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The role of bone morphogenetic proteins in vertebral development

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SUMMARY

This study first shows a striking parallel between the expression patterns of the *Bmp4*, *Msx1* and *Msx2* genes in the lateral ridges of the neural plate before neural tube closure and later on, in the dorsal neural tube and superficial midline ectoderm. We have previously shown that the spinous process of the vertebra is formed from *Msx1*- and *2*-expressing mesenchyme and that the dorsal neural tube can induce the differentiation of subcutaneous cartilage from the somitic mesenchyme. We show here that mouse BMP4- or human BMP2-producing cells grafted dorsally to the neural tube at E2 or E3 increase considerably the amount of *Msx*-expressing mesenchymal cells which are normally recruited from the somite to form the spinous process of the vertebra. Later on, the dorsal part of the vertebra is enlarged, resulting in vertebral fusion and, in some cases (e.g. grafts made at E3), in the formation of a 'giant' spinous process-like structure dorsally. In strong contrast, BMP-producing cells grafted laterally to the neural tube at E2 exerted a negative effect on the expression of *Pax1* and *Pax3* genes in the somitic mesenchyme, which then turned on *Msx* genes. Moreover, sclerotomal cell growth and differentiation into cartilage were then inhibited. Dorsalization of the neural tube, manifested by expression of *Msx* and *Pax3* genes in the basal plate contacting the BMP-producing cells, was also observed.

In conclusion, this study demonstrates that differentiation of the ventrolateral and dorsal parts of the vertebral cartilage is controlled by different molecular mechanisms. The former develops under the influence of signals arising from the floor plate-notochord complex. These signals inhibit the development of dorsal subcutaneous cartilage forming the spinous process, which requires the influence of BMP4 to differentiate.

Key words: chick embryo, *Msx* genes, BMP proteins, somitic differentiation, neural tube polarisation, vertebral cartilage formation, *Pax* genes

INTRODUCTION

In a previous study carried out on the avian embryo, we have shown that the vertebra is composed of two regions, the development of which obeys distinct regulatory mechanisms (Takahashi et al., 1992; Monsoro-Burq et al., 1994). These two regions are defined according to their position with respect to the ectoderm. The dorsalmost one, the spinous process, develops between the superficial ectoderm and the roof plate of the neural tube. The vertebral body and the neural arches are more ventrally located and generated under the influence of signals arising from the notochord and the floor plate (Pourquié et al., 1993; Brand-Saberi et al., 1993). The vertebral bodies and the intervertebral disks develop from *Pax 1* expressing sclerotomal cells (Ebensperger et al., 1995) which migrate ventrally and surround the notochord. The product of the gene *Sonic Hedgehog* (*Shh*) was shown to induce *Pax1* expression in early paraxial mesoderm in organotypic in vitro cultures (Fan and Tessier-Lavigne, 1994; Fan et al., 1995) or in vivo (Johnson et al., 1994). *Shh* was therefore proposed to play a role in ventralizing the paraxial mesoderm.

Although the notochord induces cartilage development from the ventral somitic mesenchyme, it has a strikingly different effect if grafted in a strictly dorsal position at 2 days of incubation (E2). Implanted just above the roof plate, the notochord does not hamper the dorsal migration of mesenchymal cells between roof plate and superficial ectoderm from E3 onward but prevents its differentiation into cartilage so that the neural arches remain open dorsally and no spinous process develops (Monsoro-Burq et al., 1994). The same result is obtained if the neural tube is rotated 180° dorsoventrally thus bringing the floor plate in contact with the mediodorsal mesenchyme (Takahashi et al., 1992). The lack of dorsal cartilage development is preceded by the absence of transcription of *Msx1* and *Msx2* genes which normally takes place in the dorsal neural tube, the mediodorsal mesenchyme and the overlying ectoderm, at stages preceding cartilage differentiation (from E2 to E6) (Takahashi et al., 1992; Monsoro-Burq et al., 1994, 1995).

It should be noted that expression of *Msx* genes takes place essentially in ecto-mesodermal rudiments such as the limb buds (Coelho et al., 1991a,b; Davidson et al., 1991; Robert et al., 1991; Yokouchi et al., 1991; Ros et al., 1992), the feather buds (Noveen et al., 1995) and a number of ecto-mesectodermal structures in

the head which are formed by superficial ectoderm and neural crest-derived mesenchyme (mesectoderm, see Couly et al., 1993) such as the cranial and facial skeletal structures (Takahashi and Le Douarin, 1990), and the tooth buds (MacKenzie et al., 1991a,b). In the chick, expression of *Msx2* by the neural crest-derived primordia of the mandibular membrane bones depends upon a signal arising from the superficial ectoderm of the first branchial arch. When separated from the ectoderm, the branchial arch mesenchyme stops expressing *Msx2* and fails to differentiate into membrane bones (Takahashi et al., 1991). In the mouse, complex inductive interactions between the *Msx2*-expressing dental ectoderm and the oral mesenchyme control the tightly regulated spatiotemporal pattern of *Msx* genes in teeth primordia explanted in vitro (Jowett et al., 1993).

Msx genes encode transcription factors (Catron et al., 1993; Hoffmann et al., 1994; Shang et al., 1994), and bone morphogenetic proteins (BMPs) have recently been proposed to be involved in the regulation of their expression. BMPs belong to the Transforming Growth Factor β superfamily and have primarily been isolated for their ability to induce cartilage and bone formation in subectodermic connective tissue in adult rats (Wozney et al., 1988). Among the various forms of BMPs identified so far, BMP2 and BMP4 proteins are most closely related to each other and to the *Drosophila* decapentaplegic (dpp) protein (Padgett et al., 1987). Moreover BMP2 and BMP4 bind to the same types of BMP receptors (Estevez et al., 1993; Koenig et al., 1994; Yamaji et al., 1994; Liu et al., 1995; Mishina et al., 1995; Rosenzweig et al., 1995). BMPs have been highly conserved during evolution; the human (hBMP2) and chicken (cBMP2) forms of BMP2 are 97% aa-identical. The *Bmp2* and *Bmp4* genes are expressed in several systems where *Msx* genes are also expressed under the control of ecto-mesodermal interactions. For example, in the chick, *Bmp2* and *Bmp4* expression was reported in the ectoderm and mesoderm of the branchial arches (Francis-West et al., 1994). In the mouse embryo, *Bmp4* is expressed in the dental ectoderm during the early stages of tooth formation and induces *Msx1* and *Msx2* expression in explants of dental mesenchyme (Vainio et al., 1993). In the chick neural tube, BMP4 has been implicated in the regulation of *Msx2* gene expression and neural crest cell death at the rhombencephalic level (Graham et al., 1994) and in the induction of *Msx* gene expression and neural crest formation in explants of lateral neural tube cultured in vitro (Liem et al., 1995).

