

## Control of digit formation by activin signalling

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### SUMMARY

Major advances in the genetics of vertebrate limb development have been obtained in recent years. However, the nature of the signals which trigger differentiation of the mesoderm to form the limb skeleton remains elusive. Previously, we have obtained evidence for a role of TGF $\beta$ 2 in digit formation. Here, we show that activins A and B and/or AB are also signals involved in digit skeletogenesis. *activin  $\beta$ A* gene expression correlates with the initiation of digit chondrogenesis while *activin  $\beta$ B* is expressed coincidentally with the formation of the last phalanx of each digit. Exogenous administration of activins A, B or AB into the interdigital regions induces the formation of extra digits. *follistatin*, a natural antagonist of activins, is expressed, under the control of activin, peripherally to the digit chondrogenic aggregates marking the prospective tendinous blastemas. Exogenous application of follistatin blocks physiological and activin-induced digit formation.

Evidence for a close interaction between activins and other signalling molecules, such as BMPs and FGFs, operating at the distal tip of the limb at these stages is also provided. Chondrogenesis by activins is mediated by BMPs through the regulation of the BMP receptor *bmpR-1b* and in turn *activin* expression is upregulated by BMP signalling. In addition, AER hyperactivity secondary to *Wnt3A* misexpression or local administration of FGFs, inhibits *activin* expression. In correlation with the restricted expression of *activins* in the course of digit formation, neither activin nor follistatin treatment affects the development of the skeletal components of the stylopod or zeugopod indicating that the formation of the limb skeleton is regulated by segment-specific chondrogenic signals.

Key words: Limb development, Chondrogenesis, Chick embryo, BMP-receptor, Follistatin, Noggin, BMP, TGF $\beta$ , FGF, Wnt3a

### INTRODUCTION

The developing limb constitutes a simple and useful model for the analysis of morphogenesis in vertebrates. The early limb bud consists of a core of undifferentiated mesodermal cells covered by an ectodermal jacket. At the distal margin of the bud the ectoderm exhibits a specialized thickening termed the apical ectodermal ridge (AER) which plays a pivotal role in limb development. Limb outgrowth is accomplished by proliferation of the mesoderm subjacent to the AER. This proliferating region is termed the progress zone. Chondrogenic differentiation of the limb mesoderm starts in the central core of the bud and progresses distally by the successive incorporation of cells leaving the progress zone (Summerbell et al., 1973). The primordium of the humerus/femur (stylopodium) is formed first, followed by the ulna-radius/fibula-tibia (zeugopod) and the skeletal pieces of the hand/foot (autopod) are the last to be formed. Concomitantly with the formation of the skeletal rudiments a number of mesodermal cells are eliminated by apoptotic cell death contributing to sculpt the final shape of the limb (Hurle et al., 1996). In the last few years, considerable evidence has been

accumulated indicating that the coordination between growth, cell death and differentiation of the limb mesoderm is controlled by the interaction of signalling molecules acting at the tip of the developing limb (Niswander and Martin 1993; Macias et al., 1997; Merino et al., 1998). The maintenance of the progress zone mesoderm in an undifferentiated and proliferating state is carried out by FGFs (FGF-2, -4, -8 and -9) produced by the AER (reviewed by Martin, 1998). The control of chondrogenic differentiation and cell death involves a signalling pathway mediated by BMPs (BMP-2, -4 and -7; Zou and Niswander, 1996; Kawakami et al., 1996; Yokouchi et al., 1996; Macias et al., 1997; Zou et al., 1997) and TGF $\beta$ s (Gañan et al. 1996; Merino et al., 1998). These findings point to the occurrence of a triple interactive molecular mechanism controlling the growth and differentiation of the limb mesoderm, including distal signals for proliferation (FGFs), proximal signals to establish the zones of chondrogenesis (TGF $\beta$ s), and intermediate signals (BMPs) promoting growth and differentiation in the zones committed to chondrogenesis and apoptosis in the undifferentiated mesoderm. Up to now, TGF $\beta$ 2 is the only identified signal with a potential to determine the zones of chondrogenesis (Gañan et al., 1996;

Merino et al., 1998). Exogenous application of this growth factor into the interdigital regions inhibits interdigital cell death and induces the formation of an ectopic digit through BMP signalling (Gañan et al., 1996; Merino et al., 1998). However, expression of the *tgfb2* gene at the tip of the chondrogenic digital rays occurs relatively late in development (Merino et al., 1998), and transgenic mice deficient in *tgfb2* have normal digits (Sanford et al., 1997). These findings indicate that other chondrogenic signals could be involved in limb skeletogenesis. To further understand this question we have tested the possible role of activins in the onset of chondrogenesis in the developing limb.

Activins are members of the TGF $\beta$  superfamily which play a fundamental role during embryonic development (see Stern et al., 1995). The activins are dimeric proteins, composed of disulphide-linked  $\beta$ A or  $\beta$ B subunits. Three main forms of activins, activin A ( $\beta$ A $\beta$ A), activin B ( $\beta$ B $\beta$ B) and activin AB ( $\beta$ A $\beta$ B) are distinguished by their dimeric composition, although other members of the family containing different subunits have been also identified (Hotten et al., 1995; Oda et al., 1995). Activins exert their effects by forming heterodimeric complexes with type I and type II serine/threonine kinase receptors which act as a signal transducer and ligand-binding component respectively (see Baker and Harland, 1997). Since there are different forms of activin type I and type II receptors, the biological effects of activins may be related to the receptor expressed by the responding target cells. An additional complexity of activin signalling is that other members of the TGF $\beta$  superfamily can also bind to the activin receptors (Ebner et al., 1993; Matzuk et al., 1995a), and that activin signalling may be modulated by follistatins. Follistatins, constitute a group of proteins encoded by the same gene which are generated by alternative splicing or by proteolytic cleavage. Follistatins neutralize activin bioactivity by abolishing its binding to the activin type II receptors (see, De Winter et al., 1996). Activin signalling has been implicated in early limb development (Stern et al., 1995) and in skeletal muscle differentiation (Link and Nishi, 1997), and it may be also possible that it has a role in limb skeletogenesis. Exogenous administration of activin A promotes chondrogenesis in limb bud micromass cultures (Jiang et al., 1993, see also Chen et al., 1993). In addition, although there are no detailed studies of the pattern of expression of *activin* genes in the course of limb skeletogenesis, *activin* and *activin receptor* transcripts have been identified in limb prechondrogenic aggregates (Roberts et al., 1991; Nohno et al., 1993; Feijen et al., 1994; Verschuere et al., 1995). While Inhibins are known to have chondrogenic activity in limb mesodermal cultures (Chen et al., 1993), the expression of the alpha subunit in the embryo is restricted to the developing gonads at the stages of organogenesis (Feijen et al., 1994).

