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Role of FGFs in the control of programmed cell death during limb development

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SUMMARY

We have investigated the role of FGFs in the control of programmed cell death during limb development by analyzing the effects of increasing and blocking FGF signaling in the avian limb bud. BMPs are currently considered as the signals responsible for cell death. Here we show that FGF signaling is also necessary for apoptosis and that the establishment of the areas of cell death is regulated by the convergence of FGF- and BMP-mediated signaling pathways. As previously demonstrated, cell death is inhibited for short intervals (12 hours) after administration of FGFs. However, this initial inhibition is followed (24 hours) by a dramatic increase in cell death, which can be abolished by treatments with a BMP antagonist (Noggin or Gremlin). Conversely, blockage of FGF signaling by applying a specific FGF-inhibitor (SU5402) into the interdigital regions inhibits both physiological cell death and that mediated by exogenous BMPs. Furthermore, FGF receptors 1, 2 and 3 are expressed in the autopodial mesoderm during the regression of the interdigital tissue, and the expression of *FGFR3* in the interdigital regions is regulated by FGFs and BMPs in the same fashion as apoptosis. Together our findings indicate that, in the absence of FGF signaling BMPs are not sufficient to trigger apoptosis in the developing limb. Although we provide evidence for a positive influence of FGFs on BMP gene expression, the physiological implication of FGFs in apoptosis appears to result from their requirement for the expression of genes of the apoptotic cascade. We have identified *MSX2* and *Snail* as candidate genes associated with apoptosis the expression of which requires the combined action of FGFs and BMPs.

Key words: Apoptosis, BMP, FGF receptors, *Snail*, *MSX2*, Syndactyly, Chick, Duck

INTRODUCTION

The vertebrate limb is one of the best characterized model systems for studying the molecular basis of morphogenesis in vertebrates. The early embryonic limb is a simple structure consisting of a core of mesodermal cells covered by an ectodermal jacket. In the course of development the mesodermal cells are subjected to local signals that direct proliferation, differentiation and programmed cell death according to precise spatial coordinates (Macias et al., 1999). Proliferation takes place in the progress zone (PZ) which is the most distal mesoderm of the bud, lying subjacent to the apical ectodermal ridge (AER), a specialized region of the ectoderm, encircling the distal margin of the limb bud. Differentiation into cartilage, and cell death, occur when the cells of the PZ become displaced proximally into the core of the bud (Macias et al., 1999). Differentiation of mesodermal cells into cartilage results in the formation of the limb skeleton and follows a proximodistal sequence. Cell death occurs in well defined domains and sculpts the shape of the limb, eliminating the cells located between the differentiating cartilages (Hurle et al., 1996). In the early stages of the avian limb development, the anterior (ANZ) and posterior (PNZ) necrotic zones eliminate the mesodermal cells located anterior and posterior to the zone of formation of the proximal skeletal components of the limb. At more advanced stages of development, areas of interdigital cell death (INZs) eliminate the mesodermal cells located between the developing digits.

Chondrogenesis and cell death are both controlled by BMPs (Zou and Niswander, 1996; Zou et al., 1997; Macias et al., 1997; Kawakami et al., 1996; Yokouchi et al., 1996) and each of these opposing effects appears to be related to the stage of differentiation of the mesoderm. The undifferentiated limb mesoderm undergoes apoptosis when the cells are exposed to BMPs, but if the cells have initiated aggregation into the prechondrogenic blastemas, BMPs induce growth and differentiation through the receptor BMPR1B (Merino et al., 1998). In addition, it has been found recently that interdigital BMPs play a key role in regulating the morphological identity of the digits (Dahn and Fallon, 2000). Three members of the BMP family (BMP2, BMP4 and BMP7) are widely distributed in the limb bud including the mesoderm of the ANZ, PNZ and INZs, which are destined to die and also in the proliferating mesoderm of the progress zone and in the AER (Francis-West et al., 1995). Thus, a key question to be answered is why the apoptotic effect of BMPs is restricted spatially and temporally to the zones of cell death. The presence in the limb mesoderm of the BMP antagonist Gremlin in a fashion complementary to that of BMPs may contribute to limit the spatial distribution of cell death within the limb bud (Merino et al., 1999).

FGFs have been identified as the signals responsible for mesodermal proliferation (Martin, 1998) but there is also evidence that FGFs are involved in the regulation of cell death. Exogenous administration of FGFs into the areas of physiological cell death inhibits apoptosis (Macias et al., 1996) and co-administration of FGFs with BMPs into the limb mesoderm blocks the apoptotic effect observed when BMPs are administered alone (Gañan et al., 1996; Buckland et al., 1998). In addition, syndactyly, a phenotype characteristic of defective programmed cell death, is observed in mutants with disruption in the FGF signaling pathway (Muenke et al., 1994; Wilkie et al., 1995b; Yamaguchi and Rossant, 1995; Partanen et al., 1998; Heymer and Ruther, 1999). Furthermore, local application of FGF into developing interdigital duck webs potentiates the apoptotic effect of exogenous administered BMPs (Gañan et al., 1998). These results suggest that FGFs might be at the same time survival factors and signals required for cell death. But, how FGFs may exert these apparently opposite functions awaits clarification.

We have investigated the possible function of FGFs in the regulation of the areas of programmed cell death in the developing avian limb. Our findings confirm the role of FGFs as survival factors for the limb mesoderm and provide evidence for a role of FGFs in the control of the BMP-signaling pathway responsible for establishing the areas of cell death.

MATERIALS AND METHODS

We have used Rhode Island chick embryos at between days 3 and 9 of incubation (stage 20-35, Hamburger and Hamilton, 1951) and Royal Pekin duck embryos between 7 and 10 days of incubation.

Experimental manipulation of the limbs

The function of FGFs in the control of cell death was studied by analyzing the effects of local administration of FGFs (FGF2; R&D Systems) and FGF inhibitors (SU5402, Calbiochem; and PD173074/SB-402451, a generous gift from Glaxo Smith Kline) into the limb tissues using as carriers heparin acrylic (Sigma) and ion exchange (AG1-X2, Bio-Rad) beads, respectively. The possible interactions between FGFs and BMP signaling were explored by implanting together, or at different time intervals, beads incubated in FGFs or in SU5402, and beads incubated in BMP7 (a gift from Creative Biomolecules, Hopkinton) or in a BMP antagonist (Noggin or Gremlin; both generously donated by Regeneron Pharmaceuticals Inc., Tarrytown). For these purposes the eggs were windowed at the desired stages and the right limb bud was exposed. Beads incubated in the different factors (1 hour at room temperature) or in PBS or DMSO (controls) were implanted into the limb mesoderm. The effects on the ANZ and PNZ were examined by implanting the beads in the anterior or posterior margin mesoderm of the chick wing bud at stages 20-22. The effects on the INZ were studied by implanting the beads in the third interdigital space of chick (stages 28 or 29) or duck embryos (8.5 days of incubation).