Expression of BMP4 was recently recorded at early stages, from the primitive streak up to the 25-somite stage in chick and quail embryos, and shown to occur in the neural folds as they fuse dorsally during neurogenesis and, later on, in the dorsal neural tube and superficial ectoderm (Watanabe and Le Douarin, 1996). We then thought that BMP4 could be involved in the cascade of molecular events leading to the differentiation of subcutaneous cartilage. This hypothesis was substantiated by the fact that implantation of cells infected with a retroviral construct producing BMP4 laterally within the somitic mesenchyme was able to induce *Msx1* and *2* gene expression thus producing the enlargment of the scapula (Watanabe and Le Douarin, 1996). In view of these results, we have in the present work investigated the possible role of BMPs in the development of the vertebra.

First we established the relative patterns of expression of *Bmp4* and *Msx* genes in the region where the mesenchyme forming the spinous process of the vertebra accumulates on the dorsal aspect of the neural tube. We found that *Bmp4* is expressed dorsally in the ectoderm overlying the neural tube and the most medial part of the somite at the stages when somitic mesenchyme starts its migration dorsally to the neural tube. Then, we analysed the effects of BMP4/BMP2 on vertebral development by implanting cells producing these factors either dorsally or laterally to the neural tube of 2- or 3-day old chick embryos (E2 or E3). We found that the dorsal grafts promoted the development of extra pieces of superficial dorsal cartilage, resulting in the fusion of several vertebrae and in the enlargement of the spinous process especially when the operation was realised at E3. This enhancement of superficial cartilage formation followed the overgrowth of the dorsal mesenchyme which expressed *Msx1* and *Msx2* genes from E3 up to its differentiation into dorsal cartilage. The deep lateral implantation of BMP2/4-producing cells prevented the differentiation of the lateral parts of the vertebra, preceded by an absence of *Pax1* and *Pax3* gene expression and the strong induction of *Msx1* and *Msx2* genes around the graft. In the neural tube, this lateral implantation resulted in the ectopic expression of *Pax3, Msx1* and *Msx2* genes in the lateral and ventral parts of the spinal cord.

MATERIALS AND METHODS

Chick eggs from commercial sources (JA 57 strain, Institut de Sélection Animale, Lyon, France) were incubated in a humidified atmosphere at 38°C. The embryos were staged according to, either the number of somites formed, or the criteria of the developmental tables of Hamburger and Hamilton (HH) (1951).

Construction of recombinant retroviral plasmids encoding BMPs and production of BMPs-producing cells

Quail cells were transfected with two types of retroviral constructs: (i) transiently with RCAS BP (A) (Hughes et al., 1987) in which mouse BMP4 cDNA (mBMP4) was inserted; and (ii) stably with pCRNCM (de la Pompa and Zeller, 1993) carrying human BMP2 cDNA (hBMP2). Details of the construction of hBMP2-pCRNCM, mBMP4-RCAS and control constructs with cDNA inserts in the antisense orientation, were described by Duprez et al., (1996a,b). These vectors were shown to direct production of human BMP2 and murine BMP4 which are biologically active in the chick embryo (Duprez et al., 1996a,b; Pourquié et al., 1996).

The hBMP2-pCRNCM and antisense-control viral constructs were stably transfected into the packaging cell line Q2bn (Duprez et al., 1996a). The mBMP4-RCAS viruses were used to infect QT6 cells transiently as described by Pourquié et al., (1996). The viral constructs used have the advantage of not being able to infect chick cells of the strain we used as recipient for the grafts. Thus, the effect of BMPs can be spatially controlled since they remain circumscribed to the tissues adjacent to the graft without the *Bmp* gene randomly invading the host embryo. Infected cells were prepared as described by de la Pompa and Zeller, (1993) and Duprez et al., (1996a,b). Briefly, retrovirus-infected Q2bn and QT6 cells were grown to approximately 90% confluence in 90 mm culture dishes and were trypsinized and seeded into 90 mm bacteriological Petri dishes. Q2bn cells were then incubated in 50% DMEM-50% Ham's F12 medium (v/v) supplemented with 2% chicken serum plus 8% fetal calf serum (v/v), without neomycin (G418), while QT6 line cells were incubated overnight in DMEM containing 10% (v/v) fetal calf serum. Within 24 hours, the cells in each dish formed small spherical aggregates that were selected according to their size, washed in PBS with a thin micropipette and implanted into the embryos.

Microsurgery

Cell aggregates were implanted into embryos at 2 days of incubation

(E2) of 8- to 24-somite stage after making a slit in the ectoderm at the unsegmented level. Dorsal grafts were placed above the closing neural tube (Fig. 1A), lateral grafts were inserted between the neural tube and the somites (Fig. 1B), as described by Monsoro-Burq et al., (1994). Some embryos were also grafted dorsally at E3 (stages 19-20HH) (Fig. 1C). In this case, the somite was already formed and the dermomyotome clearly visible laterally to the neural tube. At that stage the territory located dorsally to the neural tube has not yet been invaded by mesenchymal cells. Thus, insertion of the BMP-producing cell aggregates between the neural tube and the ectoderm did not directly interfere with somite development.

