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BMP-2/-4 mediate programmed cell death in chicken limb buds

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SUMMARY

During limb development, the mesenchymal cells in restricted areas of limb bud, anterior necrotic zone, posterior necrotic zone, opaque zone and interdigital necrotic zones, are eliminated by programmed cell death. The transcripts of bone morphogenetic protein (*Bmp*)-*2* and -*4* were first detected in the areas where cell death was observed, then showed overlapping expression with the programmed cell death zones except the opaque zone. To investigate the function of BMP-2 and BMP-4 during limb pattern formation, the dominant negative form of BMP receptor was overexpressed in chick leg bud via a replication-competent retrovirus to block the endogenous BMP-2/-4 signaling pathway. This resulted in excess web formation at the anterior and posterior regions of limb buds in addition to marked suppression of the regression of webbing at the interdigital regions. Significant reductions in the number of apoptotic cells in these three necrotic zones were found in the limb buds which received the virus carrying dominant negative BMP receptor. This indicates that extra tissue formation is due to suppression of programmed cell death in the three necrotic zones. Moreover, BMP-2/-4 protein induced apoptosis of mesenchymal cells isolated from the interdigital region in vitro. Other TGFβ family proteins as TGFβ1 and Activin did not show this effect. These results suggest that BMP-2 and BMP-4 are the apoptotic signal molecules of the programmed cell death process in the chick limb buds.

Key words: bone morphogenetic protein, BMP-2, BMP-4, programmed cell death, apoptosis, limb bud, chicken

INTRODUCTION

Programmed cell death (PCD) is an important process for eliminating unnecessary tissues during embryonic development for proper morphogenesis at genetically determined stages. PCD occurs in morphogenetic processes such as limb pattern formation, establishment of muscle-nerve connection, establishment of the sex-specific genital system and metamorphosis of amphibians (see review Hinchliffe, 1981).

The occurrence of PCD is a remarkable feature of limb development in the amniotes (see review Hinchliffe, 1981). The mesenchymal cells in the anterior and posterior marginal parts of chick limb buds, the anterior necrotic zone (ANZ) and posterior necrotic zone (PNZ), are eliminated by PCD at day 4-6 of development. PCD in the interdigital necrotic zone (INZ) occurs in the mesenchymal areas separating the chondrifying digits of the developing footplate in 8-day chick embryos. The INZ has been found in all amniote species with non-webbed digits indicating the presence of common mechanism for PCD. Treatment of chick embryos with bromodeoxyuridine suppressed PCD in the INZ and resulted in webs between digits remaining, indicating that PCD plays a role in shaping limb morphology (Tone et al., 1983).

If the mesenchymal tissue of the prospective PNZ at stage (st.) 17 (Hamburger and Hamilton, 1951) is transplanted to the flank, the cells die on schedule after an appropriate interval (Saunders et al., 1962). However, transplantation of the same tissue to the dorsal surface of the wing bud results in suppression of PCD (ibid.). In addition, removal of the surface ectoderm of the INZ area suppresses PCD in the INZ and results in extra digit formation (Hurle and Ganan, 1987). These observations imply that tissue interactions and programmed cell death in the INZ mesenchymal cells at developmentally programmed stages is crucial for proper PCD in the limb bud.

Several diffusible intercellular signaling molecules such as mullerian inhibitory substance (MIS), BMP-4 and Reaper were shown to mediate PCD in the regression of mullerian duct (Behringer et al., 1994), PCD in the neural crest cells from rhombomeres 3 and 5 (Graham et al., 1994), and PCD during *Drosophila* embryogenesis (White et al., 1994), respectively. In the developing limb buds, the regions of expression of *bone morphogenetic protein* (*Bmp*)-*2* and -*4* overlap with the PCD area (this paper), implying the involvement of these signaling molecules in PCD in the limb bud.

BMP-2 and BMP-4 are vertebrate homologs of Decapentaplegic in *Drosophila*, have 98% amino acid sequence identity and were shown to have invariant functions in many biological processes for tissue interactions (Kingsley, 1994a,b, and references therein) through a specific receptor

complex (Koenig et al., 1994; Suzuki et al., 1994; Yamaji et al., 1994).

The BMP-2/-4 receptor complex is composed of type I and type II receptors both carrying serine/threonine kinase activity in their cytoplasmic domains (Koenig et al., 1994; Liu et al., 1995). The type I receptor/ligand complex associates with the type II receptor whose protein kinase activity is constitutively active. This association allows the type II receptor kinase to phosphorylate GS domain of the type I receptor activating its protein kinase activity. Unlike TGFβ or Activin receptor, the type I receptor of BMP-2/-4 can bind the ligand with high affinity without coexpression of type II receptor (Koenig et al., 1994; Liu et al., 1995). Several lines of evidence indicate that the mutant forms of Activin or BMP-2/4 receptors lacking the cytoplasmic domain behave as dominant negative molecules when overexpressed (Hemmati-Brivanlou and Melton, 1992; Graff et al., 1994; Suzuki et al., 1994). This would be due to the interruption of the endogenous signaling pathway by forming abortive complexes with the endogenous type II receptor(s) in vivo.

To elucidate the roles of BMP-2/-4 during limb development, we introduced the dominant negative form of BMP-2/-4 receptor type Ia (dnBMPR-Ia; Suzuki et al., 1994) into chick leg buds via a replication-competent retrovirus system to interrupt the signaling pathway. Overexpression of this dominant negative BMP-2/-4 receptor inhibited PCD in the ANZ, PNZ and INZs. In addition, we demonstrated that the BMP-2/-4 proteins behave as mediators of cell death of the mesenchymal cells derived from the interdigital necrotic zone in vitro.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Whole-mount in situ hybridization analysis with DIG-labeled RNA probes was performed as described by Yokouchi et al., 1995). The chicken *Bmp-4* and *Bmp-2* cDNAs used as probes were isolated from a day-11 chicken embryonic cDNA library by screening with *Xenopus* probes (A. Kuroiwa, unpublished data).

Construction of the recombinant virus vector

pDS5 (Yokouchi et al., 1995), which is a variant of pDS3 (Iba et al., 1988) was used for the recombinant virus construction. The *Bsp*I-*Bam*HI fragment containing the truncated mouse BMP receptor-Ia (ΔBMPRIa) cDNA that produces a receptor protein lacking the entire cytoplasmic domain (Fig. 1B) was inserted into pCITE-3 cleaved with *Nco*I and *Bam*HI. pCITE-3 was constructed by replacing the *Apa*I-*Pst*I fragment of pCITE-1 (Novagen) with the *Apa*I-*Pst*I fragment from pCITE-2 (Novagen). The *Eco*RI-*Xba*I fragment of ΔBMPRIa-pCITE-3 was inserted into the *Eco*RV site of pDS5 after blunting with T4 DNA polymerase. *Sal*I digest of this construct was ligated with the DNA fragment carrying essential genes for virus production, and ligated product was transfected to chicken embryonic fibroblasts as described previously (Iba et al., 1988).