In this study we show that  $\beta$ A and  $\beta$ B subunits of *activin*, *activin type II receptors (ActRII)* and *follistatin* genes all exhibit a regulated pattern of expression in the developing chick limb autopod, strongly suggestive for a role of activin signalling in the establishment of the skeleton, joints and tendons of the digits. This potential function of activin signalling was explored by different experimental approaches. These include ectopic application of human recombinant activins, blocking activin signalling by follistatin treatment, and by analyzing changes in the pattern of expression of

*activin-related* genes following experimental manipulations of the limb leading to alterations in the skeletal pattern of the digits. Our findings indicate that activins play a role in the developing autopod as proximal signals that appear to specify the position of the digital rays. This effect is contrary to the role of FGFs, which maintain the mesoderm undifferentiated at the progress zone. The changes in the pattern of expression of the BMP receptor gene *bmpR-1b* following treatments with activin and the potential of the BMP antagonist, Noggin, to downregulate *activin  $\beta$ A* gene expression indicate that activin signalling acts in cooperation with BMP-signalling.

## MATERIALS AND METHODS

### Embryos and experimental manipulations of the limb

We used Rhode Island chick embryos ranging from 3 to 9 days of incubation (stages 20-35, Hamburger and Hamilton, 1951). Eggs were windowed at the desired stages and experimental manipulations of the limb were performed in the right wing or leg bud.

Local application of the different growth factor and proteins were performed using heparin or Affi-Gel blue beads. The beads were incubated in PBS or in the selected recombinant human protein solutions (see below) and implanted into the limb mesenchyme. Most experiments were performed at stages 28-29 and the beads were implanted at the tip of digit III or in the third interdigital space. In other cases, the beads were implanted at different locations and stages as indicated in Results.

Surgical removal of the AER was performed in the leg bud at stages 27-28 using fine tungsten needles. After the operation the eggs were returned to the incubator and the embryos killed at different time intervals to study changes in gene expression by *in situ* hybridization.

For ZPA grafting experiments, the posterior margin mesoderm was excised from stage 22 wing buds and grafted into the anterior wing margin of host embryos at stage 20. After the operation the embryos were returned to the incubator and used for gene expression studies.

### Morphological analysis of the limb

The morphology of the limbs was studied after cartilage staining with Alcian green as described previously (Gañan et al., 1996). The pattern of cell death was analyzed by vital staining with neutral red and by Tdt-mediated dUTP nick end labeling (TUNEL) in tissue sections as described previously (Macias et al., 1997). In some experiments the morphology of the limb was studied by scanning electron microscopy. In other experiments, the experimental and control limbs were examined histologically following fixation in 3% glutaraldehyde and Araldite embedding.

### Preparation of beads

Affi-Gel blue (Bio-Rad) or heparin acrylic beads (Sigma) were employed as carriers for administration of the selected proteins. Beads ranging between 80 and 150  $\mu$ m in diameter were selected, washed in PBS and incubated for 1 hour at room temperature in the selected protein solution. Recombinant human TGF $\beta$ 1 (R&D Systems) was used at 2  $\mu$ g/ml; recombinant human FGF-2 (R&D Systems) at 1 mg/ml; recombinant human Noggin (Regeneron Pharm Inc. Tarrytown, N.Y.) at 1 mg/ml; recombinant human activin A (obtained through the National Hormone and Pituitary Program, NHPP, of the National Institute of Diabetes and Digestive and Kidney Diseases, NIDDK) at 0.73 mg/ml. Activins B and AB (obtained from Dr S. Choe, Salk Institute) were used at 0.1 mg/ml and 0.7 mg/ml respectively. Follistatin (rhFS-288, obtained through NHPP, NIDDK) was used at 0.1 mg/ml.

### Probes and *in situ* hybridization

Specific probes for *bmpR-1b (BRK-2)*, *actRIIa* and *actRIIb* were

provided by T. Nohno. Chicken *tgfb2* and *gdf-5* probes have been described previously (Merino et al., 1998 and 1999). Fragments of chicken *activin βA* (733bp), *activin βB* (502bp) and *follistatin* (638bp) genes were obtained by RT-PCR. First strand cDNA was synthesized with a mixture of random hexamers (Promega) and 1 μg of total RNA from day 7.5 autopod. The following primers (5' to 3') were used: for chicken *activin βA* gene 5' primer 5'-CCTGAGATGGTGGGAAGCA-GTA-3' and 3' primer 5'-GCAAGCATCATCAGGAAG-3'; for chicken *activin βB* gene, 5' primer 5'-CGGACACTAGCAAC-AAGTGG-3' and 3' primer 5'-ATGCAACAGGAGTTCACGG-3'; for chicken *follistatin* gene, 5' primer 5'-ATGGAAGATCACACAG-CGC-3' and 3' primer 5'-GCTAATCCAAAGGATCTGCC-3'. PCR reactions were performed in a total volume of 100 μl using Taq DNA polymerase (Gibco BRL). The cycling conditions were 1 minute at 94°C for denaturation, 2 minutes at 55°C for annealing, 3 minutes at 72°C for elongation, and then 10 minutes at 72°C after the last cycle (35 cycles). The PCR products were subsequently cloned into pGEM-T (Promega) and the authenticity of the fragments was confirmed by dideoxy sequencing.

In situ hybridization of control and treated limbs was performed in whole-mount specimens and in tissue sections. For whole-mount in situ hybridization, samples were treated according to their size and stage of development with 10 to 30 μg/ml of proteinase K for 30 minutes at 20°C. Hybridization with digoxigenin-labeled antisense RNA probes was performed at 68°C. Reactions were developed with BCIP/NBT substrate or with purple AP substrate (Boehringer-Mannheim). In situ hybridization in tissue sections was performed using digoxigenin-labeled antisense RNA probes as described by Zou et al. (1997). Specificity of labeling was controlled using sense RNA probes.

### Retroviral constructs

A mouse *Wnt3a* cDNA encoding the entire open reading frame was cloned into the replication-competent retroviral vector RCAS (Kengaku et al., 1998). Primary chick embryonic fibroblasts were then transfected and the supernatant was collected, concentrated by ultracentrifugation and microinjected in stages 9-11 wing or leg bud primordia.

## RESULTS

### Expression of *activin β* subunits, and *activin type II receptors* in the developing autopod

All the studied members of the activin signalling pathway show precise temporo-spatial patterns of expression in the developing autopod suggesting a role of this signalling pathway in the morphogenesis and differentiation of the skeletal elements of the autopod. Expression of these components is also observed in the differentiating muscles.

Expression of *activin βA* subunit is a precocious marker of the digital rays and interphalangeal joints. *βA* transcripts start to appear at stages 25-26. As shown in Fig. 1A-D, *βA* transcripts mark the zone of formation of the prechondrogenic digital aggregates first in digits IV and III, and then in digits II and I (Fig. 1E). In the course of development, and as digits incorporate successive phalangeal elements, *βA* expression is maintained at the tip of the digits. However, it is absent at more proximal levels except in the zones of joint formation where *βA* transcripts also form prominent domains of expression (Fig. 1E-F). The appearance of *βA* transcripts in the developing joints is coincident with the expression of *gdf-5* (Fig. 1F-G) which is an early marker of the metatarso-phalangeal and interphalangeal joint regions (Storm and Kingsley, 1996;

Merino et al., 1999). As limb development proceeds and digits complete the incorporation of phalangeal elements, *βA* transcripts disappear from their distal tip. Thus, digits I and II lose their distal labeling at stages 32-33 (Fig. 1H) and digit III and IV at stages 33-34. During this period *βA* transcripts remain associated with the interphalangeal joints (Fig. 1H).