As shown by in situ hybridization, hBMP 2 or mBMP4 RNAs were strongly expressed by the grafted cells, which remained grouped together at the graft site (see Figs 5, 6). However, the position of grafts which were initially strictly dorsal were found in certain cases to be slightly modified. The grafted cells were then in dorsolateral positions, thus allowing the effects of lateral and of dorsal grafts to be analysed on the same embryo and sometimes on the same sections.

104 out of the 240 operated embryos that received hBMP2-pCRNCM-Q2bn or control-pCRNCM-Q2bn cells survived and were studied. 70 were killed between E2.5 and E6 for in situ hybridization analysis and 34 at E8-9 to examine cartilage and bone differentiation. The embryos grafted with the mBMP4-RCAS-QT6 cells were analysed by in situ hybridization at E3 for gene expression (n=3) and at E9 for skeletal formation (n=6).

In situ hybridization

The quail *Msx2* probe was cloned in the laboratory (Takahashi and Le Douarin, 1990), the chick *Msx1* probe was a generous gift from H. R. Suzuki (Suzuki et al., 1991). *Bmp2* and *Bmp4* probes were cloned by Francis et al. (1994). The *Pax3* probe (660 bp *Eco*RV cDNA fragment) used was a generous gift from P. Gruss, the *Pax1* probe from R. Balling, and the choline acetyl-transferase (*ChAT*) probe from S. Pfaff.

Non-radioactive in situ hybridization on whole embryos was performed as described by Henrique et al. (1995) on E2-3 embryos. The *Msx1*- and *Msx2*-specific fragments were prepared as described by Monsoro-Burq et al. (1995); the *Bmp2* and *Bmp4* probes were prepared as described by Francis et al. (1994). The RNA probes were labelled with digoxigenin-UTP nucleotides with a Promega RNA synthesis kit and revealed with an anti-digoxigenin antibody (Boehringer). Radioactive in situ hybridization was performed as described by Monsoro-Burq et al. (1995) on serial paraffin sections. Adjacent sections were alternatively treated with different probes or antibodies allowing several markers to be used on the same experimental or control embryo.

Immunocytochemistry

Paraffin sections were treated as described by Monsoro-Burq et al. (1995). The QCPN (for Quail non-Chick PeriNuclear antigen from Developmental Studies Hybridoma Bank) monoclonal antibody (mAb) was used to localize the quail-grafted cells on sections adjacent to those treated for radioactive in situ hybridization. The 13F4 mAb recognizes all types of muscle cells from an early developmental stage (Rong et al., 1992). The anti-BEN mAb recognizes motoneurons and floor plate in the avian neural tube (Pourquié et al., 1992).

Skeleton staining

Cartilage and bone were stained in toto in E8-9 embryos, as described by Monsoro-Burq et al. (1994) using Alcian blue and Alizarin red staining for cartilage and bone respectively, after KOH clearing of the non-skeletal tissues.

RESULTS

Early expression of *Bmp* and *Msx* genes

We first compared the patterns of expression of the *Msx1* and *2* genes to that of *Bmp2* and *4* genes during neural tube formation at the trunk level of normal embryos, from E2 (HH stage 3) to E3 (HH stage 21). At the 8- to 10-somite stage (HH stage 9+/10), *Msx1* and *Msx2* genes were expressed all along the dorsal neural tube, whether it was closed (anteriorly) or not (posteriorly to somite 10), down to the level of Hensen's node (Fig. 2A,B). *Bmp4* was expressed at high levels in the hindbrain (not shown) as noted by Graham et al. (1994) and at lower levels in the trunk, especially in the caudal parts of the neural tube and in the neural plate (Fig. 2C). The three genes were expressed at the tips of the neural folds in the neural plate (Fig. 2). The dorsal neural tube and the superficial dorsal ectoderm were also strongly labelled (Fig. 2). At E3, *Msx1, Msx2* and *Bmp4* were expressed all along the trunk in the dorsal neural tube, i.e. all three genes in the roof plate plus the alar plates for *Msx1* (Fig. 2D-F and Monsoro-Burq et al., 1995); the superficial ectoderm was also labelled by the three probes. At E3, at the levels where the somites were well differentiated into dermomyotome and sclerotome (e.g. the wing level of a stage 20 (HH) embryo, Fig. 2F-H), *Bmp4* was expressed in the ectoderm covering the medial region of the dermomyotomes (Fig. 2H). The dorsal mesenchyme migrating at E3.5-4 under the *Bmp4*-

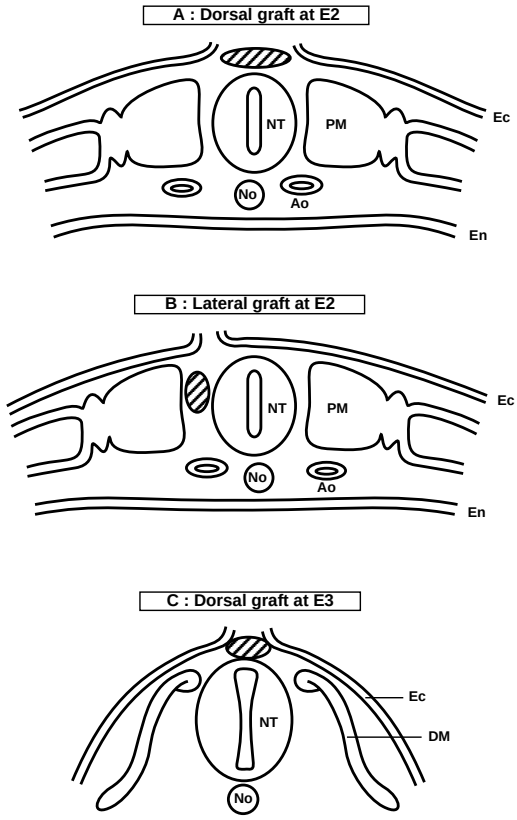

Fig. 1. Scheme of the grafts. The transfected cell aggregates (hatched) were implanted in the embryo through a slit made in the ectoderm. (A) Dorsal grafts at E2 were inserted above the neural tube in the unsegmented area. (B) Lateral grafts were inserted at E2 between the neural tube and the paraxial mesoderm of the unsegmented part of the embryo. (C) Dorsal grafts at E3 were implanted above the neural tube at the neck level (segmented area). Ao, aorta; DM, dermomyotome; Ec, ectoderm; En, endoderm; NT, neural tube; No, notochord; PM, paraxial mesoderm.

positive ectodermal area expressed *Msx1* and *Msx2* (Monsoro-Burq et al., 1995) but not *Bmp4* (not shown). *Bmp2* expression was visible in the limb bud, the branchial arches and the tail bud, but no signal was detected in the dorsal area at any stage considered in this study.