Chick strain and virus production

All animals were obtained as fertile eggs from the supplier. For injection with A-subgroup retrovirus, specific pathogen-free White Leghorn eggs (obtained from line M, Nisseiken Ltd.) were used. Recombinant viral particles were produced as described by Iba et al. (1984, 1988). Injection of the viral particles into virus-free White Leghorn embryos (Nisseiken) was performed as described previously (Yokouchi et al., 1995). As a control, we infected embryos with the virus carrying no exogenous gene sequences, in the same way.

Skeletal staining

The legs of day-7.5 embryos were stained with Alcian blue as described by Yokouchi et al. (1995). Briefly, embryos were dissected and fixed in 10% formaldehyde overnight. Embryos were immersed in 70% ethanol/1% HCl for 3 hours, stained with 0.1% Alcian blue/70% ethanol/1% HCl for 3 hours, washed with 70% ethanol/1% HCl overnight and then dehydrated. The legs of day-10 embryos were stained with hematoxylin.

Detection of cell death

Vital staining with Nile blue sulfate was performed as described by Tone et al. (1983). Briefly, embryos were stained with 0.01% Nile blue sulfate/PBS for 20 minutes at 37°C, and washed for 3 hours in PBS. The TUNEL method (Gavrieli et al., 1992) for detecting fragmented DNAs which appear following apoptosis on paraffin sections was performed using an ApopTag-FITC kit (Oncor) according to the manufacturer's protocol.

Cell culture

Micromass culture of the mesenchymal cells isolated from the third interdigital region of chick leg buds at stage 29 was performed in F-12 medium (Nissui) (Aono and Ide, 1988) with minor modifications. In this experiment, 2×10^5 cells were plated onto 24-well tissue culture plates coated with rat type I collagen (Becton Dickinson). After 24 hours, the cultures were fixed in 4% paraformaldehyde/PBS, processed by the TUNEL method using a Cell Death Detection Kit-ALP (Boehringer) or stained with DAPI. For in vitro virus infection, the mesenchymal cells from ID3 of st.28 embryos were isolated and cultured for 3 hours as described above. Then the culture medium was replaced with 70 μl of medium containing control (no insert virus) or dnBMPR-1a virus ($2\text{-}5\times10^6$ virus/ml) and incubated for 4 hours. After changing the medium to F12 containing 5 ng/ml of bFGF, cells were incubated for 20 hours. Subsequently, the medium was changed to control or BMP-2 containing medium and cultured for a further 24 hours. By this method, nearly complete infection was observed using a monoclonal antibody against the gag protein (Potts et al., 1987). Recombinant human bFGF was supplied by KAKEN. Recombinant *Xenopus* BMP-2 and *Xenopus* BMP-4 were kind gifts from TAKEDA, and recombinant human Activin was from AJINOMOTO. Recombinant human TGFβ1 was purchased from R&D systems.

Removal of dorsal ectoderm on leg buds

Fertilized egg at 5.5 days (st. 28) was windowed, and a few drops of 0.1% Nile Blue/PBS was dropped on the leg bud. The lightly stained dorsal ectoderm on interdigit 3 was peeled with the sharpened tungsten needle. Then the egg was sealed and incubated for 12 hours.

RESULTS

Introduction of the truncated BMP receptor induces the ectopic web formation

To elucidate the functions of BMP-2 and BMP-4 during limb development, we attempted to interrupt BMP-2/-4 signaling by overexpression of the truncated form of the BMP receptor using the virus vector in the leg bud. We constructed a recombinant virus carrying the truncated form of BMP receptor-Ia (dnBMPR-Ia) (Fig. 1C), which is composed of the ligand binding domain and the transmembrane domain (Fig. 1A,B). It has already been reported that the introduction of the same form of the receptor successfully inhibited signaling of the endogenous BMP-4 in the early *Xenopus* embryo (Suzuki et

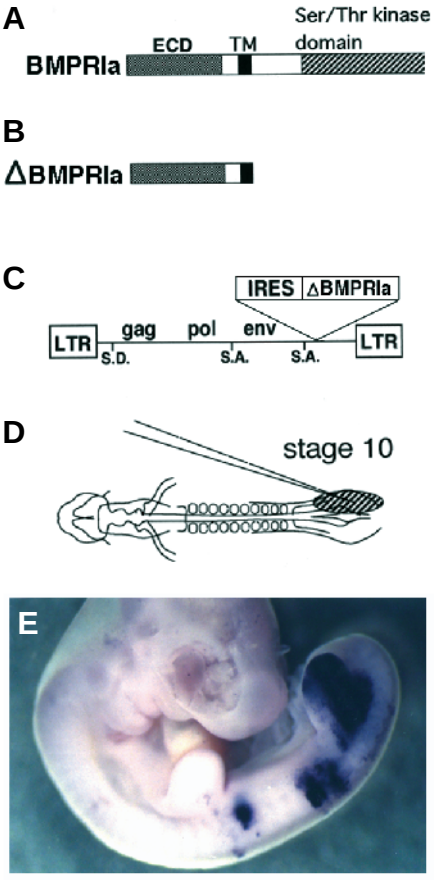

Fig. 1. Construction of the recombinant virus. (A) Structure of mouse BMP receptor type Ia (BMPR-Ia). A Ser-Thr kinase domain which is essential for signal transduction, is present in the cytoplasmic region of the receptor and a ligand binding domain is located in the extracellular region (Suzuki et al., 1994). (B) Structure of the truncated mutant BMPR-Ia (ΔBMPR-Ia). The predicted protein lacks the entire Ser/Thr kinase domain. (C) To produce a recombinant virus, the truncated BMPR-Ia cDNA was inserted into pDS5, a replication competent retroviral vector. To enhance translation, an IRES (Internal Ribosomal Entry Sequence) fragment from pCITE3 (Novagen) was joined to the initiation codon of the truncated BMPR-Ia cDNA. (D) Concentrated recombinant virus suspension was injected into the presumptive leg field of st. 10 chick embryos. (E) Histochemical staining of alkaline phosphatase activity of a stage-23 embryo which was infected with the recombinant virus carrying human placental alkaline phosphatase (Yokouchi et al., 1995). Efficient infection and expression of the inserted gene in the injected leg bud was demonstrated by this method. ECD, extracellular domain; TM, transmembrane domain.

al., 1994). We injected the recombinant viral particle suspension into the prospective right leg field of chick embryos at stage 10 (Fig. 1D) and allowed the embryos to develop to appropriate stages. By this procedure, the foreign gene contained in the recombinant virus was expressed in the mesenchymal cells of nearly the entire limb bud (Fig. 1E).

