into the limbs. However, *Pax3* expression is prematurely lost and myofibers, marked by MF20 antibody, are reduced in number and not properly oriented. Although there are many possible explanations for this phenotype, including a dependence of muscle on cartilage formation, our results are in good agreement with the hypothesis of Amthor and colleagues.

We noticed that *BmpRIB* is normally also expressed in regions coincident with *Pax3* expression, at least from stage 22 to stage 27 (not shown). It remains unclear whether *BmpRIB* is transcribed in myogenic cells or cells along their path of migration. However, the similarity between the effects of *Noggin* misexpression on *BmpRIB* and *Pax3* expression in these myogenic regions (our unpublished data) suggests the former. This raises the interesting possibility that BMPs may directly control limb myogenesis through regulation of *Pax3* expression, as could be inferred from other studies (Pourquié *et al.*, 1996; Reshef *et al.*, 1998).

Tendon. The disrupted spatial distribution of *Cek-8* and *Tgfβ2* transcripts, two autopodial tendon markers, shows that the distal tendon primordia form but do not segregate in RCAS-*Noggin*-infected limbs (see Fig. 5A). This disruption is unrelated to the muscle alterations described, since subdivision of the distal primordia is muscle-independent (Kardon, 1998; Kieny and Chevallier, 1979). It could, however, be related to the lack of a cartilage template. Based on the distal extension of tendons in the absence of the two distal most phalanges (Hurle *et al.*, 1990), distal tendon morphogenesis might not require cartilage formation. Alternatively, segregation, the proper lateral positioning of the tendon primordia along the digits (anteroposterior axis),

might respond to clues distinct from those required for their distal extension (proximodistal axis) and rely on cartilage formation. In support, when an ectopic digit is induced interdigitally, the accompanying tendons derive from a continuous lateral extension of the tendon primordia associated with the adjacent digits (Hurle *et al.*, 1990). One interpretation is that the digital skeleton provides positive signals to tendon morphogenesis, but an intact interdigit is also necessary to laterally restrict the position of the primordia to the digits. Thus, whether the suppression of the distal tendon primordia segregation by *Noggin* misexpression is a consequence of the loss of autopodial chondrogenesis or is directly linked to BMP inhibition, a role for *Bmps* expressed in the interdigital mesenchyme or the perichondrium can be considered.

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