BMPs affect vertebral development

The BMP-producing (or control) cells were implanted dorsally to the neural tube of E2 embryos (Fig. 1A). We first observed that grafting an aggregate of control cells, even of a large size, did not perturb normal skeletal development (*n*=5) (Fig. 3A). When mBMP4- or hBMP2-expressing cells were grafted dorsally to the neural tube, ectopic cartilage differentiated, and this resulted in the dorsal fusion of several consecutive vertebrae (*n*=16/22 for hBMP2-producing cell grafts, 3/6 for mBMP4-producing cell grafts; Fig. 3B-F). With the *mBMP4* construct, cartilage pieces formed were of relatively small size, while the effect was more pronounced with hBMP2-expressing cells. In some cases a large hypertrophy of the vertebral spinous process was produced at the graft site (Fig. 3G-J). This difference is likely to be due to the higher level of BMP protein produced under the control of CMV promoter in the pCRNCM viral construct (de la Pompa and Zeller, 1993). Thus, the following study was essentially based on experiments involving either lateral or dorsal grafts of hBMP2-pCRNCM-Q2bn cells.

The alterations of cartilage development varied according to the graft site. After a strictly dorsal graft, cartilage hypertrophy could affect two to three consecutive vertebrae (Fig. 3B-F), thus forming a continuous dorsal cartilaginous plate over the vertebral arches (*n*=9), while normal, separate spinous processes develop. In two embryos, operated on at E3, the dorsally fused area formed a large spinous process-like piece of cartilage (Fig. 3G-J). The vertebral body and the lateral neural arches were normal in all cases. In contrast, when the hBMP2 cells were implanted laterally between the neural tube and the somite in a deep position, profound deletions were seen in the vertebral bodies and in the neural arches of two to four consecutive vertebrae (*n*=3) (Fig. 4A), whereas the development of their dorsal spinous processes was not significantly altered.

Finally, when the laterally grafted cells occupied both a superficial and deep lateral position, extra pieces of cartilage were induced in the dermis laterally to the vertebral column, while the deep neural arches and the ipsilateral part of the vertebral body were missing (*n*=5; Fig. 4B,C). When the hBMP2 graft encompassed both the dorsal and the lateral positions, hypertrophy and fusion of the spinous processes coexisted with large deletions of the lateral and ventral parts of the vertebra (*n*=2; Fig. 4D-F).

One can therefore conclude from these observations that the effects of BMPs vary in a spectacular manner according to subtle changes in their position with respect to the superficial