Human recombinant FGF2 and BMP7 were diluted in PBS at a concentration of 0.5 mg/ml; human recombinant Noggin and Gremlin diluted in PBS were employed at 1 mg/ml; SU5402 and PD173074 were diluted in DMSO and employed at 4 mg/ml and 2 mg/ml, respectively.

In some experiments FGF2 was substituted for FGF4 or FGF8, and BMP7 for BMP2. No significant changes in the observed effects were apparent from these substitutions.

Morphology, cell death and cell proliferation

The morphology of the limbs following the different treatments was studied in whole-mount specimens after cartilage staining with Alcian Green as described previously (Gañan et al., 1996) and by scanning electron microscopy. The pattern of cell death was analyzed by vital staining with Neutral Red (see Macias et al., 1997) and by Tdt-mediated dUTP nick end labeling (TUNEL) in paraffin sections following the instructions of the manufacturer (Boehringer Mannheim).

Cell proliferation was analyzed by anti-bromodeoxyuridine immunolabeling. For this purpose 100 µl of bromodeoxyuridine (BrdU) solution (100 µg/µl) was pipetted directly over the limb. After 30 minutes of further incubation, the embryos were fixed in 70% ethanol. The autopod was then dissected free, dehydrated and embedded in paraffin wax. Immunocytochemistry to detect BrdU incorporation was carried out in tissue sections according to the instructions of the manufacturer (Becton Dickinson) using anti-BrdU and rhodamine-conjugated secondary antibody.

Micromass cultures

High density (2×10^7 cells/ml) micromass cultures were set up from stage 25 progress zone leg bud mesoderm. Cells were incubated in serum free medium (DMEM) for 5 days and the medium was changed daily. After 24 hours of incubation, SU5402 at 50, 200, 500 or 800 ng/ml, BMP7 at 50 ng/ml or Noggin at 100 ng/ml were added to the medium. The chondrogenic outcome of control untreated and cultures treated with different combinations of SU5402, BMP7 and Noggin was evaluated by Alcian Blue staining or by studying the expression of the type II collagen gene by whole-mount in situ hybridization. In all cases SU5402 was added 12 hours before BMP7 to ensure that FGF signaling was blocked prior to the administration of BMP7.

Probes and in situ hybridization

The probes for *BMP2*, *-4* and *-7*, and *Fgf-8* were provided by P. Francis-West and J. C. Izpisua-Belmonte. *MSX2* was provided by A. Kuroiwa; type II collagen was provided by W. Upholt. Probes for FGF receptor genes 1, 2 and 3 were provided by J. M. Richman. *Fgf12* was provided by I. Muñoz-Sanjuan. For *Snail* we employed a chick probe corresponding to nucleotides 258-767 (Sefton et al., 1998).

In situ hybridization of control and treated limbs was performed in whole-mount specimens and in tissue sections. For whole mount, samples were treated with 10 µg/ml of proteinase K for 20-30 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). Micromass cultures were processed in the same way, but proteinase K treatment was performed at 7 µg/ml for 8 minute at 20°C. For in situ hybridization in tissue sections, we employed paraffin wax sections (8 µm) and radioactive probes labeled with ^{35}S.

RESULTS

FGF signaling is required for interdigital cell death

The possible physiological implication of FGFs in cell death was first analyzed by blocking FGF signaling by local application of the FGF inhibitor SU5402 (Mohammadi et al., 1997). Beads incubated in SU5402 at 4 mg/ml were inserted into the interdigital mesoderm at stage 28. Under these conditions cell death was inhibited (11/14; Fig. 1A,B) leading to soft tissue syndactyly (8/10; Fig. 1E,F). To check whether inhibition of cell death was transitory or permanent, interdigits treated with SU5402 were subsequently treated with BMP7. For this purpose a bead soaked in BMP (BMP-bead) was implanted 24 hours after implantation of a SU5402-bead and the interdigit was examined for cell death 12-16 hours later. Under these conditions BMPs failed to induce apoptosis except at the most distal part of the interdigit (9/9; Fig. 1C,D). In some experiments we employed the FGF inhibitor PD173074 (Mohammadi et al., 1998) as an alternative to SU5402. Interdigital cell death was also inhibited by PD173074 (11/17), but the inhibition was only appreciable during the first 24 hours after the treatment.

To rule out any potential effect of SU5402 on the BMP receptors, we studied in vitro whether SU5402 inhibited the chondrogenic response of limb mesoderm micromass cultures to exogenous BMPs. It has been well documented that BMPs are the mediators of chondrogenesis in micromass cultures (Pizette and Niswander, 2000). While Noggin intensely inhibited chondrogenesis (Fig. 1G-K), SU5402 added to the culture medium did not modify significantly the pattern of chondrogenesis (24/24, Fig. 1K,L). Furthermore, the intense chondrogenic effect of exogenous BMP7 (Fig. 1O,P) was not inhibited by the addition to the culture medium of SU5402 (24/24; Fig. 1M,N). Similar results were obtained by specific cartilage staining with Alcian Blue (Fig. 1G,I,K,M,O) and by analyzing the expression of the type II collagen gene (Fig. 1H,J,L.N,P). Since both chondrogenesis and apoptosis by BMPs are mediated by the receptor BMPR1B (Zou et al., 1997), these findings show that there is no direct effect of SU5402 on BMP signaling. To additionally confirm these findings in vivo, local treatments with SU5402 and BMP7 were applied to the tip of digit 3. As expected, outgrowth was blocked in the digits treated with SU5402 (Fig. 1Q). However, when a bead incubated in BMP7 was implanted 12-24 hours after local application of a SU5402 bead the growth promoting effect caused by BMP7 in the developing cartilage (Macias et al., 1997) was not inhibited (12/12; Fig. 1Q,R).

These results are indicative for a role of FGFs in the control of interdigital cell death. Potential candidates of the FGF family involved include FGF8, expressed in the AER until the stages of interdigital cell death (Fig. 2A; Gañan et al., 1998) and FGF12 (FHF-1), which is expressed in the interdigital mesoderm (Fig. 2B; Muñoz-Sanjuan et al., 1999).