Expression of *activin βB* subunit showed a spatial pattern of expression similar to that of *βA*, but its temporal appearance was different (Fig. 1I-J). Up to stage 30, *βB* transcripts were not detected in the autopod. From stage 30, *βB* transcripts appeared associated with the zones of joint formation (Fig. 1I). The appearance of these joint domains followed the same sequence than that observed for *βA*. However, they were always detected some hours after the appearance of *βA* transcripts. Expression of *βB* transcripts at the condensing tip of the digits was nearly correspondent with the period in which the digits stop distal outgrowth, thus showing a sequence of appearance opposite to that of *βA*. The first digital tip showing *βB* transcripts was digit I at stage 31 (Fig. 1I), followed by digit II and the tips of digits III and IV lacked *βB* transcripts up to stage 32 (Fig. 1J).

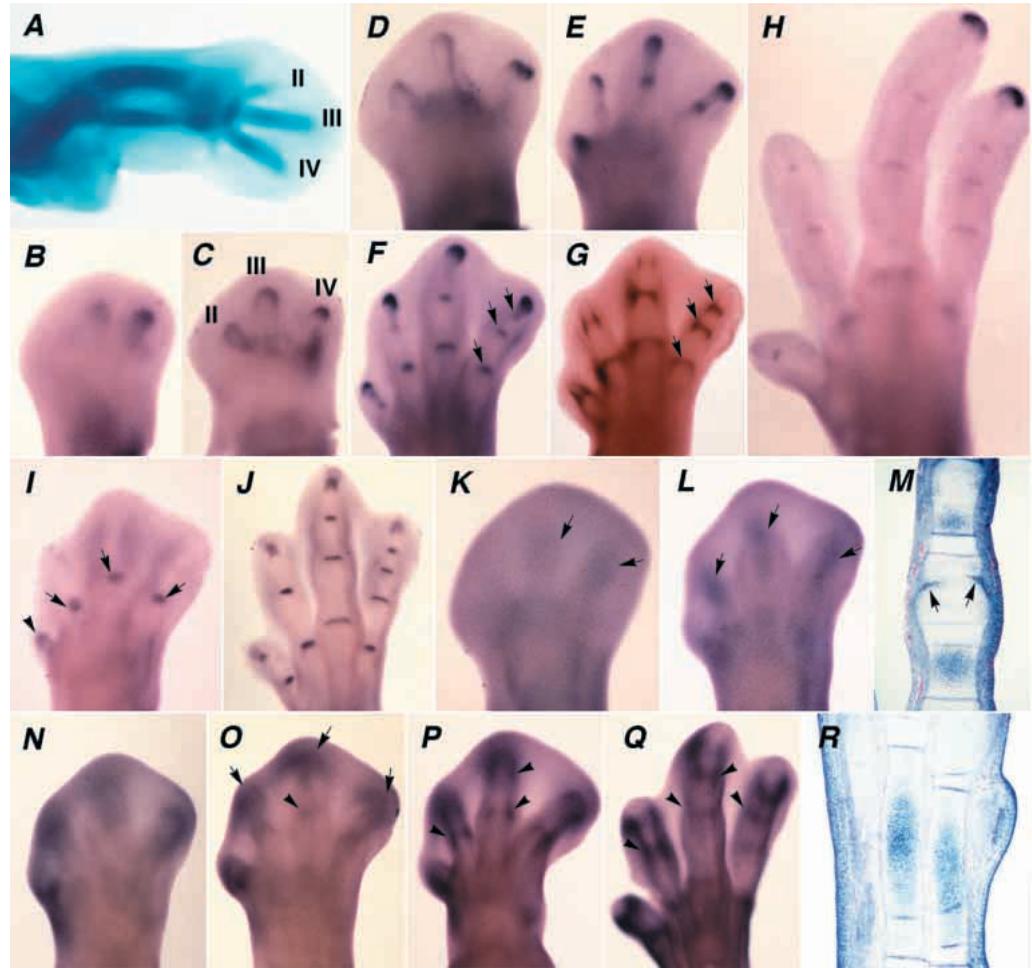
While transcripts of both *actRIIA* and *actRIIB* are associated with the zones of expression of *activin β* subunits, they also show additional domains of expression in the differentiating cartilage of the phalanges. *ActRIIA* is mainly expressed in the developing muscles. However, at stage 28, low expression levels are also detected in the undifferentiated mesenchyme surrounding the digital rays (Fig. 1K-M). Well defined domains of expression of this receptor in the skeletal elements of the autopod are only detected in the diaphysis of the skeletal pieces as they initiate differentiation into hypertrophic cartilage (Fig. 1M). At these relatively advanced stages, *actRIIA* transcripts are also intense in the peripheral mesenchyme of the joints when they initiate the process of cavitation (Fig. 1M). An additional region of expression of *actRIIA* is the myotendinous junction of the autopodial tendons (not shown).

*ActRIIB*, as observed for *actRIIA*, is expressed in the developing muscles (not shown) and in later stages in the hypertrophic cartilage and in the developing joints in the course of cavitation. However, unlike *actRIIA*, *actRIIB* exhibits prominent domains of expression in the stages of digit formation in the mesenchyme surrounding the distal tip of the digit and the zone of joint formation (Fig. 1N-Q). At stage 28, *actRIIB* expression is low and appears concentrated around the condensing tip of the developing digits (Fig. 1N). From this stage, the expression domains associated with the distal tip of the digits are more intense and surround the precartilaginous condensation (Fig. 1O-Q). Well defined domains of *actRIIB* expression are also detected in these stages in the mesenchyme around the zones of joint formation (Fig. 1P-Q). From stages 31-32, expression is intense in the hypertrophic cartilage (Fig. 1R) and in the mesenchyme of the joint interzone (not shown). At these stages, expression of *actRIIB* is also detectable at low levels in the boundary of the autopodial tendons (not shown).