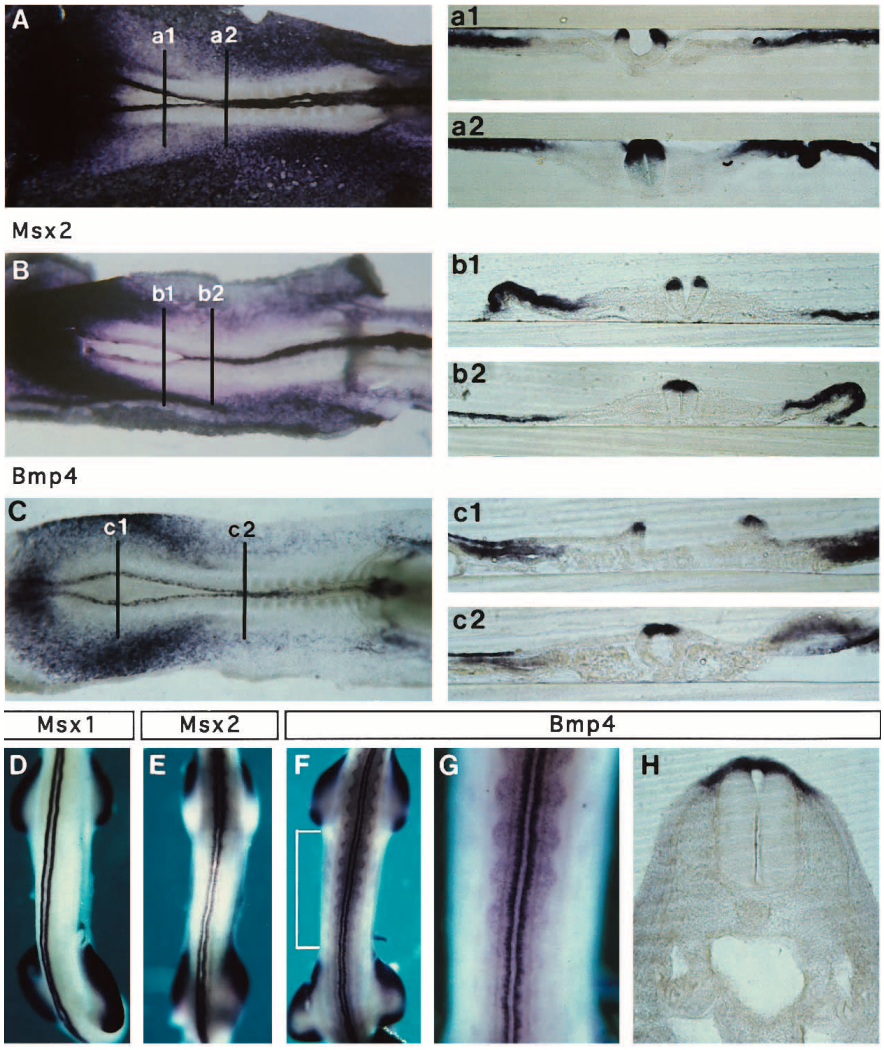

Fig. 2. Expression of *Msx1*, *Msx2*, *Bmp4* in normal E2-E3 embryos. Expression of the three genes was compared by in situ hybridization analysis on whole embryos. At 10-somite stage, at the trunk level, *Msx1* (A), *Msx2* (B) and *Bmp4* (C) genes were expressed in the neural folds at the level where the tube is not yet closed (a1, b1, c1) and in the dorsal neural tube and superficial dorsal ectoderm in more anterior regions (a2, b2, c2). Laterally, the somatopleure and the ectoderm were also labelled. (A,B,C) dorsal views of the embryos; rostral side on the right; the levels of the sections a1-c2 are indicated. At E3 (Stage 19-20 HH), the dorsal spinal cord and ectoderm expressed *Msx1* (D), *Msx2* (E) and *Bmp4* (F). Anteriorly, from the level of the 4th somite visible, up to the mid-trunk level of the stage 20 HH embryo, *Bmp4* gene expression expanded laterally (G, enlargement of the boxed area shown in F). This expression expands progressively from rostral to caudal in the dorsal ectoderm covering the most median region of the dermomyotome to the dorsal midline at the level of each somite. Note (in H) that *BMP4* expression is restricted to the dorsal area above the intersomitic spaces. (H) Transverse section (60 μm) at the wing level. Only the ectoderm is labelled, no mesenchyme being present dorsally at this stage and level. Scale bars, (A,B) 500 μm; (C) 400 μm; (a_1-c_2, H) 120 μm; (D) 700 μm; (E) 732 μm; (F) 770 μm; (G) 250 μm.

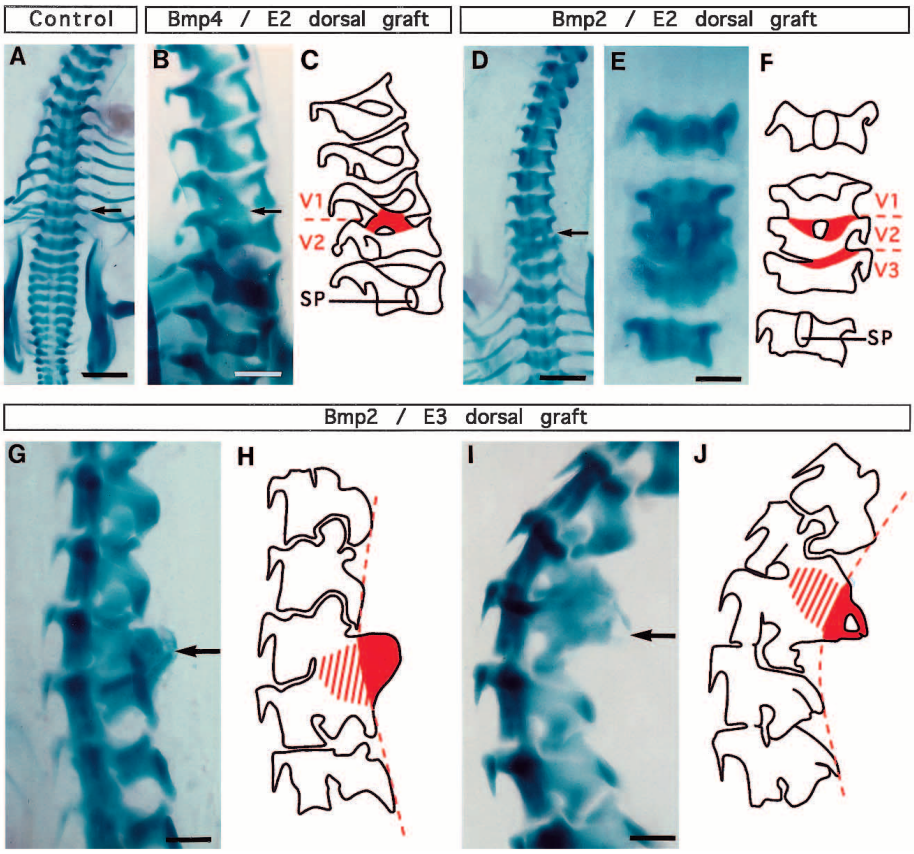

Fig. 3. Dorsal graft of BMP-producing cells induce ectopic superficial cartilage formation. The cells were grafted dorsally above the neural tube at E2 and E3 (see Fig. 1). The skeleton was observed at E9. After the graft of control cells (A), the vertebral column was normal at the level of the operation (arrow) (A: dorsal view). The dorsal graft of BMP4-producing cells at E2 (B,C) resulted in the partial fusion of two consecutive vertebrae V1 and V2 (arrow) as schematized in C (the ectopic cartilage of the fused area is colored red). The spinous process (SP) indicates the dorsal midline (B: dorsolateral view). The dorsal graft of BMP2-producing cells resulted in larger fusions of the dorsal vertebral cartilage (D,E,F). The three consecutive fused vertebrae (V1,V2,V3) of the embryo presented in D (dorsal view), were dissected (E), and the fused areas drawn in red in scheme F. When the BMP2-producing cells were implanted dorsally at E3 at the neck level, we observed the fusion of two consecutive vertebrae and the development of a large spinous process-like piece of cartilage (arrow in G,I) (G,I) Side views; the spinous processes are on the right, the vertebral bodies on the left, the fused area is hatched in red in H and I; the ectopic spinous process-like cartilage located above the fused area is colored red H and I. The red dotted line indicates the level of normally developed spinous processes. Scale bars, (A, D) 1.5 mm; (B, G, I) 0.7 mm and (E) 0.5 mm.