It has been reported that FGF receptors 1-3 are expressed in association with the differentiation of the limb mesoderm into cartilage. We have analyzed the distribution of these receptors in the interdigital regions during the stages of cell death. *FGFR1*, *FGFR2* and *FGFR3* were expressed in the autopod before and during the stages of interdigital cell death. *FGFR1* was expressed at low levels in the undifferentiated mesenchyme of the autopod and showed domains of higher expression in the differentiating cartilages and in the the

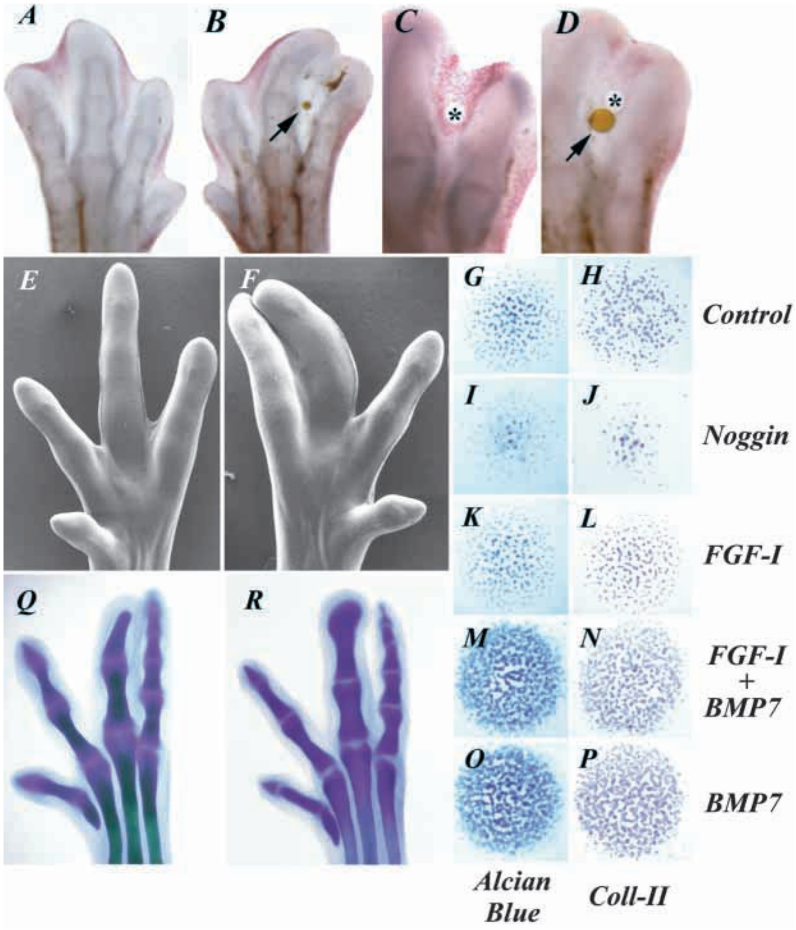

Fig. 1. Inhibition of interdigital apoptosis by treatment with SU5402. (A,B) Left side, control (A) and right side (B) treated chick leg autopodes vital stained with Neutral Red 48 hours after implantation of a SU5402-bead (arrow). (C,D) Interdigital apoptosis 12 hours after application of a BMP-bead (*) in a normal limb interdigit (C) and in an interdigit 24 hours after implantation of a SU5402-bead (arrow; D). Note the reduced apoptotic effect of BMPs in limbs previously treated with the FGF inhibitor. (E,F) Scanning electron micrographs showing the presence of soft tissue syndactyly 4 days after interdigital application of SU5402 (F); E is the left control autopod of the same embryo. (G-P) Micromass cultures of stage 25 progress zone mesoderm showing chondrogenesis by Alcian Blue staining after 5 days of incubation (G,I,K,M,O) or by in situ hybridization for the type II collagen gene expression after 4 days of incubation (H,J,L,N,P). Control untreated cultures (G,H) and cultures treated with Noggin (100 ng/ml; I,J); SU5402 (800 ng/ml; an FGF inhibitor; K,L); SU5402 and BMP7 (800 and 50 ng/ml respectively; M,N); and BMP7 (50 ng/ml; O,P). Note that chondrogenesis is intensely inhibited by Noggin (I,J versus G,H) but not by SU5402 (K,L versus G,H) and that addition of SU5402 does not inhibit the chondrogenic effect of BMP7 (M-N vs O-P). (Q,R) Digit morphology 3 days after implantation of only a SU5402-bead at the tip of digit 3 (Q) and after combined treatment of SU5402 and BMP7 (R). Note that in both cases digit outgrowth is blocked by the inhibition of FGFs, but the growth promoting effect of BMP7 on the cartilage is not blocked giving rise to a dramatically enlarged phalanx (R).

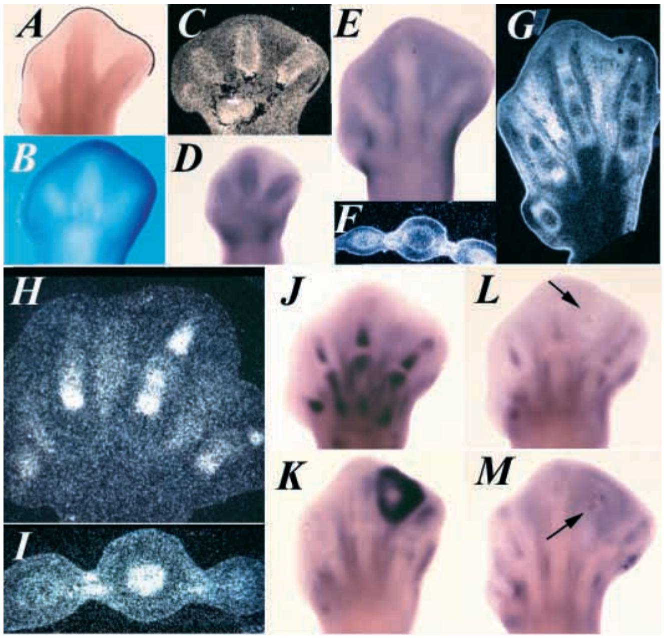

Fig. 2. Expression of FGFs and FGF receptors in the developing autopod during the stages of interdigital cell death. (A) Expression of *Fgf8* in the AER at stage 31. At this stage interdigital apoptosis is present in the second and third interdigits. (B) Expression of *Fgf12* at stage 29. This member of the FGF family is expressed in the progress zone mesoderm and in the interdigital regions showing a predominant distribution in the posterior part of the autopod. (C,D) Expression of *FGFR1* at stages 30 (C) and 29 (D). This receptor is predominantly expressed in the surface of the condensing mesenchyme of the digital rays and in the developing tendons, but a low level of expression is also present in the undifferentiated mesenchyme. (E-G) Expression of *FGFR2* in the developing autopod. (E) Whole-mount in situ hybridization after short digestion with proteinase K (20 minutes) in a stage 30 autopod showing the ectodermal expression of *FGFR2* in the ectoderm. (F) Tranverse and (G) longitudinal sections of the autopod at stage 31. Note the intense expression of this receptor in the interdigital mesoderm. (H-M) Expression and regulation of *FGFR3* in the developing autopod. (H) Longitudinal and (I) transverse sections of the autopod at stage 30 showing the intense expression of this receptor in the diferentiating cartilage and in the interdigital regions. (J-M) Whole-mount in situ hybridization showing the regulation of *FGFR3* after interdigital application of BMP7 (K) and FGF2 (L,M). (J) Control autopod at stage 30. (K) Experimental autopod 6 hours after implantation of a BMP-bead. Note the intense up-regulation of this receptor in the treated interdigit. (L,M) Experimental autopodes 10 hours (L) and 20 hours (M) after implantation of an FGF-bead (arrow). Note that *FGFR3* is initially down-regulated (L) and then up-regulated (M) by the application of FGFs.