We analyzed the phenotypes at day 7.5 and day 10. On the left side limbs, the interdigital webs began to regress, as observed in normal development, at day 7.5 (Fig. 2A). In contrast, on the infected right side, ectopic mesenchymal bulges were formed at a region anterior to digit 1, and at a

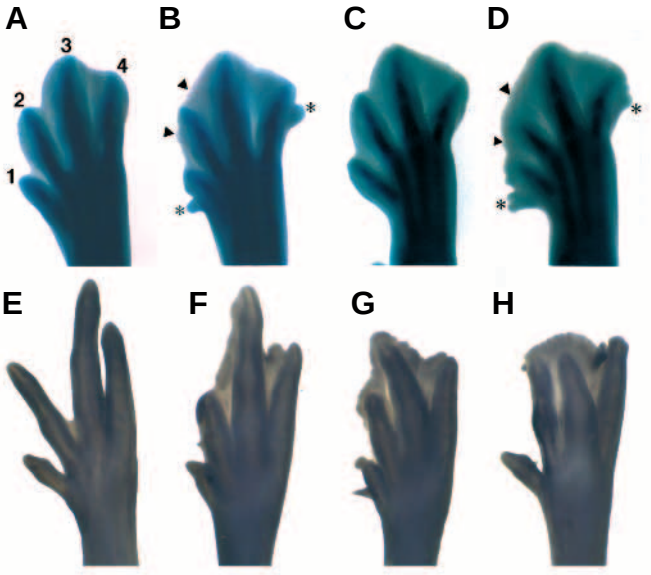

Fig. 2. Induction of extra webs in the leg buds following infection with the dnBMPR-Ia virus. (A-D) Leg buds at st. 32 (day 7.5) of development. (A,B) Dorsal view of control left leg (A) and virus-infected right leg (B). For ease of comparison, the control side was reversed photographically. In the infected side, ectopic bulge formation (asterisks) and suppression of web regression (arrowheads) were observed. (C,D) Dorsal view of another sample. The frequency of extra web formation was 17/30. No visible alterations were seen in limb buds infected with virus containing dnBMPR-Ia (data not shown). (E-H) Leg buds at day 10 of development. (E) Dorsal view of the control left side. (F-H) Dorsal view of the infected side. At this stage, interdigital webs completed regression (E), but on the infected side, webs remained in each interdigital region (F-H). The frequency of residual webs was 20/40.1-4, digit number.

region posterior to digit 4 at high efficiency (Fig. 2B,D asterisks, Table 1). Moreover, regression of the web was suppressed (arrowheads) and the digits were more separated from each other because of the presence of extra intervening tissues. At day 10, on the control side, complete regression of webs was observed in all of the interdigital regions (Fig. 2E). In contrast, on the infected side, webs that should have regressed normally remained in the interdigital region (Fig. 2F-H, Table 1). In embryos infected with the control virus, these morphological changes were not observed (data not shown, Table 1). The ectopic bulges formed at day 7.5 (Fig. 2B,D) and which remained as webs at day 10 did not contain any cartilaginous tissue (Fig. 2G). Thus, interruption of BMP-2/-4 signaling resulted in extra tissue formation in the leg buds. In addition, formation of the second and third phalanges was inhibited at high frequency (Fig. 2F-H). This indicates that BMP-2/-4 have different functions in cartilage formation in the digital region. It is possible that persistent webbing is a secondary consequence of a shortening of the digits. However this is unlikely because webbing was observed even in the specimen in which no significant reduction of digits was detected (Fig. 2F digit 3 and 4).

The truncated BMP receptor causes suppression of programmed cell death

As shown above, regions where the ectopic bulges and webs

Table 1. Effects of truncated BMP receptor Ia virus on the chick leg

	Number of virus injected limb buds	Morphology		Phenotype		
		Normal	Affected	Ectopic bulges in the ANZ/PNZ	Supression of web-regression	Maintenance of webs
stage 31						
IRES-ΔBMPRIa	19	11	8	8	8	*
control	10	10	0	–	–	*
stage 36						
IRES-ΔBMPRIa	31	15	16	3	–	15
control	10	10	0	–	–	–

To analyze the effects of the dnBMPR-Ia, infected limbs were dissected 6 or 9 days after virus injection and stained as described in Materials and Methods. Limbs showing any alterations in morphology were scored. As a control, virus carrying no insert was injected.

*Since the webs of the leg bud just begin to regress in the interdigital region at this stage of normal development, the effects of infection could not be determined.

were formed by overexpression of the dnBMPR-Ia correlated well with the regions where mesenchymal tissue was eliminated by PCD. The same phenotype was seen in the BrdU-applied limb bud, in which PCD has been suppressed (Tone et al., 1983). To analyze whether PCD was suppressed by overexpression of dnBMPR-Ia, the infected embryos were allowed to develop to day 7.5 and the dead cells were visualized by Nile blue vital staining (Tone et al., 1983). On the control side, large numbers of dead cells were detected in the ANZ, PNZ and INZs (Fig. 3A,C,E). On the infected side, no dead cells were detected within the ectopic mesenchymal bulges that formed in the ANZ and PNZ (Fig. 3B,E) and the number of dead cells in the INZs was reduced by half (Fig. 3B,D). We then examined the apoptotic cells in the INZs by the TUNEL method (Gavrieli et al., 1992). In the INZs of the control side, many labeled cells were found in the central part of the INZs (Fig. 3G,I). On the infected side, the number of labeled cells was reduced by half (Fig. 3H,J). No suppression of apoptosis was observed when the control virus was used (data not shown). These results demonstrate that dnBMPR-Ia suppresses apoptosis in the leg bud, strongly supporting the hypothesis that BMP-2/-4 mediates apoptotic signals.

Bmp-2/-4 expression and PCD in the leg bud

The results shown above indicate that BMP-2/-4 function as the apoptotic signal during limb development. We next analyzed the relationship between the spatial and temporal expression profiles of *Bmp-2* and *Bmp-4*, and progression of apoptosis in the leg buds.

Apoptotic cells in the ANZ were detected from st. 23 (day 3.5) to st. 32 (day 7.5; blue dots in Fig. 4A-E). Apoptotic cells in the PNZ appeared at st. 29 (day 6) and was present to st. 32