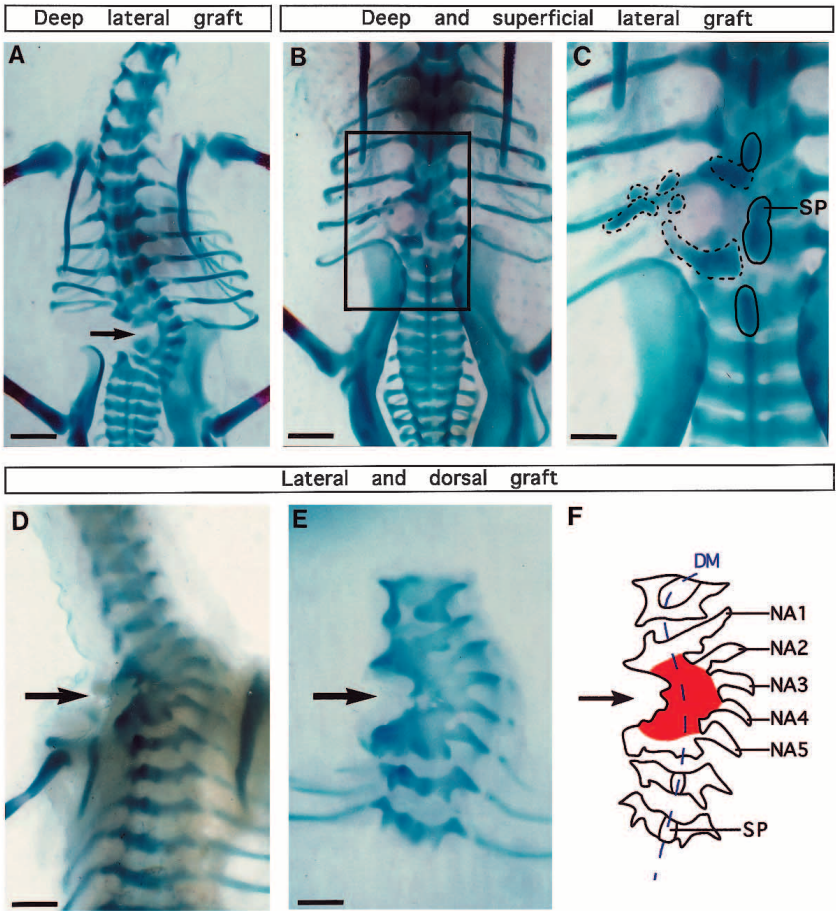

Fig. 4. Lateral BMP2-producing cells grafts**.** The effect of the BMP-producing cells was analysed after lateral grafts between the neural tube and the somite at E2. The effects varied according to the position of the graft towards the ectoderm. (A) Deeply implanted cells prevented the lateral parts of the vertebrae from forming along the length of several consecutive vertebrae (arrow in A: dorsal view of the spinal column) whereas control grafts were normal (not shown). The spinous processes of the affected vertebrae formed normally. (B,C) If the grafted cells were located laterally, in deep as well as in superficial positions, the absence of lateral cartilage was accompanied by the development of several pieces of ectopic superficial cartilage, fused or not with the spinous processes, under the ectoderm (B: dorsal view of the spinal column, C: the area boxed in B is enlarged in C, the ectopic cartilage pieces are outlined with a dashed black line, the spinous processes with black lines). (D,E,F) When the BMP2-producing cells were placed in lateral and dorsal positions, we observed a lack of the lateral parts of the vertebrae (arrow) whereas the dorsal area was formed by the fusion of 5 vertebrae presenting distinct neural arches (NA1 to NA5) on the contralateral side (D: dorsal view; E: dissected vertebrae; F: the fused areas are colored red). The spinous processes (SP) and the fused area are located in the dorsal midline (DM), drawn in blue. Scale bars, (A,B,D) 1.4 mm and (C,E) 0.7 mm.

ectoderm, the dorsal or ventral parts of the somite (dermomyotome or sclerotome) and the neural tube. We therefore decided to explore the effect of these signalling molecules earlier in development on the expression of genes known to play a role in patterning both the paraxial mesoderm and the neural tube.

Local effect of BMP2 on gene expression in the paraxial mesoderm derivatives and in the neural tube

The embryos were analysed from E3 (24 hours after grafting) to E6 by in situ hybridization with the following probes and antibodies: *Msx1* and *Msx2*, which normally label neural tube, dorsal ectoderm and dorsal mesenchyme (Takahashi et al., 1992; Monsoro-Burq et al., 1995); *Pax1* and *Pax3*, which normally label the sclerotome and the dermomyotome respectively (Goulding et al., 1994; Ebensperger et al., 1995); 13F4 mAb (Rong et al., 1992) to assess muscle cell differentiation. The dorsoventral patterning of the neural tube was monitored under the experimental conditions by looking at dorsal markers *Msx2* (normally expressed in the roof plate), *Msx1* and *Pax3* (normally expressed in the roof and alar plates), the motoneuron marker *ChAT* (Monsoro-Burq et al., 1995) and the motoneuron and floor plate marker anti-BEN mAb (Pourquié et al., 1992). Lateral or dorsal implantation of control cells did not perturb either the development of neural and somitic structures or the expression of the markers (n=12, data not shown).

Dorsal implantation of hBMP2-producing cells

25 embryos that received hBMP2-expressing cells dorsally at the 8- to 24-somite stage were examined (Figs 5, 6). The grafted cells located dorsally above the neural tube were covered by the healing host ectoderm. Sections treated for in situ hybridization with a probe for the *hBMP2* gene showed that the gene was expressed exclusively by the grafted quail cells and that the chick strain used as the host (JA57 strain) is not susceptible to infection by the virus used in this study (Fig. 5E).