### Formation of extra digits by exogenous administration of activins

To analyze the function of activins in the formation of the limb skeleton, beads incubated in recombinant activins were implanted into the limb bud at different stages and positions. Implantation of activin A-beads at stages 22 to 24 was not

**Fig. 1.** (A) Control leg bud at stage 26 whole-mount stained with Alcian green showing the skeletal pattern of the limb at this stage. Digital rays II, III and IV are identifiable at this stage. (B-F,H) Whole-mount in situ hybridizations showing the pattern of expression of *activin  $\beta$ A* in the leg bud autopod at stages 25 (B), 26 (C), 27 (D), 29 (E), 31 (F) and 33 (H). Note the dynamic expression of  $\beta$ A at the digital tips and in the developing joints. Compare A and C to note the correlation between the establishment of the digital rays and the expression of  $\beta$ A. Note also the absence of  $\beta$ A transcripts from the tips of digits I and II at stage 33 once the whole set of phalangeal elements have been established (H). (G) Leg bud autopod at stage 31 showing the expression of *gdf-5* in the developing joints. Compare with F to note that the appearance of  $\beta$ A transcripts in the joints is coincident with *gdf-5* (arrows). (I-J) Expression of *activin  $\beta$ B* in the autopod at stage 30 (I) and 32 (J). Note that  $\beta$ B transcripts appear first in the joints (arrows in I) and then at the digit tips (arrowhead in I). Expression of  $\beta$ B transcripts at the tip of the digits is coincident with the formation of the last phalanx of each digit (J). (K,L) Expression of *actR11a* in the leg bud autopod at stages 28 (K) and 30 (L). Low levels of *actR11a* are detected at these stages associated with the developing digital rays (arrows). (M) Longitudinal section of digit III at stage 33 showing the expression of *actR11a*. Note intense expression in the differentiating cartilage of the diaphyses of the phalanges and in the developing joint mesenchyme (arrows). (N-Q) Expression of *actR11b* in the autopod at stage 29 (N), 30 (O), 31 (P) and 32 (Q). Note expression at the tip of the digital rays (arrows) and in the developing joints (arrowheads). (R) Longitudinal section of a stage 32 autopod showing the presence of *actR11b* transcripts in the diaphyses of the developing metatarsal cartilages.



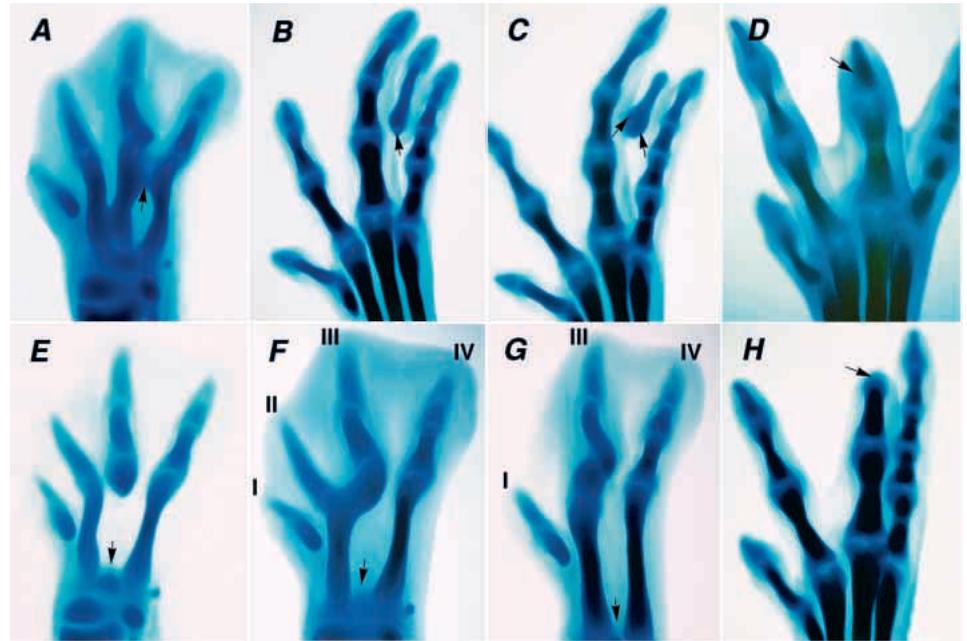
followed by skeletal alterations ( $n=22$ ). These experiments were performed both in wing and leg buds, and the beads were implanted in the anterior margin, posterior margin and progress zone mesoderm. From stages 25-26, the beads were implanted in the progress zone mesoderm corresponding to the interdigital regions. In all cases the treated limb exhibited a stage dependent pattern of skeletal alterations. Implantation of activin A-beads at stages 25 or 26 was followed by excrescences in the metatarsal cartilages which, in the most severe cases, caused the fusion of two adjacent skeletal pieces (Fig. 2A;  $n=6$ ). When the beads were implanted at stage 27, cartilage excrescences were also formed but they were associated with the proximal phalanges ( $n=7$ ). When the beads were implanted at stages 28 or 29, an extra digit consisting of two or three phalangeal elements was formed in 23 out of 29 treated embryos (Fig. 2B) and ectopic chondrogenesis lacking an identifiable morphology was observed in 5 of the remaining 6 embryos. At stage 30, in the majority of the cases, activin A-beads induced a small ectopic cartilage lacking digit morphology ( $n=8$ ). By stage 31, when the interdigital mesoderm begins to be removed by

programmed cell death, ectopic chondrogenesis was only found in 2 out of 5 experimental embryos.

Since the digital expression of the *activin  $\beta$ A* and  *$\beta$ B* subunits shows differences indicative of the presence of activin A at the beginning of digit formation and/or activins AB or B at the end of digit formation, we analyzed the effect of local administration of these activins in the interdigital region. No significant differences in the ability to induce ectopic chondrogenesis were detected between activin AB or B and activin A. Ectopic chondrogenesis or extra digit formation was observed in 12 out of 16 embryos following implantation of activin B-beads in the interdigital region at stages 28-29, and in 6 out of 8 embryos after implantation of activin AB-beads.

In view of the similarities in the chondrogenic inducing effects among the different activins and between activins and TGF $\beta$ s (Gañan et al., 1996) we analyzed possible molecular differences preceding the appearance of cartilages following the application of these growth factors (activins or TGF $\beta$ s). In these experiments, we analyzed the induction of *bmpR-1b* and *activin  $\beta$ A* and *tgfb2* genes themselves. BMPR-1b is a type I

**Fig. 2.** Alcian green stained autopods showing the skeletal alterations induced by treatments with activins (A,B) TGF $\beta$ 1 (D), follistatin (E-G) and combinations of TGF $\beta$ 1 and follistatin (C). The location of the bead is indicated by arrows. (A) Fusion of the metatarsal cartilages induced by the implantation of an activin A-bead in the third interdigital space at stage 26. (B) Extra digit induced by the implantation of an activin A-bead in the third interdigit at stage 28. (C) Rudimentary extra digit lacking joints following implantation of a TGF $\beta$ 1-bead and a follistatin-bead at stage 28. (D) Truncation of digit III following implantation of a TGF $\beta$ 1-bead at the tip of digit III at stage 27. Note the claw morphology of the second phalanx in the truncated digit. (E-G) Different skeletal patterns of the autopod following implantation of a follistatin-bead at the tip of digit III at stage 25. In all the three specimens, formation of the third metatarsal is inhibited. In E a digit III lacking continuity with the proximal metatarsal element is formed. In F digit III is in contact with a bifurcated second metatarsal cartilage. In G, digit III is associated with the second metatarsal and digit II is missing. (H) Truncations of digit III after implantation of a follistatin-bead at the tip of digit III at stage 27. Note the different morphology of the distal phalanx of the truncated digit in this case in comparison with that shown in D caused by implantation of a TGF $\beta$ 1-bead.