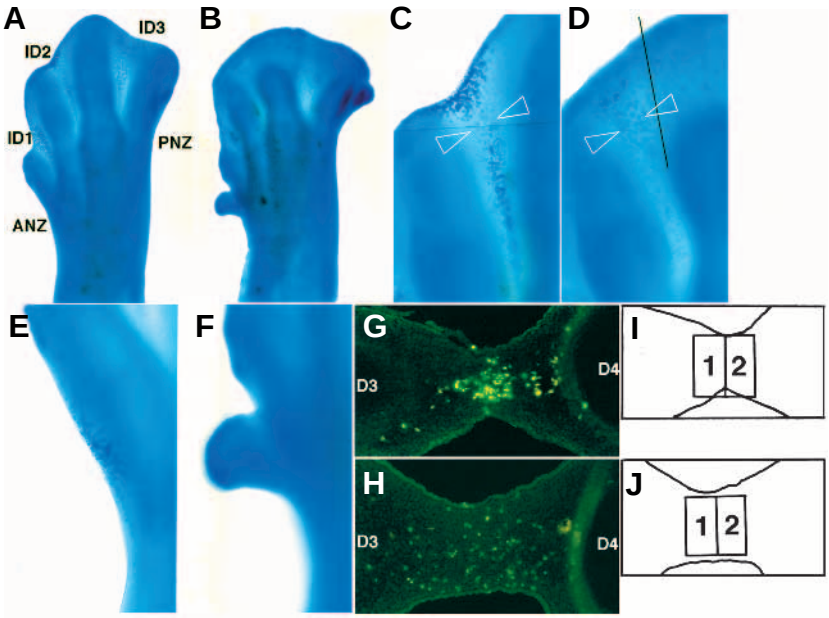

Fig. 3. The dnBMPR-Ia suppresses programmed cell death in the leg bud. (A-F) Assessment of cell death in leg buds using vital staining (Tone et al., 1983). (A,B) Dorsal view of a stained whole-mount control leg (A, reversed) and an infected leg (B) at day 7.5. (C,D) Higher magnification of ID2 of the control side(C, reversed) and of the infected side(D). (E,F) High magnification view of the ANZ in the control limb bud (E, reversed) and in the infected limb bud (F). In the control limb bud (A,C,E), many dead cells, stained by Nile blue, were observed in the ANZ, PNZ and INZs. In contrast, in the infected limb bud (B,D,F), a significant reduction in the number of stained cells was observed. Frequency of cell death suppression was 7/15. The number of dead cells in the distal region (peripheral region, distal to the two arrowheads) of ID2 in control limb (C) was 139 and that of the infected limb (D) was 74. The number of dead cells in the proximal region (proximal to the two arrowheads) of ID2 in control limb (C) was 123 and that of the infected limb (D) was 57. Thus the number of dead cells was reduced by half in the infected limb. For dead cell measurement in D, we counted Nile blue-stained cells in the area left of the line. The number of the dead cells in the ANZ of the control limb (E) was 111 but no dead cells were observed in the area corresponding to the ANZ of the infected limb (F). (G,H) Detection of apoptotic cells by the TUNEL method. Cross sections in interdigit 3 of the control side (G) and of infected side (H). Yellow-green signal shows the labeled apoptotic cells. I and J indicate the area for the apoptotic cell counting of the specimens in G and H respectively. For quantitative measurement of the apoptotic cells in ID3, we counted the number of apoptotic cells in the unit area of both control (I) and infected (J) limb. Numbers of apoptotic cells in compartment 1 and 2 of control ID3 were 38 and 39 respectively. In contrast, numbers of apoptotic cells in compartment 1 and 2 of infected ID3 were17 and 20 respectively. Thus numbers of apoptotic cells in infected interdigits was reduced by half. ID, interdigit.

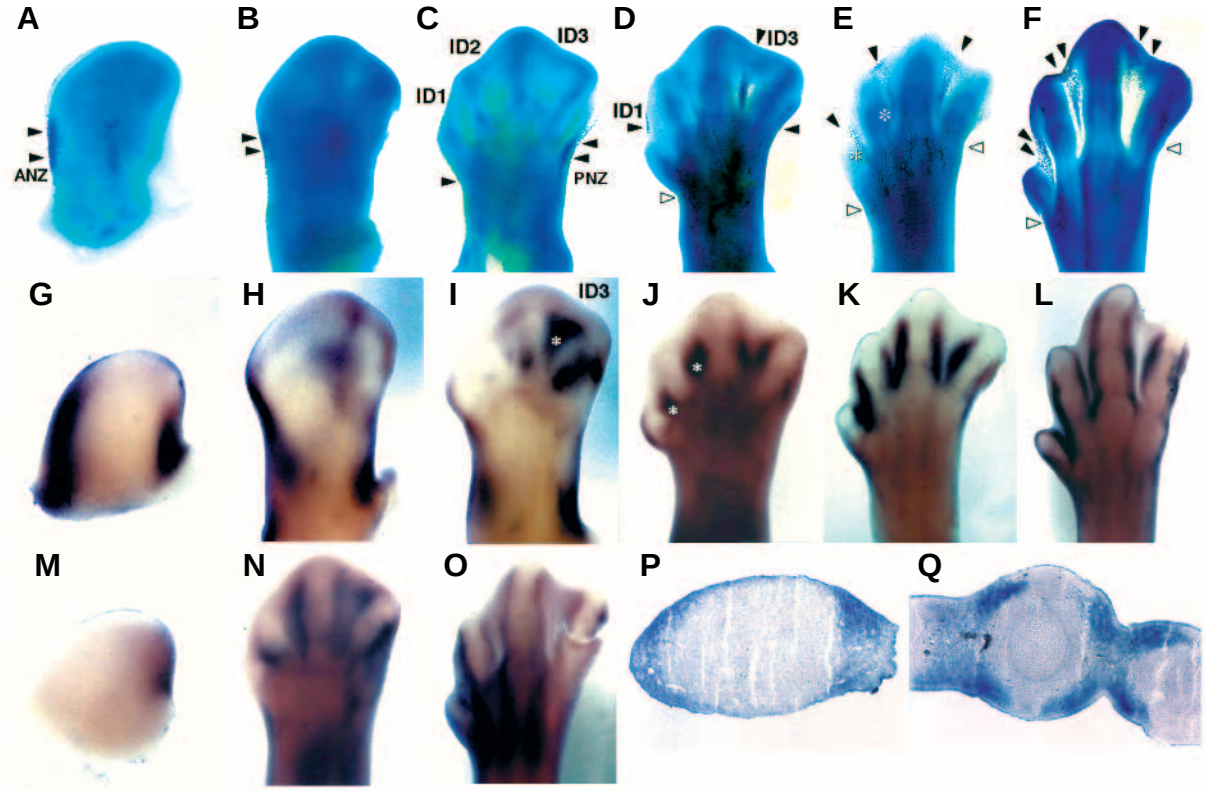

Fig. 4. Distribution of apoptotic cells and expression patterns of *Bmp-2* and *Bmp-4* in the developing chick leg bud. (A-F) Distribution of dead cells visualized as blue dots by Nile Blue vital staining at st. 23 (day 3.5; A), st. 25 (day 5; B), st. 29 (day 6; C), st. 31 (day 7; D), st. 32 (day 7.5; E) and st. 33 (day 8; F). Filled arrowheads show the areas of PCD. (G-L) Distribution of *Bmp-4* mRNA detected by whole-mount in situ hybridization at st. 22+ (day 3.5; G), st. 25 (day 5; H), st. 29 (day 6; I), st.30 (day 6.5; J), st. 32 (day 7.5; K) and st. 33 (day 8; L). At early stages, *Bmp-4* expression was found in the areas which later become ANZ and PNZ. At later stages, *Bmp-4* expression in the proximal ID3 at st. 29 (I, asterisk) preceded proximal apoptosis in the ID3 at st. 31 by 24 hours. (D). This expression of *Bmp-4* (J; asterisks) prior to the cell death was also observed in the proximal ID2 and ID1 from st. 30 to st. 32 (E). During apoptosis in INZ, *Bmp-4* was expressed in the regions adjacent to those showing apoptosis (K,L). (M-O) Whole-mount in situ hybridization for *Bmp-2* at st. 22 (day 3.5; M), st. 29 (day 6; N) and st. 31 (day 7.5; O). *Bmp-2* was expressed in the presumptive INZ(N). (P,Q) Cross-sections of legs hybridized with *Bmp-4* probes at st. 23 (P) and at st. 31 (Q). ANZ, anterior necrotic zone; PNZ, posterior necrotic zone; ID, interdigit. Open arrowheads indicate the positions of apoptotic cells in the ventral side which are not visible in this view.