Perturbations of gene expression were observed after the graft of hBMP2-expressing cells and varied with the amount of implanted cells. Small-sized grafts had no effect either on gene expression or on subsequent morphological development. In contrast, larger grafts placed on top of the roof plate at E2 induced spectacular reactions in the mesenchyme located dorsally to the neural tube. At E4, the graft induced a large hypertrophy of the dorsal mesenchyme which strongly expressed *Msx1* (n=4) (not shown) and *Msx2* (n=7) genes (Fig. 5B). This effect was observed up to E6, the stage when the normal *Msx* gene expression starts to disappear as cartilage differentiation begins in the dorsal part of the vertebra (n=5) (Fig. 6A-F). Moreover, at E3, an abnormal expression of *Msx2* occurred in the dorsomedial region of the dermomyotome which also expressed *Pax3* as in normal embryos (not shown). *Msx2* expression in the dermomyotome was transient and had disappeared at E4. At this stage, the dermomyotome still expressed *Pax3* and was pushed laterally by the hypertrophy of the *Msx*-expressing dorsal mesenchyme (Fig. 5A,F) and the sclerotome expressed *Pax1* normally (not shown). At E6-7, formation of muscle masses (*Pax3*- and 13F4-positive) and of *Pax1*-positive sclerotome was not perturbed (n=6) (Fig. 6H,I). In the spinal cord, *Msx* gene expression was increased in the dorsal neural tube, especially at late stages, when compared to controls (Fig. 6A-F). Expression of the ventral markers such as the *ChAT* gene (Fig. 5C) and BEN protein by motoneurons (not shown) remained unchanged.

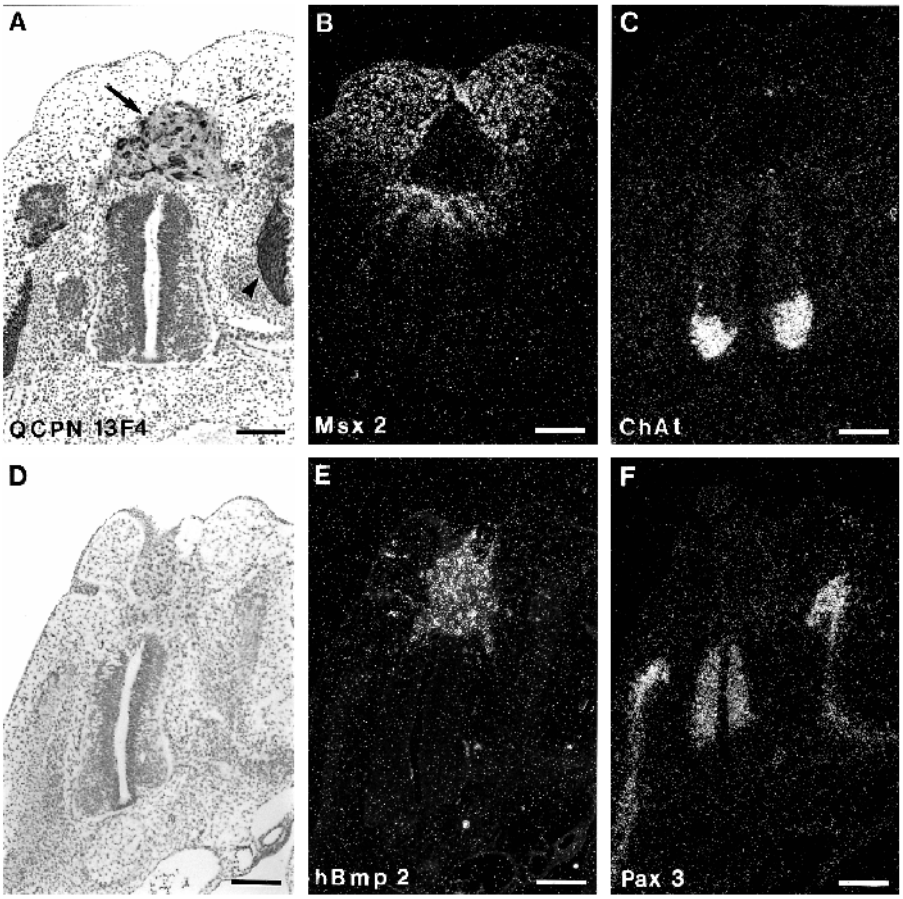

Fig. 5. Effects of dorsal grafts of BMP-producing cells analysed at E4. The BMP2-producing cells were grafted dorsally at E2. Transverse sections of E4 embryos show that the grafts remained located on top of the neural tube. In A, the grafted cells, labelled in black with QCPN mAb (arrow), are located on top of the neural tube and still strongly express the *hBMP2* gene (E); the myotomes, labelled in grey with 13F4 mAb, are seen on each side of the neural tube in A (arrowhead). The graft is surrounded by overdeveloped dorsal mesenchyme, strongly expressing *Msx2* (B). The roof plate is *Msx2* positive. The motoneurons, labelled by the *ChAT* probe, are normally developed (C). Laterally to the neural tube, the dermomyotomes are present (A,F) and express *Pax3* normally (F). A,B,C and D,E,F are adjacent sections. A,D, bright fields; B,C,E,F, dark fields. Scale bars, 120 μm.

Effect of the lateral implantation of BMP-producing cells on the paraxial mesoderm

When hBMP2-producing cells were implanted laterally to the neural tube (i.e. between the neural tube and the paraxial mesoderm) in the unsegmented region of 8-24 somite stage embryos, development of somitic derivatives was deeply perturbed. At the most affected levels, the dermomyotome did not form and *Pax3* expression was either absent or restricted to a few dispersed cells (Fig. 7G,H). As early as 24 hours after grafting, *Pax1* transcripts were absent in the mesenchymal somitic cells located in the area normally occupied by the sclerotome (Fig. 7I,J), this effect being still observed at E5 (Fig. 7K-L). The overall somite size was reduced on the grafted side. In contrast, *Msx1* and *Msx2* were strongly expressed ectopically in the somitic mesenchyme closely apposed to the graft in embryos at E2.5-E4 (n=14) (Fig. 7A-D). The expression was seen around the grafted cells, that is in superficial as well as in deep positions, while during normal development (as in the contralateral non-operated side) *Msx* genes are expressed only superficially, between the neural tube and the ectoderm. Although in most cases strongest expression was seen between the grafted cells and the superficial ectoderm, the ectoderm was not necessary to obtain *Msx* expression, which always occurred in close proximity to the graft. The induction of *Msx1* and *Msx2* genes was also observed after implanting mBMP4-expressing cells (n=3) (not shown).