tendon-forming mesenchyme running dorsal and ventral to the developing digits (Fig. 2C,D). *FGFR2* was highly expressed in the developing autopod, including the autopodial ectoderm (Fig. 2E) and the digital and interdigital mesenchyme (Fig. 2F,G). In the developing digits, *FGFR2* transcripts were abundant in the perichondrium, in the developing joints and in the differentiating cartilage of the tip of the digits (Fig. 2F,G). Expression in the interdigital mesenchyme was also intense before and during the stages of cell death (Fig. 2G). *FGFR3* exhibited well defined domains of expression in the interdigital regions and in the digital rays (Fig. 2H-J). The interdigital domains were closely coincident with the areas of cell death and its expression was regulated by local treatments with BMP7 and FGF2. Interdigital implantation of a BMP-bead caused a rapid (2 hours) and dramatic up-regulation of *FGFR3* gene expression (Fig. 2K). Interestingly, local treatments with FGF2 regulated the interdigital expresion of *FGFR3* in a temporal fashion paralleling the effects on cell death described below. Ten hours after the interdigital implantation of the FGF-bead, expression of *FGFR3* was inhibted (Fig. 2L), but by 20 hours after the treatment expression of this gene was up-regulated (Fig. 2M).

Exogenous FGFs potentiate apoptosis in INZ

To further analyze the potential influence of FGFs in apoptosis we studied the effects of exogenous FGFs in the interdigital mesoderm. FGF-beads implanted in the interdigital mesoderm at stage 28 or 29, caused an initial inhibition of programmed cell death detectable by 12 hours after the treatment (8/8; Fig. 3A,B). However, a dramatic increase in cell death was apparent 24 hours or later after the application of FGF-beads (12/12; Fig. 3C,D). This feature was particularly evident in the duck interdigits where in physiological conditions cell death is restricted to the most distal mesoderm (12/12; Fig. 3E,F). As in physiological conditions dying cells were TUNEL positive and during the first 30 hours exhibited a characteristic distribution at some distance from the bead (Fig. 3G, see also Fig. 8A). Analysis of cell proliferation by BrdU assay revealed an intense inhibition of cell proliferation in the zone of cell death coincidently with the onset of apoptosis (Fig. 3H).

Cell death mediated by FGFs occurs through BMP signaling

Since physiological cell death in the limb is mediated by BMPs, we decided to check whether inhibiting BMP signaling could inhibit cell death mediated by FGFs. In all the experiments described above implanting a bead incubated in the BMP antagonist Noggin, in association with the FGF-bead, inhibited cell death (8/8; Fig. 3I-J), indicating that the mediation of cell death by FGFs occurs through BMP signaling. In view of this result we next analyzed the effect of FGF on BMP gene expression. Our findings indicate that FGFs regulated positively the expression of BMPs in the interdigital regions.

When FGF-beads were implanted in the interdigital regions, *BMP*s were up-regulated intensely (11/12; Fig. 4A-C). As described previously in the chick epiblast (Streit and Stern, 1999), up-regulation of BMPs occurred at some distance from the bead forming a characteristic crescent-like domain of expression concentric to the bead (see Fig. 4B).

Blocking FGFs by implanting SU5402-beads in the interdigits did not cause a significant change in BMP gene expression during the first 20 hours after the treatment (Fig. 4E). After longer intervals (30-40 hours) changes in the expression of BMPs were detected. As shown in Fig. 4D and F, *bmp* transcripts were expressed in the marginal ectoderm of the interdigit and in the proximal mesenchyme close to the bead, while they were absent from the distal region of the interdigital mesoderm. The maintenance of *BMP* gene domains in the experimental limbs at these advanced stages of development could be explained by the survival of the interdigital mesoderm resulting from this treatment and

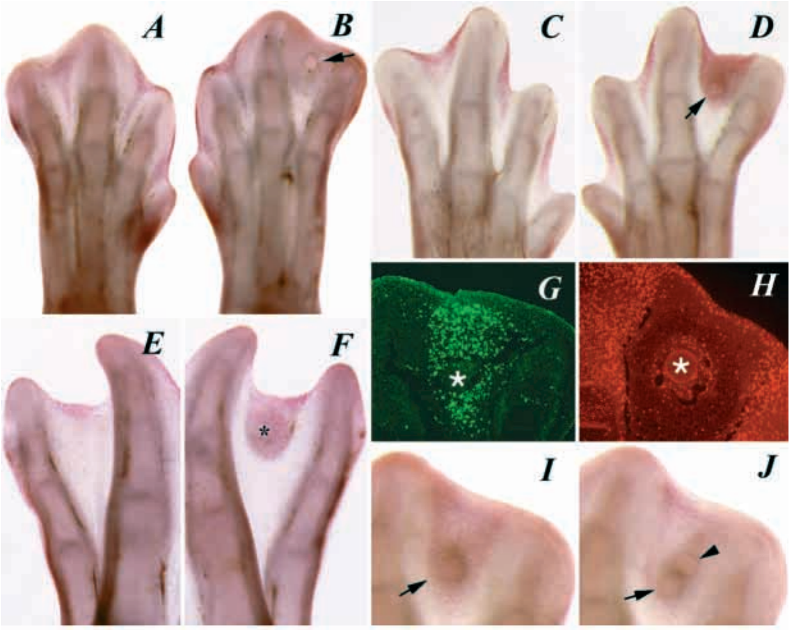

Fig. 3. Effects of FGF2 on interdigital cell death. (A,B) Interdigital cell death (vital stained with Neutral Red) in left control (A) and right treated (B) chick autopods 14 hours after implantation of a FGF-bead into the third interdigit (arrow). Note that at this time period of treatment cell death is fully inhibited by FGFs. (C) Left control and (D) right treated chick autopodes 48 hours after implantation of an FGF-bead (arrow) into the third interdigit. Note the intense increase of cell death at this time period after the application of FGF2. (E) Control and (F) experimental duck interdigital webs showing the increase in apoptosis 50 hours after implantation of a FGF-bead (*; F). (G) TUNEL labeling of interdigital cell death 30 hours after the implantation of an FGF-bead (*). (H) BrdU incorporation into the interdigital mesoderm 30 hours after the implantation of a FGF-bead. Note that cell proliferation is inhibited in the zone of induced apoptosis (compare with G). (I,J) Illustrate the inhibition of FGF-mediated cell death by co-administration of a Noggin-bead. (I) Cell death 30 hours after the implantation of an FGF-bead (arrow) and (J) its inhibition when a Noggin-bead (arrow) is co-implanted with a FGF-bead (arrowhead).

indicates that expression of *BMP*s does not require the presence of FGF.