(Fig. 4C-E). At st. 23, the *Bmp-4* transcripts were distributed in the mesoderm of the anterior marginal region and that in the posterior proximal region (Fig. 4G,P). From st. 25 (day 5) to st. 32, the expression domains overlapped with the ANZ and the PNZ (Fig. 4H-K).

From st. 31(day 7) to st. 33 (day 8), apoptotic cells appeared in the INZs. Each INZ is composed of two subregions, a proximal and a distal region and the order of appearance of which is strictly regulated (Pautou, 1975). At st. 31(Fig. 4D), the first apoptotic zone was detected in the proximomedian part of interdigit (ID)3 and the second zone in the distal part of ID1 6 hours later. Around st. 32 (Fig. 4E), the third and fourth zones were found in the proximomedian parts of ID2 and of ID1, and the fifth zone was in the distal part of ID2. At st. 33 (Fig. 4F), their domains expanded to encompass whole of the interdigital region.

During this period, the mesenchymal expression of *Bmp-4* preceded the appearance of apoptosis in the proximal interdigital region. At st. 29, *Bmp-4* transcripts were first detected in the proximal ID3 (Fig. 4I, asterisk), 24 hours prior to the beginning of cell death in the proximal ID3 at st. 31 (Fig. 4D). At st. 30, the *Bmp-4* transcripts were detected in the proximal ID1 and ID2 (Fig. 4J, asterisks), 24 hours prior to the appearance of cell death in the same regions at st. 32 (Fig. 4E. asterisks). In the course of PCD progression, the expression of *Bmp-4* in the proximomedial IDs, where apoptosis is progressing, was diminished. The *Bmp-4* expression continued in the mesenchymal cells surrounding the phalanges that are adjacent to the dying cells (Fig. 4K-L). At this stage, the *Bmp-4* transcripts were expressed in the mesoderm (Fig. 4Q).

Bmp-2 was expressed in all of the interdigital region at st. 29 (Fig. 4N) and continued at st. 32, the expression domain became biased distally (Fig. 4O). The expression of *Bmp-2* was weaker than that of *Bmp-4*. Thus, the expression domains of *Bmp-4* and *Bmp-2* precede PCD appearance and then overlap to PCD regions in the leg buds. These observations strongly support BMP-2/-4 mediation of apoptosis of the mesenchymal cells in the developing leg bud in vivo.

Factors influencing *Bmp-4* expression in the limb buds

In the tooth bud, ectodermal BMP-4 activate mesenchymal *Bmp-4* expression (Vanio et al., 1993). Moreover, mesenchymal expression of homeobox containing genes, *Msx1* and *Msx2*

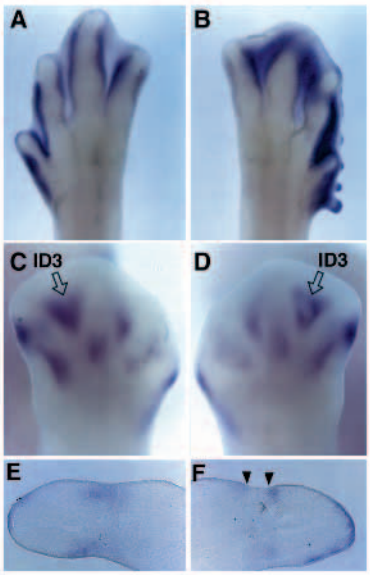

Fig. 5. Effects of overexpression of dnBMPR-Ia and of the removal of the ectoderm on *Bmp-4* expression. (A) Expression of *Bmp-4* in the leg bud of control side. At this stage, the medial expression of *Bmp-4* in each interdigital region disappeared, whereas *Bmp-4* is expressed in the mesenchymal cells surrounding the phalanges. (B) Expression of *Bmp-4* in the leg bud of the infected side. In addition to the expression in the mesenchymal cells surrounding the phalanges, the expression was observed in the medial region of the interdigit. (C-F) Expression of *Bmp-4* in the leg bud after removal of the dorsal ectoderm. The dorsal ectoderm of ID3 at st. 28 was removed (see Material and Methods) and expression of *Bmp-4* was analyzed 12 hours later. (C) Dorsal view of control side. Transcripts of *Bmp-4* were detected in the proximal part of ID3 (arrow). (D) Dorsal view of the experimental side. The *Bmp-4* transcripts disappeared in the ectoderm-removed mesenchyme (arrow). (E,F) Cross section of ID3 on the control side (E) and on the experimental side (F). On the experimental side the of *Bmp-4* transcripts in the dorsal half disappeared (between two arrowheads).

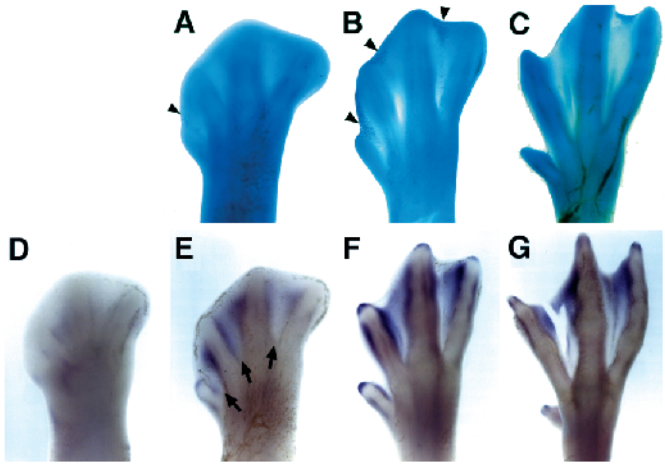

Fig. 6. Cell death and of *Bmp-4* expression in duck leg bud. (A-C) Distribution of dead cells visualized by Nile Blue staining at stages equivalent to chicken st. 31, st. 33– and st. 35–. In the interdigital regions of the duck leg bud at st. 31, first apoptotic cells were detected in the distal part of ID 1(A, arrowhead). At st. 33–, the cell death zone in the ID 1 expanded proximally and cell death occurred in the distal parts of ID 2 and 3 (B, arrowheads). At st. 35–, the entire ID 1 and distal parts of ID 2 and 3 were regressed by cell death (C). (D-G) Expression pattern of *Bmp-4* at st. 29, st. 31, st. 33 and st. 35. *Bmp-4* transcripts were not detected at st. 29 (D). At st. 31, *Bmp-4* transcripts were detected in the interdigital region except the proximal medial region (E, arrows) and this expression was still observed at st. 33 (F) and st. 35 (G).

were affected by ectodermal BMP-4 (Vanio et al., 1993). In the interdigital regions of the leg bud, the same genes are also expressed (Ros et al., 1994) suggesting the same regulatory cascade is conserved in the limb buds.