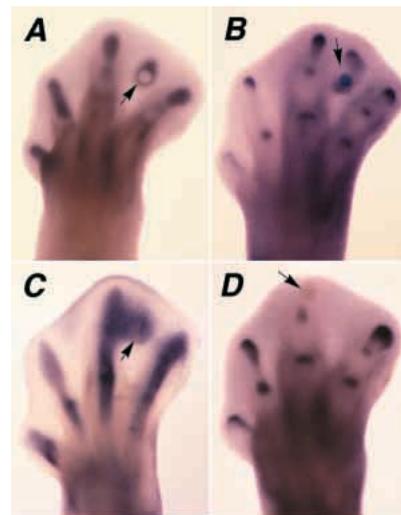


receptor for BMPs. Previous retrovirus-mediated overexpression experiments using either dominant negative (Zou and Niswander, 1996) or constitutive active constructs (Zou et al., 1997) indicated that this receptor was the transducer of the chondrogenic signals in the limb bud. Our findings showed that the implantation in the interdigital space of beads incubated with either TGF $\beta$ 1 ( $n=30$ ), activin A ( $n=34$ ), activin B ( $n=28$ ), or activin AB ( $n=20$ ) was followed by the ectopic expression of the above mentioned genes in a similar temporal pattern (Fig. 3A-C). Ectopic expression of *bmpR-1b* was detected between 10 and 15 hours after implantation of beads incubated with activins (Fig. 3A) or TGF $\beta$ 1. Ectopic expression of *activin  $\beta$ A* (Fig. 3B) and *tgfb2* (Fig. 3C) occurred between 15 and 20 hours following the implantation of the various beads. This suggests that activation of these genes occupied a subsequent step in the molecular cascade triggered by the application of TGF $\beta$ 1- or activin-beads. In accordance with this interpretation, implantation of beads incubated with the BMP antagonist Noggin at the tip of the digits downregulated the expression of *activin  $\beta$ A* (Fig. 3D;  $n=8$ ). A similar finding was previously observed for the *tgfb2* gene (Merino et al., 1998).

#### Alterations of digit morphogenesis following implantation of TGF $\beta$ -beads or activin-beads at the tip of the digits

Our present findings, along with previous studies on the role of TGF $\beta$ 2 in digit formation (Gañan et al., 1996; Merino et al., 1998), showed that  *$\beta$ A*, *tgfb2* and  *$\beta$ B* transcripts were progressively and additionally expressed at the tip of the digits and, further, that the proteins encoded by those genes were able to induce extra digits when they were exogenously administered into the interdigital mesoderm. To analyze

possible functional implications of these redundant chondrogenic signals, beads bearing activins or TGF $\beta$  at the concentration required to induce extra digits were implanted at the tip of the developing digits between stages 27-29.



**Fig. 3.** (A) Ectopic interdigital expression of the *bmpR-1b* gene 15 hours after implantation of an activin A-bead (arrow) in the third interdigit at stage 28. (B) Ectopic interdigital expression of *activin  $\beta$ A* 20 hours after implantation of a TGF $\beta$ 1-bead (arrow) in the third interdigit at stage 28. (C) Ectopic interdigital expression of *tgfb2* 20 hours after implantation of an activin A-bead (arrow) in the third interdigit at stage 28. (D) Downregulation of  *$\beta$ A* expression at the tip of digit III 15 hours after implantation of a Noggin-bead (arrow) at the tip of digit III (compare with the expression of  *$\beta$ A* at the tip of digit III in B).

Implantation of TGF $\beta$ -beads was followed by truncation of the digits at proximodistal levels dependent on the stage of treatment. However, regardless of the level of truncation, in 11 out of 21 experimental limbs the distal phalanx of the truncated digits exhibited the characteristic claw morphology of the distal phalanges (Fig. 2D). The remaining truncated digits were characterized by the absence of a number of phalanges but the tip of the digit was rounded. Histological examination of the treated limbs showed that truncation was due neither to abnormal cell death nor to the disintegration of the AER (not shown).

Treatments with activins caused minor phalangeal alterations, including joint fusion and shortening or thickening of the phalanx that the bead was associated with (not shown). However, at difference of TGF $\beta$ -treatments, truncations were unusual (2 out of 6 experimental embryos treated with activin A; 3 out of 9 embryos with activin B; and 0 out of 10 embryos with activin AB) and when present the distal phalangeal element showed a rounded tip rather than the claw morphology observed after TGF $\beta$  treatment.

#### Regulation of *activin* expression following experimental manipulation of the anteroposterior and proximodistal signalling pathways of the limb

Since the expression of *activin*  $\beta$ A (which is the first molecular marker of the digits) appeared first in the most posterior digits, we explored whether ZPA influences its expression. To test this, a small fragment of stage 22 limb mesoderm containing the ZPA was grafted in the anterior margin of the wing bud of stage 20 host embryos. This operation resulted in the induction of extra anterior domains of the  $\beta$ A gene, which were detectable 48 hours after the ZPA graft, showing a clear association with the mirror digit duplication induced by the graft (Fig. 4A).

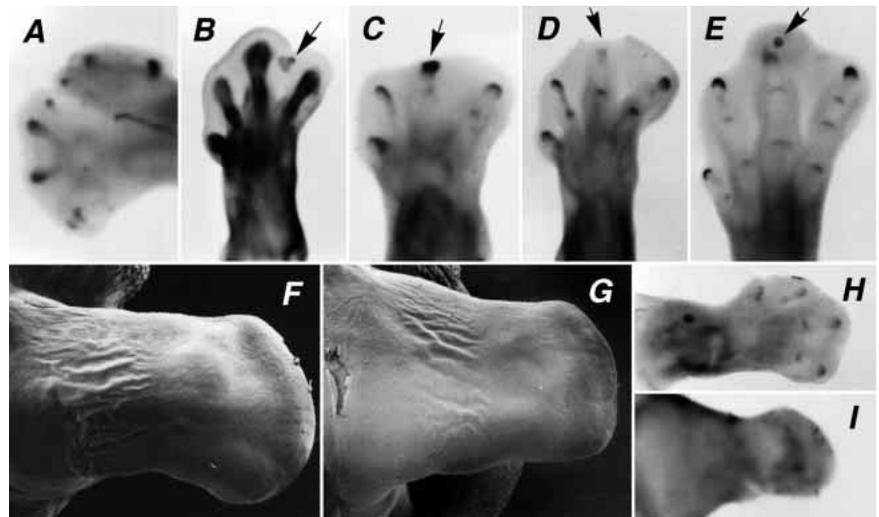
In previous studies we observed that extra digits can be induced by surgical removal of the AER from the interdigital regions (Hurler and Gañan, 1987). This finding suggested a role of AER in inhibiting the expression of chondrogenic signals in the subjacent mesoderm. To test this hypothesis, AER was removed from the interdigital regions at stage 28 ( $n=14$ ), which induced ectopic *tgfb2* (Fig. 4B), that was followed by a small domain of *activin*  $\beta$ A (not shown) that preceded the appearance of the ectopic cartilage. In addition, surgical removal of the AER from the tip of digit III at stage 28 ( $n=12$ ) caused an initial upregulation of  $\beta$ A *activin* expression at the tip of the treated digit (Fig. 1C) followed by intense downregulation detectable 24–30 hours thereafter (Fig. 4D). However, since these findings could be related to a local production of TGF $\beta$ s in the course of healing of the ectodermal wound (Martin et al., 1993), complementary experiments to increase AER function were also performed.