Regulation of *MSX2* and cell death mediated by FGFs

It has been shown that apoptosis by BMPs requires the expression of the homeobox-containing gene *MSX2* in the limb mesoderm (reviewed by Chen and Zhao, 1998). Hence, we have studied the possibility that this gene is the target of FGFs in the control of interdigital cell death. Implantation of FGF-beads into the limb mesoderm expanded the domain of *MSX2* (7/7; Fig. 5A,B). This effect was observed 12 hours or later after the treatment and was more noticeable in the duck webs where expression of *MSX2*, as interdigital apoptosis, is physiologically restricted to the most distal region of the interdigit (Fig. 5C,D). Interestingly, blockage of FGF signaling by SU5402 was accompanied by severe downregulation of *MSX2* in the interdigits detectable 10 hours or later after the treatment (7/8; Fig. 5E). Considering that, in spite of the inhibition of cell death, the interdigits treated with SU5402 maintain a considerable level of *BMP* expression it seems likely that FGFs control cell death through the regulation of *MSX2*. Moreover, we have also found evidence for a major role of BMPs in *MSX2* gene expression. BMP-beads were potent upregulators of *MSX2* (not shown) whereas Noggin- or Gremlin-beads (Fig. 5F) downregulated *MSX2* gene expression. In addition, coimplantation of FGF- and Noggin-beads reduced considerably the induction of *MSX2* by FGFs (Fig. 5G), indicating that the induction of *MSX2* by FGFs requires BMP sgnalling. However, BMP treatments failed to increase the expression of *MSX2* in interdigits previously treated with the FGF inhibitor (Fig. 5H). Together all these findings indicate that *MSX2* gene expression requires the action of both FGFs and BMPs.

Expression and regulation of *Snail* and cell death

Members of the *Snail* family of zinc finger transcription factors have been implicated in the negative regulation of programmed cell death in *C. elegans* (Metzstein and Horvitz, 1999) and vertebrates (Inukai et al., 1999). In the chick, two members of this family, *Snail* and *Slug*, have been identified. *Slug* is expressed in the developing limb, including the interdigital regions, but its possible involvement in cell death has been discarded on the basis of its expression and regulation (Ros et al., 1997). *Snail* expression has been implicated in the process of limb induction (Isaac et al., 2000) and in the onset of chondrogenesis (Sefton et al., 1998) but its expression pattern in the course of digit morphogenesis has not been studied. Here we have explored whether *Snail* regulates cell death in cooperation with FGFs/BMPs.

Snail transcripts are present in the wing and leg bud

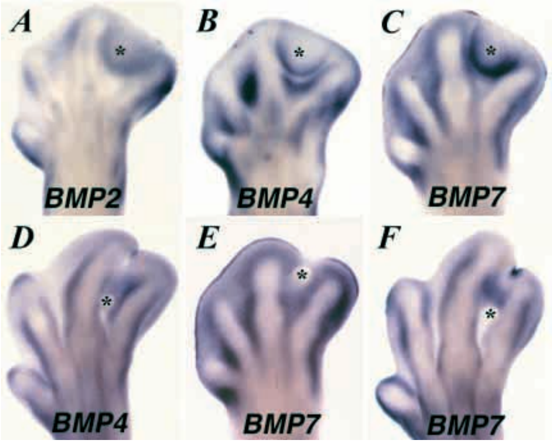

Fig. 4. (A-C) Effect of FGF-beads (*) implanted into the third interdigit at stage 28 on the expression of *BMP2* (A) *BMP4* (B) and *BMP7* (C). (D-F) Effects of beads (*) soaked in SU5402 implanted into the third interdigit at stage 28; (D,F) The expression of *BMP4* and *BMP7*, 36 hours after the treatment; (E) expression of *BMP7*, 20 hours after the treatment. Note that *BMP*s continue to be expressed at high levels in the treated interdigits.

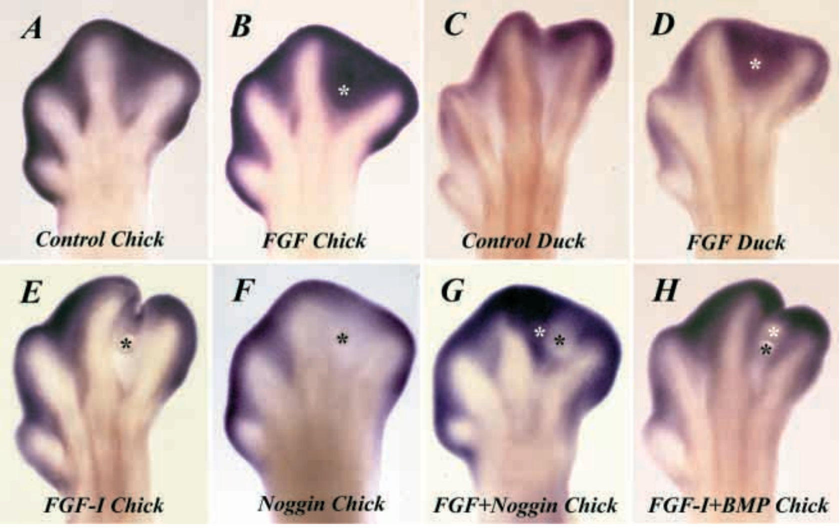

Fig. 5. Regulation of the interdigital expression of *MSX2* in chick and duck limbs by FGFs and BMPs. (A) Control chick at stage 30. (B) Upregulation of *MSX2* 24 hours after the implantation of a FGF-bead (*). (C) Control duck at day 9.5 of incubation. Note that *MSX2* expression in the duck is restricted to the most distal region of the interdigital webs. (D) Upregulation of *MSX2* in the duck 14 hours after interdigital implantation of a FGF-bead (*). (E) Down-regulation of *MSX2* in the chick 30 hours after implantation of a bead incubated in SU5402. (F) Down-regulation of *MSX2* in the chick 14 hours after implantation of a Noggin-bead (*). (G) Expression of *MSX2* in the chick 14 hours after implantation of a FGF-bead (white asterisk) and a Noggin-bead (black asterisk). Note the up-regulation by FGFs is inhibited in the zone of influence of the Noggin-bead. (H) Expression of *MSX2* in the chick 8 hours after implantation of a BMP-bead (white asterisk) 24 hours after implantation of a SU5402-bead (black asterisk). Note that in these conditions BMPs fail to upregulate *MSX2*.

mesoderm throughout the whole period studied here. At stages 25-26 *Snail* is expressed in the anterior and posterior margins of the zeugopod and in the autopodial region (Fig. 6A). From stage 27 *Snail* is expressed at the tip of the growing digits, in the perichondrium of the developing phalanges, excluding the zones of joint formation, and is strongly expressed in interdigital domains, which correlates closely with the areas of interdigital cell death (Fig. 6B-D).