We analyzed whether *Bmp-4*, *Msx1* and *Msx2* expression in the leg buds were affected by the infection with the dnBMPR-Ia virus. On the control side, *Bmp-4* was expressed in the mesenchymal cells surrounding the phalanges in a 'V' shape because of the disappearance of *Bmp-4* transcripts in the apoptotic regions at st. 32 (Fig. 5A). In contrast, *Bmp-4* was expressed in both the mesenchymal cells surrounding the phalanges and the medial region of the interdigits on the infected side (Fig. 5B). This apparent ectopic medial *Bmp-4* signal was due to continuous expression of *Bmp-4* in the mesenchymal cells that was rescued from PCD pathway by the dnBMPR-Ia. The expression pattern of *Msx1* and *Msx2* was not affected by infection by the dnBMPR-Ia virus (data not shown). These results suggest that dnBMPR-Ia did not interrupt the autoregulatory circuit of *Bmp-4* expression, while it did suppress apoptosis.

Removal of the dorsal ectoderm of the interdigital region at st. 28 induces repression of apoptosis in the underlying mesenchyme in that region (Hurle and Ganan, 1986). This implies the involvement of the ectodermal signal in the expression of *Bmp-4* in the interdigital mesenchyme. To examine this possibility, we removed the dorsal ectoderm of ID3 of the leg bud at st. 28. At this stage, *Bmp-4* has just begun to be expressed in ID3 mesenchyme beneath the dorsal and ventral ectoderm. At 12 hours after the operation, in the control side, the expression domain of *Bmp-4* in ID3 expanded to central region (Fig. 5C,E). In contrast, in the operated side, dorsal expression of *Bmp-4* disappeared (Fig. 5D,F). This result suggests that the ectodermal signal is involved in the interdigital cell death through controlling the expression of *Bmp-4*.

Difference in *Bmp-4* expression between chick and duck limb buds

To ascertain whether *Bmp-4* is responsible for the difference in the death pattern between chick leg and duck leg, we analyzed the spatiotemporal expression profile of *Bmp-4* in the duck leg bud.

In the chick leg bud at st. 29, *Bmp-4* was first expressed in the proximal part of ID3 (Fig. 4I). In contrast, in the duck leg bud at the equivalent limb development stage, *Bmp-4* transcripts were not detected (Fig. 6D). In the chick leg bud at st. 31, *Bmp-4* was expressed in all of the proximal interdigital regions in a 'V' shape (Fig. 4K). In the duck leg bud at the equivalent stage, *Bmp-4* expression pattern was similar to that of chick except for the most proximal part of the V (Fig. 6E arrows). At this stage, cell death in the distal region of ID1 was detectable (Fig. 6A arrowhead). In the chick leg bud at st. 33, *Bmp-4* was expressed in the interdigital region except the distal and medial part where apoptosis is going on (Fig. 4L). In the duck leg bud at the equivalent stage, *Bmp-4* was expressed in

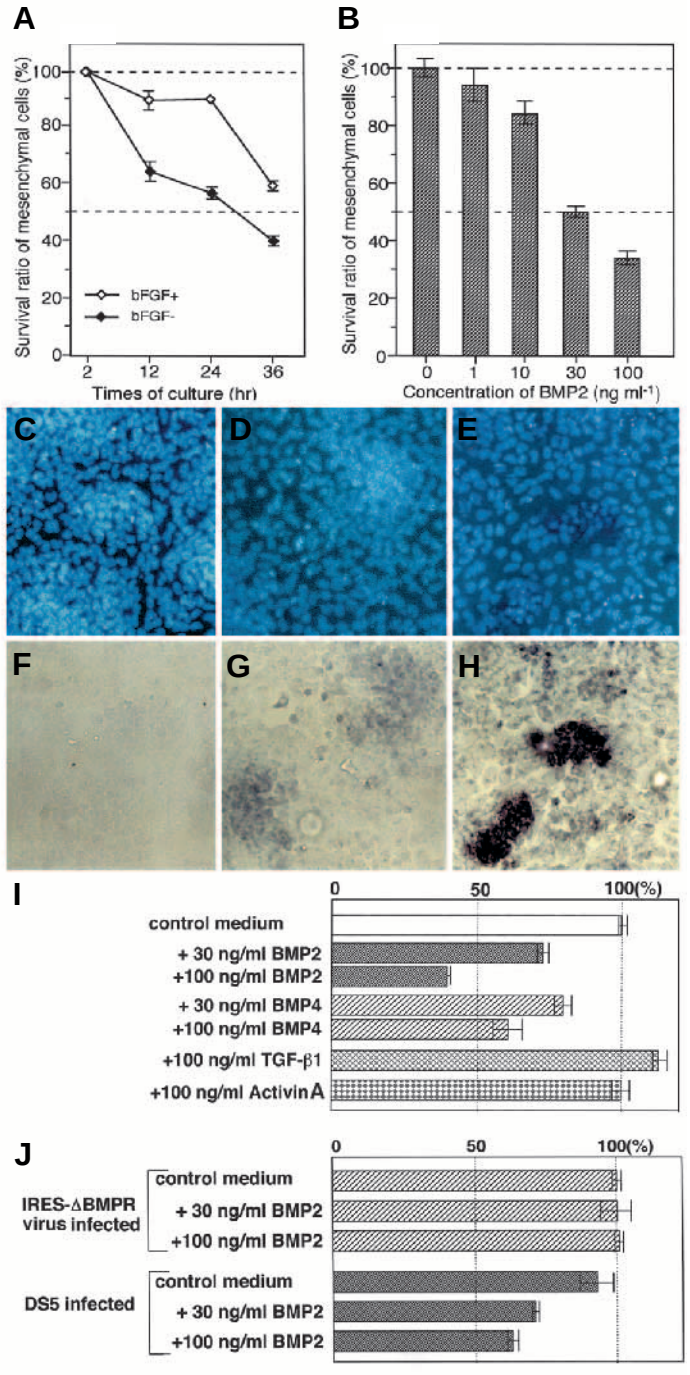

Fig. 7. BMP-2 and BMP-4 reduce the viability of the interdigital mesenchymal cells through stimulation of apoptosis in vitro. (A) Addition of 5 ng/ml of FGF-2 to F12 medium maintained 90% of the web mesenchymal cells for 24 hours in vitro (open box). In contrast, half of the cells were dead in F12 alone within 24 hours (filled box). (B) Effects of a range of concentrations of BMP-2 on the survival of web-mesenchymal cells. Attached cells died and peeled off the dish within 24 hours after addition of BMP-2. To count the remaining cells on the dish, the cultures were stained with DAPI. (C-E) DAPI staining of culture treated with BMP-2 for 24 hours. Cells were cultured in F12 supplemented with 5 ng/ml of recombinant FGF-2 (control medium; C), in control medium with 30 ng/ml of recombinant BMP-2 (D) or with 100 ng/ml of BMP-2 (E). (F-H) TUNEL staining of the apoptotic cells in culture with BMP-2. (F) Cells cultured in control medium. (G) Cells in control medium with 30 ng/ml or (H) with 100 ng/ml of BMP-2. (I) Effects of other members of the TGFβ protein family on the viability of the interdigital mesenchymal cells. (J) Effect of BMP-2 on control virus (no insert) or dnBMPR-1a virus infected mesenchymal cells from ID3 in culture (see Materials and Methods for experimental procedure).