The effects of increasing AER function

were initially analyzed by implanting beads bearing FGF-2 at the tip of the growing digits ( $n=6$ ). Under these conditions, undifferentiated cells lacking *activin*  $\beta$ A expression accumulated at the tip of the digit and its physiological expression remained restricted proximally in association with the previously formed digit condensation (Fig. 4E). To further analyze this antagonistic effect of FGFs, *activin*  $\beta$ A expression and digit formation were studied in limbs in which the function of the AER was experimentally increased by retrovirus-mediated misexpression of the *Wnt3a* gene ( $n=16$ ). Under these conditions non-ridge limb ectoderm is induced to express functionally active AER genes leading to the formation of limbs characterized by a hypertrophied AER function (Kengaku et al., 1998). In the most severe phenotypes, limbs misexpressing *Wnt3a* did not form digital cartilages and were characterized by a narrow autopod lacking *activin*  $\beta$ A expression (Fig. 4F–I).

#### Expression of *follistatin* in the developing autopod

The possible function of follistatin in modulating *activin* signalling moved us to analyze whether it was expressed during the development of the autopod. Expression of *follistatin* in early stages of limb development has been studied by Amthor et al., (1996). At these early stages (prior to stage 23), *follistatin* transcripts are associated with the developing somites and with the myogenic cells invading the limb bud. From stages 25–26, in addition to the myogenic masses of the zeugopodial region, *follistatin* transcripts are also observed in the autopod associated with the zones of formation of the



**Fig. 4.** (A) Expression of *activin*  $\beta$ A in a duplicated wing bud induced by the grafting of a ZPA in the anterior margin of the bud at stage 20. Note that the digital domains of  $\beta$ A parallel the skeletal duplication of the wing. (B) Ectopic induction of *tgfb2* (arrow) 20 hours after surgical removal of the AER from the interdigital region at stage 28. (C) Upregulation of *activin*  $\beta$ A at the tip of digit III (arrow) 20 hours after local AER removal from the tip of the digit. (D) Downregulation of *activin*  $\beta$ A at the tip of digit III (arrow) 30 hours after local AER removal from the tip of the digit. (E) Downregulation of  $\beta$ A expression at the tip of digit III 36 hours after implantation of a FGF-bead at the tip of digit III (arrow). (F,G) Scanning electron micrographs showing the morphology of the leg bud at stage 27 in an experimental limb misexpressing *Wnt3a* (G) and in the contralateral control limb (F). (H,I) Expression of *activin*  $\beta$ A in an experimental limb misexpressing *Wnt3a* (I) and in its contralateral control limb (H). Note the absence of digital domains of *activin*  $\beta$ A expression in the experimental limb.

digital rays (Fig. 5A-D). These digital domains of *follistatin* expression are arranged dorsally and ventrally to the differentiating cartilage marking the future regions of tendon formation (Fig. 5B-D), and are particularly abundant at the distal tip of the digit close to the zone of expression of *activin*  $\beta$ A transcripts (Fig. 5D). The appearance of the different domains of *follistatin* expression follows the same temporal sequence as digit formation, appearing first associated with digits IV and III (stages 25-26) and then with digits II and I (stages 28-29). Up to stages 32-33, expression of *follistatin* is most intense in the distal region of the digit dorsally and ventrally to the zone of expression of *activin*  $\beta$ A, but considerable labeling is also present along the entire digital trajet of the tendons (Fig. 5D). From these stages, *follistatin* transcripts are concentrated in the mesenchyme surrounding the tendinous blastema, in the outer layer of the perichondrium and less intensively in the mesenchyme of the joint interzone (Fig. 5E-F).

In view of the temporal and spatial coincidence in the expression of *follistatin* and *activin*  $\beta$ A subunit at the distal growing tip of the digits, we explored a possible causal relationship between both genes by analyzing the effect of exogenous administration of activin A on the expression of *follistatin* ( $n=7$ ). In these experiments ectopic expression of *follistatin* was detected following implantation of activin A-beads in the interdigital regions. This domain of *follistatin* expression was first detectable 20 or 24 hours after the treatment when a mesodermal condensation was apparent around the bead (not shown), and expression was maintained in association with the extra digits (Fig. 5G).

### **Follistatin blocks digit outgrowth and neutralizes activin A-induced chondrogenesis but not TGF $\beta$ -induced chondrogenesis or interdigital cell death**

Follistatin treatments were designed to block the function of activin A. Implantation of follistatin-beads into the limb bud (anterior margin, posterior margin or progress zone mesoderm) prior to stage 25 was not followed by alterations in limb skeletogenesis ( $n=29$ ). From stage 25 up to stage 30, which corresponds to the period in which  $\beta$ A transcripts are expressed in the digital rays, implantation of follistatin-beads at the tip of the growing digits was followed by intense inhibition of chondrogenesis. Treatments at stages 25-26 inhibited, in all cases ( $n=13$ ), the formation of the metatarsal cartilage but digit formation was often restored at the level of the phalangeal elements (Fig. 2E-G). An interesting feature of these autopods lacking the metatarsal cartilage of digit III, was that, in most cases (9 out of 13), the phalangeal pieces of digit III became aligned with the metatarsal of digit II (Fig. 2F-G) leading to partial or total truncation of digit II (Fig. 2G). From stage 27 implantation of follistatin-beads at the tip of the growing digits resulted in the formation of truncated digits lacking all the phalangeal pieces that should be formed distally to the bead (Fig. 2H).