The above described pattern of gene expression in the interdigital regions is suggestive of a positive, rather than negative, involvement of *Snail* in cell death. This possibility was further analyzed by studying its expression in the duck leg bud, which is characterized by a reduced extension of the areas of interdigital cell death. Expression of *Snail* in the duck interdigits was similar to that in the chick only prior to the onset of interdigital cell death (not shown). From day 9 of incubation (equivalent to stage 30-31 in the chick) *Snail* expression became restricted to the distal margin of the interdigits in a fashion closely coincident with the pattern of cell death present in the duck interdigits (Fig. 6E, see also Fig. 3E).

In view of the positive correlation between *Snail* expression and cell death, we analyzed the regulation of this gene in the limb by FGFs and BMPs. Indeed, we first analyzed whether the increase in cell death induced by the interdigital application of FGF-beads modifies the expression of this gene. Under these experimental conditions, *Snail* expression was upregulated (Fig. 6,F,J). This upregulation was particularly dramatic in the duck interdigit (Fig. 6F) showing a close correlation with the increase in the areas of cell death (Figs 6F and 3F). Implantation of BMP-beads was also followed by upregulation of *Snail* (Fig. 6H). In addition, the physiological expression of this gene was downregulated locally following implantation of beads bearing a BMP antagonist (Noggin or Gremlin; Fig. 6I). Both effects were detected very quickly (between 2 and 5 hours after the implantation of the beads) and preceded the induction and inhibition of cell death induced respectively by BMPs and by BMP antagonists. To check whether this effect was mediated by BMPs, FGF-beads were implanted together with a Noggin-bead. Under these conditions, the effects of FGF-beads were inhibited in the mesoderm close to the Noggin-bead (Fig. 6G) indicating that, as observed for *MSX2*, upregulation of the expression of this gene by FGF requires the presence of BMPs. Also as observed for *MSX2*, interdigital expression of *Snail* was intensely downregulated by treatments with SU5402 (Fig. 6K), and this inhibition could not be abolished by the subsequent application of a BMP-bead (Fig. 6L).

Regulation of cell death in the ANZ and PNZ by FGFs

To check whether the involvement of FGFs was a specific feature of INZs or if it represented a common mechanism controlling cell death in the limb bud, experiments were performed in the anterior and posterior mesoderm of the early wing bud. For this purpose FGF-beads were implanted into stage 20-22 wing buds. In agreement with previous studies (Riley et al., 1993; Akita et al., 1996; Nikbakht and McLachlan, 1999) FGF treatments caused a significant mesodermal overgrowth around the bead and was followed by alterations in the cartilages of the zeugopod and by the formation of extra cartilaginous elements in the zone of bead application detectable 3-4 days after treatment (Fig. 7A-C).

As observed in INZs, analysis of cell death following these treatments revealed a double effect of FGFs on cell death. During the first 12 hours after treatment, physiological cell death was inhibited (8/10; Fig. 7D-F), but this initial inhibition was followed by increased cell death detectable by Neutral Red vital staining 24 hours or later after treatment (18/18; Fig. 7G-H) and by TUNEL labeling (Fig. 8A). The increase in cell death was more accentuated in the ANZ than in the PNZ. The molecular basis for this difference is out of the scope of this study although it may be caused by the influence of FGFs on *Shh* gene expression. *Shh* is also involved in the regulation of cell death in the PNZ (Sanz-Ezquerro and Tickle, 2000) and in the expression of *Gremlin* (Zuñiga et al., 1999), a BMP antagonist able to inhibit cell death (Merino et al., 1999). When Noggin beads were implanted in combination with FGF beads, cell death was intensely inhibited (8/8; Fig. 7I), indicating that as observed for INZs, BMPs mediated cell death induced by FGFs.

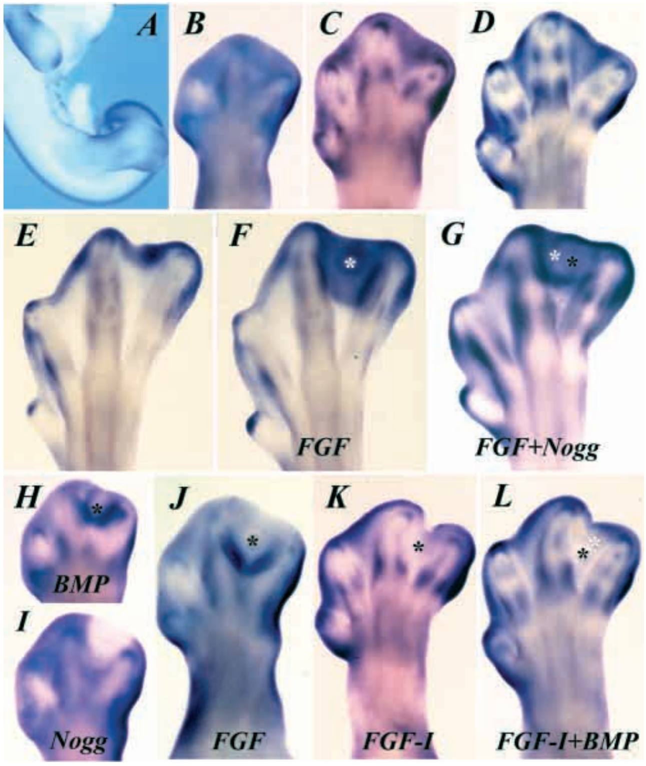

Fig. 6. (A-D) Expression of *Snail* in the embryonic chick limb at stages 25 (A), 26 (B), 29 (C), and 31 (D). Note the progressive increased labeling of the interdigital regions at stage 31 (D) in correlation with the establishment of INZs. (E) Expression of *Snail* in the duck leg autopod at day 9.5 of incubation. Note that the interdigital domains of *Snail* become restricted distally coincidently with the zones of interdigital cell death in the duck (compare with Fig. 3E). (F) Upregulation of *Snail* in the third interdigit of the duck leg 14 hours after implantation of a FGF-bead (*). (G) Duck leg bud 14 hours after implantation of a FGF-bead (white asterisk) and a Noggin-bead (black asterisk). Note that the positive effect of FGFs on *Snail* expression is inhibited in the zone of influence of the Noggin-bead. (H) Upregulation of *Snail*, 6 hours after implantation of a BMP-bead (*) into the third interdigit of the chick. (I) Down-regulation of *Snail* expression 6 hours after implantation of a Noggin-bead (*) into the third interdigit of a stage 28 chick embryo. (J) Upregulation of *Snail* in the chick 10 hours after interdigital implantation of a FGF-bead (*) at stage 28. Note that the upregulation of *Snail* parallels that observed for BMPs (see Fig.4B). (K) Down-regulation of *Snail*, 24 hours after interdigital implantation of a bead (*) incubated in SU5402. (L) Expression of *Snail* in the chick interdigit 8 hours after implantation of a BMP-bead (white asterisk) 24 hours after implantation of a SU5402 bead (black asterisk). Note that in these conditions BMPs are unable to upregulate *Snail* expression (compare with H and K).