the whole of the interdigit region except the distal part where cell death is progressing (Fig. 6B,F). At st. 35, when the distal apoptosis was completed in the duck leg (Fig. 6C), *Bmp-4* continued to be expressed in all of the webs (Fig. 6G). The later expression profile is inconsistent with the correlation of *Bmp-4* expression and cell death in the interdigital regions in leg bud. We will discuss this inconsistency later.

BMP-2/-4 proteins can induce apoptosis of the mesenchymal cells from the presumptive necrotic zone in vitro

To demonstrate directly that BMP-2/-4 are the apoptotic signals in the limb bud, we tested whether these proteins can induce apoptosis of the mesenchymal cells from the presumptive necrotic zone of the leg bud in vitro. We chose the mesenchymal cells derived from interdigit 3 at stage 29, which are programmed to die 24 hours later in vivo (Fig. 4C,D). Prior to this test, we established the conditions necessary to maintain these cells in vitro for 24 hours by adding 5 ng/ml of bFGF to the F-12 medium and by using collagen-coated dishes with no addition of serum (Fig. 7A). Under these conditions, we tested the effects of BMP proteins on the mesenchymal cells after 24 hours culture. Following addition of more than 10 ng/ml of BMP-2, reduction in viability was observed (Fig. 7B). After addition of more than 30 ng/ml of BMP-2, apoptotic cells were detected (Fig. 7F-H). BMP-4 also reduced the viability of the cells in the same range as BMP-2 (Fig. 7I), and this effect was attributed to apoptosis (result not shown). In contrast, other members of the TGFβ superfamily such as TGFβ1 and Activin showed no effect on cell viability (Fig. 7I).

In order to determine that dnBMPR-1a virus infected cells are not responsive to BMP, mesenchymal cells from ID3 were isolated and infected with the recombinant virus in vitro and analyzed for BMP sensitivity. The number of living cells was decreased after treatment with BMP-2 in control virus infected culture (Fig. 7J). In contrast, the number of living cells was not significantly changed in dnBMPR1a infected culture (Fig. 7J). This indicates that cells expressing dnBMPR-1a are not responsive to BMP.

These results suggest that BMP-2/-4 are the specific factors that mediate the apoptosis of the interdigital mesenchymal cells.

DISCUSSION

Expression of *Bmp-2/-4* starts at a very early stage of embryonic development (Winnier et al., 1995) and these genes are also expressed and have roles in many tissue interactions at later stages of development (Kingsley, 1994a,b). As *Bmp-2/-4* play crucial roles from the beginning of embryonic development, disruption of this system causes early embryonic

lethality (Mishina et al., 1995; Winnier et al., 1995). Use of local introduction of dominant negative type receptor for BMP allowed us to elucidate the function of BMP-2/-4 at later stages by mimicking a regional loss-of-function mutation.

Presence of BMP receptor type Ia and lb mRNA in the limb bud was confirmed by RT-PCR (results not shown). It was very difficult to visualize their mRNA by in situ hybridization indicating that these transcripts are not abundant in the limb bud. In contrast, transcription from the recombinant virus in the infected limb was very easily detected by in situ hybridization. In addition, we have shown IRES sequence efficiently enhances the translation of given mRNA in the infected limb mesenchymal cells (Yokouchi et al., 1995). These results indicate that the amount of truncated BMP receptor type Ia protein in infected limb mesenchymal cells could be in great excess compared to the endogenous normal type I receptor and sufficient to inactivate endogenous type II receptor forming an abortive complex as in the case of *Xenopus* embryos (Suzuki et al., 1994).

BMP-2 and BMP-4 are apoptotic factors in the developing limb bud

Introduction of the dominant negative BMP receptor resulted in formation of ectopic bulges both in the anterior and posterior sides of the leg bud and formation of ectopic webs in the interdigital regions. The regions in which extra tissues were formed correspond to those in which the mesenchymal tissues are eliminated by PCD in normal development. The programmed cell death in the ANZ, PNZ and INZs of the leg bud was suppressed by the dnBMPR-Ia. These results suggest that BMP-2/-4 mediate apoptosis in the necrotic zone of the developing limb bud. This is supported by the evidence that the expression of *Bmp-2/-4* and the distribution of the apoptotic cells is closely related, as discussed below (Fig. 4). Finally, we demonstrated that BMP-2 and BMP-4 proteins can induce apoptosis of the mesenchymal cells of the leg bud in vitro. These observations indicate that BMP-2 and BMP-4 are the apoptotic factors in the programmed cell death zone of developing limb bud. Recently Zou and Niswander (1996) demonstrated that overexpression of the dominant negative form of BMPR type Ib also induced suppression of PCD in limb bud. Their observations are similar to ours, however there are several differences and discussion of the differences provide more information on the function of BMPs and their receptors during PCD, as discussed below.

Mechanism of regulation of PCD in the leg bud by BMP-4

We observed the level of expression of *Bmp-4* was higher than that of *Bmp-2* in the INZs of the limb buds (Fig. 4I,K,N,O). In addition, BMP-4 protein can reduce the viability of the web mesenchymal cells (Fig. 7I) in the same dose range as BMP-2 in vitro. This suggests that BMP-4 is a major apoptotic signal in the leg bud. We also found that the expression of *Bmp-4* precedes apoptosis and continues in the same regions as apoptosis in the ANZ and PNZ (Fig. 4G). This result suggests that BMP-4 is necessary for both initiation and maintenance of the apoptotic state in the ANZ /PNZ in an autocrine manner. In contrast, during the apoptotic stage in the INZ, the spatiotemporal *Bmp-4* expression pattern is different from the ANZ/PNZ. The expression of *Bmp-4* precedes the proximomedial apoptosis in the interdigital regions (Fig. 4I,J), and was diminished in that region when the apoptotic cells appeared (Fig. 4K). These observations suggest that BMP-4 is necessary only for determination of apoptosis of the proximal INZ in an autocrine manner. BMP-4 may be involved in the maintenance of apoptosis of the proximal INZs in a paracrine manner because *Bmp-4* expression continued in the regions adjacent to the proximal medial apoptosis in the INZs (Fig. 4K,L).