Chondrogenesis induced by activin A was inhibited in all cases when the activin A-beads were postincubated in follistatin prior to their implantation in the interdigits ( $n=11$ ). Similarly, activin-A-induced chondrogenesis was inhibited, although to a lesser extent (5 out of 8 cases), when a bead of follistatin was co-implanted with a bead of activin A (not shown). To exclude the possibility that the inhibition of

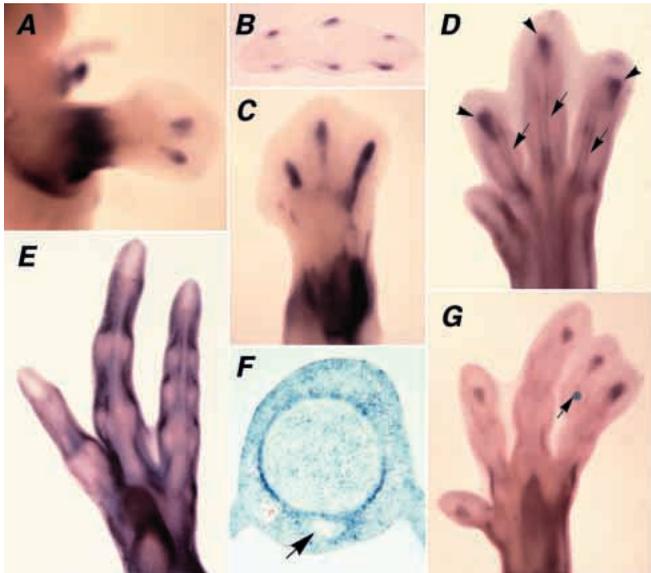
chondrogenesis by follistatin was not specifically due to its antagonistic effect on *activins*, we tested the effect of follistatin on TGF $\beta$ -induced chondrogenesis. For this purpose two beads, one incubated in TGF $\beta$ 1 and one incubated in follistatin, were implanted in the same interdigit ( $n=14$ ). In these experiments, ectopic chondrogenesis was never abolished, although the extra digits observed following this double treatment were in general thinner and smaller than those induced by TGF $\beta$ s alone. Interestingly, joints were in most cases absent from these extra digits (Fig. 2C).

Implantation of follistatin-beads in the interdigital regions ( $n=20$ ) was followed by normal development and interdigital cell death was not modified even when two beads were implanted in the same interdigit within a 12-hour interval (not shown).

## **DISCUSSION**

### **Activin A induces the formation of digits**

Previous studies have reported the presence of *activin*  $\beta$ A transcripts in mouse limb prechondrogenic condensations (Feijen et al., 1994). Here, we show that the expression of  $\beta$ A in the limb chondrogenic condensations is restricted to the digits while it is absent from the condensations of more proximal skeletal elements. We further show that this gene constitutes the earliest marker for the digital condensations (metatarsal and phalanges). Its expression starts at stage 25 coincidentally with the formation of the metatarsal pieces and is maintained at the growing tip of the digits until the last phalanx is formed (stages 32-34). Expression of *actRIIb* is intense at these stages in the mesodermal cells surrounding the distal tip of the digit indicative of a role of this receptor in the transduction of the activin signals. The lack of  $\beta$ B transcripts in the digits until the formation of the last phalanx indicates that only activin A is present in the initial stages of digit formation. This pattern of gene expression, along with the chondrogenic promoting effect of activin A in limb mesoderm micromass cultures (Jiang et al., 1993), is strongly indicative of a role of activin A in the formation of the digits. The induction of ectopic digits observed here following implantation of beads bearing activin A into the interdigital limb mesoderm and the inhibition of digit formation after treatments with follistatin, which is a natural antagonist of *activins* (de Winter et al., 1996), are in full agreement with this interpretation. In addition, and most importantly, our findings provide evidence for a stage-dependent specificity of the chondrogenic function of activin A in the course of limb development. Prior to stage 25, treatments with activin A-beads are ineffective in promoting ectopic chondrogenesis. From this stage, and coincidentally with the presence of  $\beta$ A transcripts in the digital prechondrogenic aggregates and *actRIIb* in the mesenchyme surrounding the digital condensations, activin A-beads implanted in the interdigital regions induce the formation of extra digits or ectopic chondrogenesis. Conversely, implantation of follistatin-beads blocks digit growth but has no effect on the formation and growth of the proximal skeletal elements of the limb (zeugopod and stylopod). As for activin-mediated chondrogenesis, inhibition of chondrogenesis by follistatin begins at stage 25. In addition, the chondrogenic effect of activin A-beads is fully blocked when the beads are postincubated in follistatin prior to their implantation. Although



**Fig. 5.** (A-E) Expression of *follistatin* in the developing leg bud at stages 26 (A), 29 (B,C), 32 (D) and 34 (E). Note the presence of precise digital domains of *follistatin* gene expression from stage 26 (A) which mark the position of the developing tendons (indicated by arrows in D). Note also the increased expression of *follistatin* at the tip of the digits (arrowheads). (B) Transverse section of the autopod at stage 29 showing the arrangement of the *follistatin* transcripts dorsally and ventrally to the developing digits. (F) Transverse section of digit III at stage 33 illustrating the distribution of *follistatin* transcripts in the perichondrium and around the ventral tendons (arrow) also observed in whole-mount preparations (E). G shows the presence of *follistatin* expression in association to an extra digit induced by implantation of a TGF $\beta$ 1-bead (arrow) in the third interdigit at stage 28.

*follistatin*, in addition to antagonizing activins (de Winter et al., 1996), is also able to neutralize some BMPs (Fainsod et al., 1997; Iemura et al., 1998), it is unlikely that the inhibitory effect of *follistatin* in the formation of normal and activin-induced digits is only explained by its potential to antagonize BMPs. *follistatin* does not block chondrogenesis induced by treatment with TGF $\beta$ s, which also involves BMP-signalling (Merino et al., 1998), and in contrast to other specific BMP-antagonists such as Noggin (Merino et al., 1998) or Gremlin (unpublished observations), does not inhibit interdigital cell death, a biological process present in the autopod at these stages which is also mediated by BMPs (Zou and Niswander, 1996; Yokouchi et al., 1996; Macias et al., 1997).

Previous *in vitro* studies indicate that the function of activin A in chondrogenesis is to recruit cells to form the chondrogenic condensation by increasing cell adhesion (Jiang et al., 1993). As will be discussed below, our study shows that the role of activin A in digit chondrogenesis is also to induce the expression of *bmpR-1b*, thus making the cells sensitive to the chondrogenic influence of BMPs (Zou et al., 1997).

#### Participation of activins B, A/B and TGF $\beta$ 2 in the course of digit formation

An additional finding of this study is that the progressive incorporation of phalangeal elements into the digits is accompanied by the progressive expression of genes encoding

factors with chondrogenic potential. In a previous study, we observed that *tgfb2* is expressed at stage 29 when the distal part of the digits are being formed (Merino et al., 1998), and TGF $\beta$ s administered exogenously are able to induce extra digits (Gañan et al., 1996). Here, we show that coincidentally with the appearance of the condensation of the last phalanx of each digital ray, *activin  $\beta$ B* is also expressed at the tip of the digits, suggesting that activins AB and/or B are also involved in this advanced period of digit formation. Again, our functional approach indicates that both activin B and activin AB are able to induce extra digits. Thus, the progressive establishment of the digit skeleton involves the successive addition of activin A, TGF $\beta$ 2 and activin B and/or AB all of them with strong chondrogenic potential. This redundancy in chondrogenic signals may explain why the experimental disruption of *tgfb2* (Sanford et al., 1997) or *activin* genes (Matzuk et al., 1995b) in the mouse does not result in a mutant digit phenotype. A comparable degree of redundancy is also observed in the control of the proximodistal outgrowth of the limb which is mediated by at least 4 different FGF isoforms (FGF-2, FGF-4, FGF-8 and FGF-9) produced by the AER (see review by Martin, 1998) and in BMP signalling associated with the control of proliferation, differentiation and cell death of the progress zone mesoderm (Niswander and Martin, 1993; Macias et al., 1997) which includes at least three different BMPs (BMP-2, BMP-4 and BMP-7; Francis-West et al., 1995; Laufer et al., 1997).