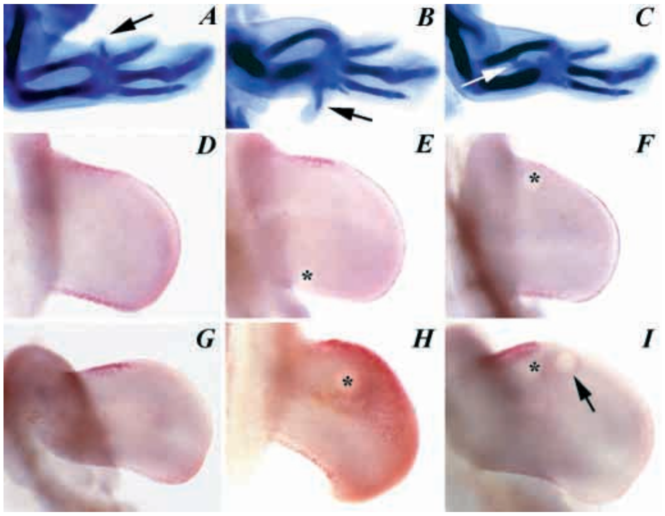

Fig. 7. (A-C) Skeletal alterations after treatments with FGFs in specimens stained with Alcian Green showing the formation of extra cartilaginous elements after implantation of FGF-beads at stage 20 in the anterior margin mesoderm (A), posterior margin mesoderm (B) and in the progress zone (C). Arrows indicate the position of the induced extra cartilaginous elements. (D-H) Changes in the pattern of cell death after treatments with FGFs in specimens vital stained with neutral red. (D-F) Control (D) and experimental wing buds showing the inhibition of apoptosis in the PNZ (E) and in the ANZ (F) 12 hours after the implantation of FGF-beads (*). (G,H) Control and experimental limbs showing the normal pattern of ANZ (G) and the increased apoptosis 24 hours after implantation of an FGF-bead (*, H) in the anterior margin mesoderm. (I) Limb bud vital stained for cell death 24 hours after implantation in the anterior margin mesoderm of an FGF-bead (*) and a Noggin-bead (arrow). Note the intense inhibition of apoptosis in the zone of influence of the Noggin-bead (compare with H).

Also, as observed in INZs the mesodermal domains of *Snail* (Fig. 8B), *MSX2* (Fig. 8C) and *BMP2* (Fig. 8D), *BMP4* (Fig. 8E-F) and *BMP7* (Fig. 8G-H) were increased by the application of FGF-beads. Furthermore, in accordance with the negative influence of BMPs on the maintenance of the AER (Gañan et al., 1998; Pizette and Niswander, 1999), the increased expression of *BMP*s was accompanied by flattening of the AER in the zone close to the bead (Fig. 8I).

As expected, the most significant effect of SU5402-beads at early stages of development was the impairment of limb outgrowth. Implantation of SU5402-beads in the progress zone mesoderm at stages 20-22 caused a rapid degeneration of the AER followed by limb truncation.

DISCUSSION

The progress zone mesoderm plays a central role in limb morphogenesis. In this region the mesodermal cells are subjected to the influence of the AER which is responsible for outgrowth and proximodistal patterning of the limb. Two signals of opposite functional significance, BMPs and FGFs, play a critical role in the outcome of the PZ mesoderm (Niswander and Martin, 1993). FGFs support limb outgrowth by inducing proliferation in the PZ mesoderm while BMPs block limb outgrowth and promote apoptosis. It has also been shown that BMPs exert a negative influence on the maintenance of an active AER expressing FGFs (Gañan et al, 1998; Pizette and Niswander, 1999). We provide new evidence of molecular interactions between FGFs and BMPs in the control of limb outgrowth. We show that BMPs exert a positive influence on the expression of FGFR3. Interestingly, this receptor has been implicated in the inhibition of cell proliferation by FGFs (Sahni et al., 1999). In addition, we have also noted that FGFs have a positive effect on the mesodermal expression of *BMP* genes accompanied by intensification of

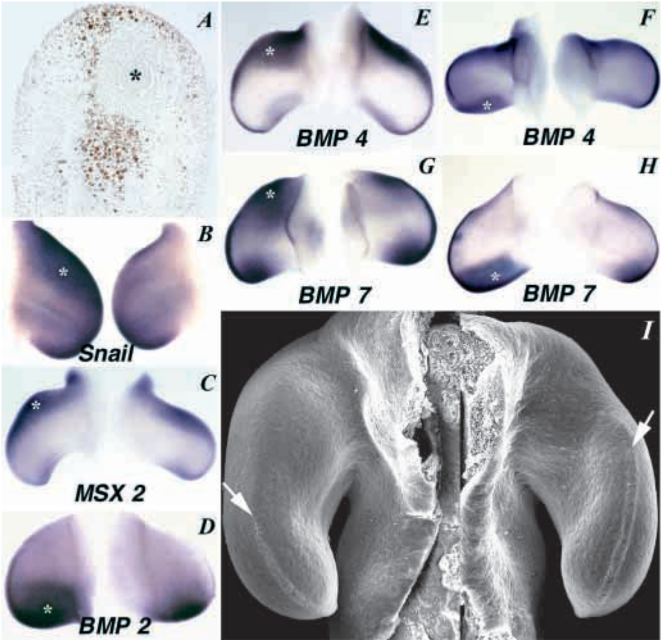

Fig. 8. (A) Transverse section of the limb bud processed for TUNEL showing the increased cell death 24 hours after the implantation of an FGF-bead into the anterior margin mesoderm. Note that apoptosis is induced at some distance form the bead (*). (B,C) Upregulation of *Snail* (B) and *MSX2* (C) 16 and 24 hours, respectively, after implantation of an FGF-bead (*) into the anterior margin mesoderm of the chick wing bud. (D-H) Regulation of *BMP* gene expression between 5 and 20 hours after implantation of FGFs in the anterior (E-G) or posterior margins (D,F,H) of the bud at stages 20-22: *BMP2* (D); *BMP4* (E,F); and *BMP7* (G,H). Note the expanded domains of *bmp*s in the treated limbs in comparison with the contralateral control limb. (I) Scanning electron micrograph showing the flattening of the AER 24 hours after the implantation of an FGF-bead in the anterior margin of the wing bud. Arrows indicate the anterior end of the AER.

BMP signaling (deduced by the flattening of the AER and the increase in BMP-mediated apoptosis). The absence of a significant downregulation of BMPs in the interdigits following blockage of FGF signaling indicates that FGFs are not necessary to maintain BMP gene expression. However, as will be discussed below, our findings indicate that FGFs are required for appropriate functioning of BMPs. In addition, the positive influence of FGFs on BMP gene expression might be important in maintaining BMPs and FGFs in equilibrium to ensure normal outgrowth of the limb.