It was reported that PCD in ID3 was inhibited if the overlying ectoderm has been removed (Hurle and Ganan, 1986). In this work we showed that this manipulation eliminated the *Bmp-4* expression in the interdigital mesenchymal cells (Fig. 5D). This result suggests that initiation or maintenance of mesodermal *Bmp-4* expression in the INZs is somehow mediated by the signal(s) from the overlying ectoderm. Considering the role of BMP-4 in PCD, this observation also gives the solution for the cascade of above phenomenon.

BMP-4 and web formation

Different avian legs have a variety of web pattern which depends on the cell death pattern in the interdigital region of the leg bud. In the chick leg bud, all of the interdigital regions suffer cell death so that no web is formed. In contrast, in the duck leg bud, the first interdigital region and only the distalmost parts of the second and third interdigital regions are removed by cell death and result in the formation of the webs in interdigit 2 and 3. As shown in this study, the expression of *Bmp-4* in the duck leg bud was not detected at st. 29 and began to express later than that of chick. Moreover, the proximal medial expression was not detected at st. 31. These spatiotemporal differences in *Bmp-4* expression are consistent with the difference of cell death pattern between chick and duck. These results suggest that the difference in the expression pattern of *Bmp-4* is essential for the formation of the interspecies differences in cell death pattern in the leg bud. In addition, curiously, *Bmp-4* was expressed in the web mesenchyme during the later stages nevertheless this region does not die. Perhaps, the component of the intracellular signal transducing system changes in later stages and BMP-2/-4 signal is used for controlling other biological systems, i.e. the phalangeal chondrogenesis, rather than cell death.

Heterochrony and morphological diversification

Considering the relationship between timing of *Bmp-4* expression and progression of cell death in the interdigital zone, initiation of *Bmp-4* expression seems to be the rate-limiting step for PCD. BMP-4 signaling seems to be required for the phalangeal chondrogenesis because introduction of both dnBMPR-Ia (this study) and dnBMPR-Ib (Zou and Niswander, 1996) resulted in phalangeal defects. As shown in Fig. 6E-G, *Bmp-4* is also expressed in the mesenchymal cells surrounding phalanges of both chick and duck leg bud indicating that BMP-2/-4 have a role in phalangeal chondrogenesis. These indicate that the *Bmp-4* gene has a *cis* regulatory element responsible for the phalangeal and ID expression, in both chick and duck, for proper development of the phalanges. As shown in Fig. 4I,J, *Bmp-4* expression in chick ID2 and 3 begins earlier than in duck ID2 and 3 (Fig. 6D). This indicates that the *cis* regulatory element responsible for timing for gene expression is changed in chick and this allows expression of *Bmp-4* earlier

than duck. This time point would be crucial for the interdigital mesenchymal cells to choose between proliferation or differentiation. Perhaps the presence of BMP-4 at this stage opens the pathway for cell death to the interdigital mesenchymal cells in such a situation. If this is true, it is a good example of local heterochrony for gene expression being a driving force for interspecies morphological diversification (Gould, 1977).

Autoregulatory signaling pathway and death signaling pathway of BMP-2/-4

The presence of an autoregulatory loop of *Bmp-4* expression in several tissues has been demonstrated and the involvement of *Msx* gene expression in this loop has also been shown (Vanio et al., 1993; Zou and Niswander, 1996). However, interruption of BMP-2/-4 signaling in the limb bud in this study altered neither *Bmp-4* nor *Msx* gene expression in the mesenchymal cells surrounding the phalanges. Zou and Niswander reported that interruption of BMP-4 signaling by dnBMPR-Ib represses both *Bmp-4* and *Msx2* (Zou and Niswander, 1996). There are two points of difference between our experiment and theirs. The first point is that we used BMPR-Ia and they used BMPR-Ib. The second is that in our case, the truncated form of BMPR-Ia does not have any cytoplasmic domain whereas in the case of BMPR-Ib a single amino acid change has been introduced in the kinase domain of the cytoplasmic region. The second point seems to be more crucial. Not so much has been reported on the factor(s) that interact with the cytoplasmic domain of BMPR, however several such molecules have been reported for the TGFβ receptor complex, which has similar receptor structure to the BMPR complex. Association of TRIP-1 to the TGFβ receptor is limited only when the receptor complex is formed then phosphorylated by S/T kinase in type I receptor (Chen et al., 1995). On the other hand, p21(RAS) farnesyltransferase alpha subunit associates only with type I receptor (Kawabata et al., 1995; Wang et al., 1996). In addition, it has been demonstrated that a certain domain in the cytoplasmic region is responsible for TGFβ dependent growth inhibition among many responses to TGFβ (Saitoh et al., 1996). These factors which interact with different domains of the receptor complex would be responsible for intracellular multiple signal transducing pathways. It is easily to imagine that BMPR complex also interacts with different intracellular factors responsible for individual signaling pathways. Complex formation of BMPR-II with kinase negative type Ib receptor which still has a cytoplasmic domain, blocks both the cell death signaling pathway and signaling for *Bmp-4* and *Msx* regulatory loop. The same truncated receptor used in our study (BMPR-Ia) blocks ventral mesoderm formation when introduced in early *Xenopus* embryos (Suzuki et al., 1994). This effect was not alleviated by addition of BMP-4, indicating that the effect is unlikely to be due to depletion of extracellular ligand but is due to interruption of BMP-2/-4 signal by forming abortive receptor complex (Suzuki et al., 1994). The same situation would also be true in limb mesenchymal cells infected by dnBMPR-Ia virus. So it is possible that the ligand binding extracellular domain and transmembrane domain of BMPR-I are enough to interrupt the cell death signal by forming the abortive complex with BMPR-II, whereas the *Bmp-4* and *Msx* regulatory loop is still activated by this form of BMPR complex. In summary, different results between two laboratories suggest that the intracellular signal transduction pathway for cell death and for regulating *Bmp-4* and *Msx* expression through BMPR complex is different in the limb bud.

Death signaling pathway

In the limb bud, the apical ectodermal ridge (AER) is the main source of FGF -2, -4, -8, and functional assays have shown that these FGFs can substitute for the AER to maintain limb outgrowth (Niswander and Martin, 1993; Fallon et al., 1994). At later stages of limb development, the AER undergoes flattening, loses its activity and FGF expression decreases significantly (Niswander and Martin, 1992; Dono and Zeller, 1994). At the same time, *Bmp-2/-4* expression increases in the ID mesenchyme then apoptosis occurs. In the early limb bud, BMP-4 was demonstrated to be antagonistic to FGF activity (Niswander and Martin, 1993). It is possible that the balance of FGF signal(s) and BMP signal(s) is also important for the balance of cell proliferation and differentiation in the later stages of limb development. Recently, the BMP-4 signal was reported to result in phosphorylation of MAPKK-p38 through activation of novel MAPKKK-TAK1 (Yamaguchi et al., 1995). Signal transduction to p38 also occurs in the apoptotic process in PC12 cells induced by withdrawal of the trophic factor NGF (Xia et al., 1995) whose receptor also possesses tyrosine kinase activity in the cytoplasmic domain similarly to the FGF receptor. Similar pathways may balance death or viability status in the limb bud.

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