The occurrence of truncated digits in which the last phalanx, in spite of its proximal location, displays a distal claw morphology, which is observed after application of TGF $\beta$  at the tip of the digit, suggests that the progressive addition of chondrogenic signals might contribute to the distal determination of the phalanges. Also remarkable in this study is the specificity of activin/TGF $\beta$  signalling in the formation of digits. This indicates that other signals may account for the onset of chondrogenesis at the zeugopod and the stylopod segments of the limb. From this observation, it is tempting to propose that changes in the nature of the chondrogenic signals may lead to the formation of morphologically different prechondrogenic aggregates, thus contributing to the establishment of differences in the skeletal morphology of the different segments of the limb. This may establish a link between the early patterning of the limb skeleton by homeobox genes and the morphology of the skeleton. The mesoderm cells responding to activin/TGF $\beta$  signalling corresponding to the domains of expression of *hoxA13* and *5'hoxD* genes (Nelson et al., 1996). In addition, digit duplication following the grafting of ZPA in the anterior margin of the limb (which is accompanied by rearrangement in the domain of expression of *hoxd* genes; Izpisua-Belmonte and Duboule, 1992) is followed by the anterior duplication of the digital domains of expression of *activin  $\beta$ A* subunit. Also, in support of that interpretation are recent studies reporting differences in the chondrogenic behaviour of the limb mesoderm in relation with the pattern of *hoxd* gene expression observed *in vitro* (Jung and Tsonis, 1998) and in retrovirus-induced misexpression experiments (Goff and Tabin, 1997).

#### Interactions between activin A, TGF $\beta$ 2 and BMP signalling

Previous work has established that BMPs (BMP-2, BMP-4 and BMP-7) play a double role in the developing autopod promoting cartilage growth and interdigital cell death (Zou and

Niswander, 1996; Macias et al., 1997; Capdevila and Johnson, 1998; Merino et al., 1998). Signalling through the type I receptor BMPR-1B appears to account for the chondrogenic effects of BMPs (Kawakami et al., 1996; Zou et al., 1997; Merino et al., 1998). Here we show that, as observed previously for TGF $\beta$ s (Merino et al., 1998), the formation of extra digits following treatment with activins is preceded by the induction of an ectopic domain of *bmpR-1b*. This expression is followed by the appearance of *activin  $\beta$ A* and *tgf $\beta$ 2* transcripts either in treatments with TGF $\beta$  or with activins. In addition, treatment with the BMP antagonist Noggin downregulates the physiological expression of  *$\beta$ A* at the tip of the digits, a feature also observed previously for *tgf $\beta$ 2* (Merino et al., 1998). These findings indicate the occurrence of a complex cascade of molecular events accounting for the formation of digits which is triggered by the activation of *bmpR-1b*. All the tested growth factors (activins A, B, and AB, and TGF $\beta$ s) are able to induce *bmpR-1b*, but the pattern of expression of these factors in vivo points to activin A as the physiological signal for this function. The activation of *bmpR-1b* makes the cell sensitive to the chondrogenic effect of local BMPs, thus blocking its alternative function promoting cell death. In addition, signalling through BMPR-1b positively regulates the expression of *tgf $\beta$ 2* and *activin  $\beta$ A* establishing a loop which ensures the progressive outgrowth of the digits. The appearance of  *$\beta$ B* transcripts in the induced cartilages requires longer periods, suggesting that other mechanisms account for the regulation of activins B and/or AB.

### Interactions between activin signalling and AER function

Limb outgrowth is sustained by proliferation of the mesodermal cells in the progress zone. In this region, the mesodermal cells are maintained in an undifferentiated and proliferating state due to the influence of FGFs produced by the AER (see review by Martin, 1998). The above results indicate that activin A signalling triggers differentiation of the progress zone cells when they leave the influence of the AER. Our experimental designs to analyze possible interactions between activins, AER and FGFs clearly shows that FGFs play an opposite but complementary function to activins. Application of FGFs at the tip of the digits downregulates  *$\beta$ A* expression and delays chondrogenesis. Most remarkably, expression of *activin  $\beta$ A* subunit and digit chondrogenesis are both fully inhibited in limbs overexpressing *Wnt3a*. Previously, it has been demonstrated that *Wnt3a* is an inducer of the AER and its misexpression causes a hyperactive ridge with *fgf-8* being expressed at the limb bud margin and in a considerable number of cells of the dorsal and ventral ectoderm (Kengaku et al., 1998). Changes of  *$\beta$ A* expression following local removal of the AER from the tip of the digits illustrates the complementary roles of FGFs and activins. A few hours after AER removal,  *$\beta$ A* is upregulated, thus showing the antagonistic effect of FGFs and activin. However, by 24 hours after AER removal,  *$\beta$ A* expression undergoes progressive downregulation which can be explained by the arrest in the incorporation of cells coming from the progress zone caused by AER removal.

### Activin signalling in joint and tendon formation

The formation of joints is a complex process which involves at

least three successive steps: first, chondrogenic differentiation is arrested in the zone of joint formation; second, cell death removes mesenchymal cells from the joint interzone; and third, differentiation of the capsulo-synovial apparatus including vascular invasion (Mitrovic, 1977; Mori et al., 1995). Several signalling molecules, including GDF-5 (Storm and Kinsley, 1996; Brunet et al., 1998; Merino et al., 1999), BMP-2 (Macias et al., 1997), TGF $\beta$ 2 (Merino et al., 1998), and BMP-6 (unpublished observations) have been identified in the developing joints of the digits, but their precise function in the different steps of joint formation awaits clarification. Our present observations show that *activin  $\beta$ A* and  *$\beta$ B* subunits and *actRIIa* and *actRIIb* exhibit a regulated pattern of expression in the different steps of joint formation indicative of a function of activin signalling in digit arthrogenesis. In this study, the absence of joints in TGF $\beta$ -induced extra digits after treatment with follistatin is the only functional evidence for a role of activins in joint formation.

We have observed that *follistatin* gene expression constitutes a precocious marker of tendon formation. In addition, although activins are not detected in the early developing tendons, maximal expression of *follistatin* is always observed at the distal tip of the digits close to the domain of *activin* expression, and ectopic administration of activins induces *follistatin* expression followed by tendon formation. Thus, our finding may be indicative of a molecular coordination between the formation of the skeletal primordia and their corresponding tendons. However, at the present we can only speculate about the possible role of follistatin in tendon formation. Since the tendinous blastemas are composed of cells able to chondrify, (Milz et al., 1998) it can be proposed that follistatin avoids the excessive extension of the prechondrogenic aggregates thus establishing the limit between tendon and cartilage. Alternatively, the presence of *follistatin* in the tendinous primordia may be related to the BMP-antagonic effect of follistatin (Fainsod et al., 1997; Iemura et al., 1998).

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