Signaling by FGFs occurs through different tyrosine kinase receptors (FGFR1-4; Wilkie et al., 1995a). In the avian limb bud three FGF receptor genes (1,2 and 3) exhibit a specific pattern of expression in association with the different stages of cartilage differentiation (Noji et al., 1993; Szebenyi et al., 1995). Here we show that *FGFR1*, *FGFR2* and *FGFR3* are expressed in the autopodial mesoderm in a pattern compatible with a role in the control of cell death. Furthermore, permanent inhibition of cell death and syndactyly was induced by local treatment with SU5402. SU5402 inhibits FGF signaling by interacting with the catalytic domain of FGF receptors (Mohammadi et al., 1997). The specificity of this FGF inhibitor is supported by the absence of inhibitory effect (insulin and EGF receptors) or by the weak inhibitory effect (PDGF receptor) on other tested receptors with tyrosine kinase activity (Mohammadi et al., 1997). In addition, we have observed here that the chondrogenic promoting effect of BMPs is not affected by SU5402 excluding a potential direct effect on BMP signaling. In previous studies it has been found that syndactyly is a common result of spontaneous or induced mutations in *FGFR1* (Partanen et al., 1998; Muenke et al., 1994) and *FGFR2* (Wilkie et al., 1995b; Cohen and Kreiborg, 1995) in both mouse and humans (Apert and Pfeiffer syndromes), thus suggesting a role of these receptors in the control of interdigital apoptosis. However, those mutations appear to mediate a gain-of-function of the receptors (Yu et al., 2000; Zhou et al., 2000) rather than inhibiting FGF signaling. Therefore, syndactyly in those mutants might be explained by the inhibitory effect on cell death observed in our experiments shortly after the exogenous application of FGFs (see below). In this study the expression and regulation of *FGFR3* in the interdigits is suggestive of a positive function of this receptor in programmed cell death. Constitutive activation of *FGFR3* causes apoptosis in chondrocytes (Legeai-Mallet et al., 1998). In addition, owing to the inhibitory effect on cell proliferation mediated by *FGFR3* (Sahni et al., 1999), the upregulation of this gene observed here following treatments with BMPs might explain the cell cycle arrest associated with interdigital apoptosis. This interpretation is reinforced by the down- and up-regulation of *FGFR3* caused after short and long term treatments with FGFs, which are accompanied by inhibition and increased cell death, respectively.

In agreement with previous studies (MacCabe et al., 1991; Macias et al., 1996; Gañan et al., 1996; Buckland et al., 1998; Ngo-Muller and Muneoka, 2000) we have observed that FGFs are able to temporally inhibit cell death in the ANZ, PNZ and INZ. However, the initial inhibitory effect is later followed by potentiation of apoptosis in the treated mesoderm. Noggin was very potent in blocking this process of cell death, indicating that BMPs, as in physiological conditions, were the mediators of the apoptosis induced by FGFs. Furthermore, the inhibition of interdigital cell death and subsequent syndactyly observed after blocking FGF signaling by treatment with SU5402 indicates that FGFs are physiologically required for programmed cell death. In addition, the absence of cell death in the interdigits treated with SU5402, in spite of the maintenance of considerable levels of *BMP* gene expression, indicates that BMPs alone are not sufficient to induce cell death.

Our findings point to a cooperative role of FGFs with BMPs in the regulation of genes implicated in the molecular cascade responsible for apoptosis. We have identified *MSX2* and *Snail* as candidate genes associated with apoptosis whose expression requires the combined action of FGFs and BMPs.

It has been proposed that *MSX2* is required for BMP-mediated apoptosis (Graham et al., 1994; Chen and Zhao, 1998). Furthermore, experimental misexpression of *MSX2* in the limb bud induces apoptosis (Ferrari et al., 1998). In agreement with these findings, this study has shown that inhibition of interdigital cell death following blockage of FGF signaling by SU5402 is accompanied by intense downregulation of *MSX2*. Furthermore, our results indicate that both FGFs and BMPs are required for the induction and maintenance of *MSX2* expression. While both FGFs and BMPs alone are able to intensely upregulate *MSX2* gene expression in the intact limb, these individual effects are blocked in treatments with FGFs in combination with Noggin and in treatments with BMPs in combination with SU5402.

The mechanism responsible for the initial inhibition of cell death by FGFs remained elusive in this study. Since members of the *Snail* family of zinc finger transcription factors have been identified as antiapoptotic factors conserved in *C. elegans* (Metzstein and Horvitz, 1999) and in vertebrates (Inukai et al., 1999) we analyzed whether *Snail* was also involved in this process in the chick. Our observations strongly suggest that *Snail* plays a role in apoptosis in the developing limb. However, all our findings point to a positive role of this gene in apoptosis. *Snail* transcripts are present in the limb mesoderm at the same stages as ANZ and PNZ, and precise interdigital domains are also observed that closely coincide with the appearance of interdigital cell death. Furthermore, in duck interdigits, characterized by reduced extension of interdigital cell death, *Snail* expression is restricted to the zones of cell death instead of being increased as would be expected if it were an antiapoptotic factor. Moreover, in both the chick limb and duck interdigits, all treatments performed to increase cell death were accompanied by a parallel induction of *Snail* expression while expression was inhibited by interdigital application of SU5402 in correlation with the inhibition of cell death. In addition, we have also observed that BMPs and FGFs regulate the expression of this gene in a similar fashion to that described above for *MSX2*. The most likely explanation for a role of *Snail* in apoptosis is its potent activity in decreasing cell adhesion through the repression of the expression of cadherins (Cano et al., 2000). Cell survival requires an appropriate cell-cell and cell-extracellular matrix adhesion (Lin and Bissell, 1993) and apoptosis following a loss of cell adhesion has been observed in epithelial cells and non-epithelial cells (Martin-Bermudo et al., 1998; Sakai et al., 2000). The interdigital regions exhibit specific domains of expression of different cadherins (Kimura et al., 1995; Kitajima et al., 1999; Inoue et al., 1997) and prior to cell death contain a highly organized network of extracellular matrix (Hurle et al., 1994) which is disrupted concomitantly with the onset of cell death (Hurle and Fernandez-Teran, 1983). In consequence, expression of *Snail* in the interdigital regions may regulate changes in cell adhesion which must necessarily occur during involution of the interdigital tissue.

In conclusion, this study shows that FGF signaling is necessary for apoptosis during limb development. In its absence, BMPs are not sufficient to induce cell death indicating that the establishment of the apoptotic areas requires the presence of the two signaling pathways. In addition, we have identified *MSX2* and *Snail* as potential players in the apoptotic cascade whose expression requires the convergence of the signals mediated by both FGFs and BMPs. Thus, our study unravels a putative functional link between these two signaling pathways in the control of morphogentic outgrowth of the limb.

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