In conclusion, the lateral implantation of hBMP2, performed at E2 in the non-segmented area, led to the ectopic induction of *Msx* gene expression in the somitic cells, while preventing expression of the normal somitic developmental program. These effects resulted later in the lack of the lateral and ventral parts of the vertebrae and to hypertrophy of their dorsal aspect.

Effect of lateral implantation of hBMP2-producing cells on the neural tube

The grafting of hBMP2-expressing cells induced the expression of *Msx1*, *Msx2* and *Pax3* in the lateral and ventral regions of the neural tube, in areas which normally do not express these genes (Fig. 8). Examination of 12 embryos showed that a close contact between the graft and the neural epithelium was required for the induction to occur. Moreover, *Pax3*, *Msx1* and *Msx2* could be expressed by the basal plates themselves when the graft position was ventral, whereas the floor plate was always excluded from the induced area. The expression of dorsal genes resulted in the lack of motoneurons, as revealed by the anti-BEN antibody (Fig. 8G); the floor plate and the contralateral basal plate were normally BEN-positive. Thus the lateral BMP grafts induced the dorsalization of the neural tube in vivo.

DISCUSSION AND CONCLUSIONS

The observation that in the avian embryo *Bmp4* is expressed in the neural folds before the neural tube closes and is later maintained in the superficial dorsal ectoderm and in the dorsal part of the neural tube at early stages of avian development has been made by several authors (Liem et al., 1995; Watanabe and Le Douarin, 1996). In the present study we have shown that in the trunk region, at E2, expression of *Bmp4* is parallel to that of *Msx* genes in the neural tube and the ectoderm. Moreover, at E3 (HH stage 20), we show here that *Bmp4* expression expands laterally in the ectoderm overlying the neural tube and the dorsal part of the somites. Thus, the somitic mesenchyme, that migrates above the neural tube shortly after this stage, is likely to receive the BMP4 signal. This dorsal mesenchyme expresses *Msx* genes which have previously been proposed to play a role in the development of the dorsal part of the vertebra (Takahashi et al., 1992; Monsoro-Burq et al., 1994). Several reports have revealed that BMP4 can induce *Msx* gene expression, this is the case, for example, during tooth morphogenesis (Jowett et al., 1993). Moreover BMP4-producing cells grafted into the paraxial mesoderm in a lateral position were shown to induce *Msx1* and *Msx2* in the subectodermal mesenchyme, resulting in the enlargment of the scapula (Watanabe and Le Douarin, 1996).

The work presented here was aimed at investigating the role of BMP4 and its closely related factor BMP2 in the cascade of molecular events leading to the formation of the spinous process of the vertebra.

We used two types of viral constructs harboring either mBMP4 or hBMP2 and producing these signalling molecules. Cell lines containing either of these molecules turned out to be active in the in vivo assay that we devised. The hBMP2-pCRNCM-express-

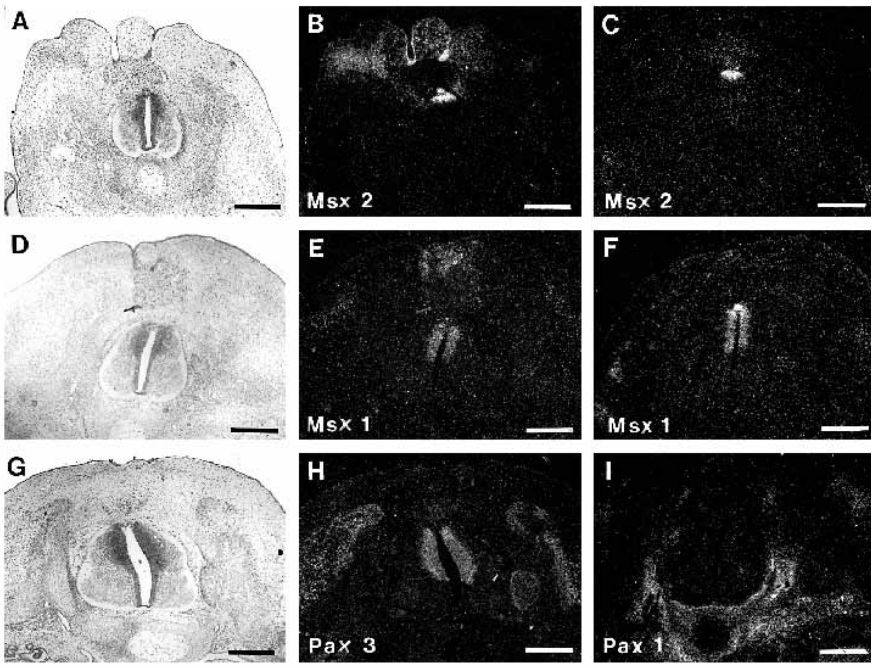

Fig. 6. Effects of dorsal grafts of BMP-producing cells analysed at E6. Transverse sections of E6 embryos which have received grafts of hBMP2-producing cells at E2 show that *Msx2* (A,B) and *Msx1* (D,E) gene expression is enhanced in the dorsal mesenchyme and ectoderm. Compare to the normal expression which is vanishing at this developmental stage (C,F). After grafting hBMP2-producing cells, *Pax3* (G,H) and *Pax1* (I) genes were normally expressed in the muscle masses and the sclerotomes respectively. (A,D,G: bright fields; B,C,E,F,H,I: dark fields). Scale bars, 250 μm.

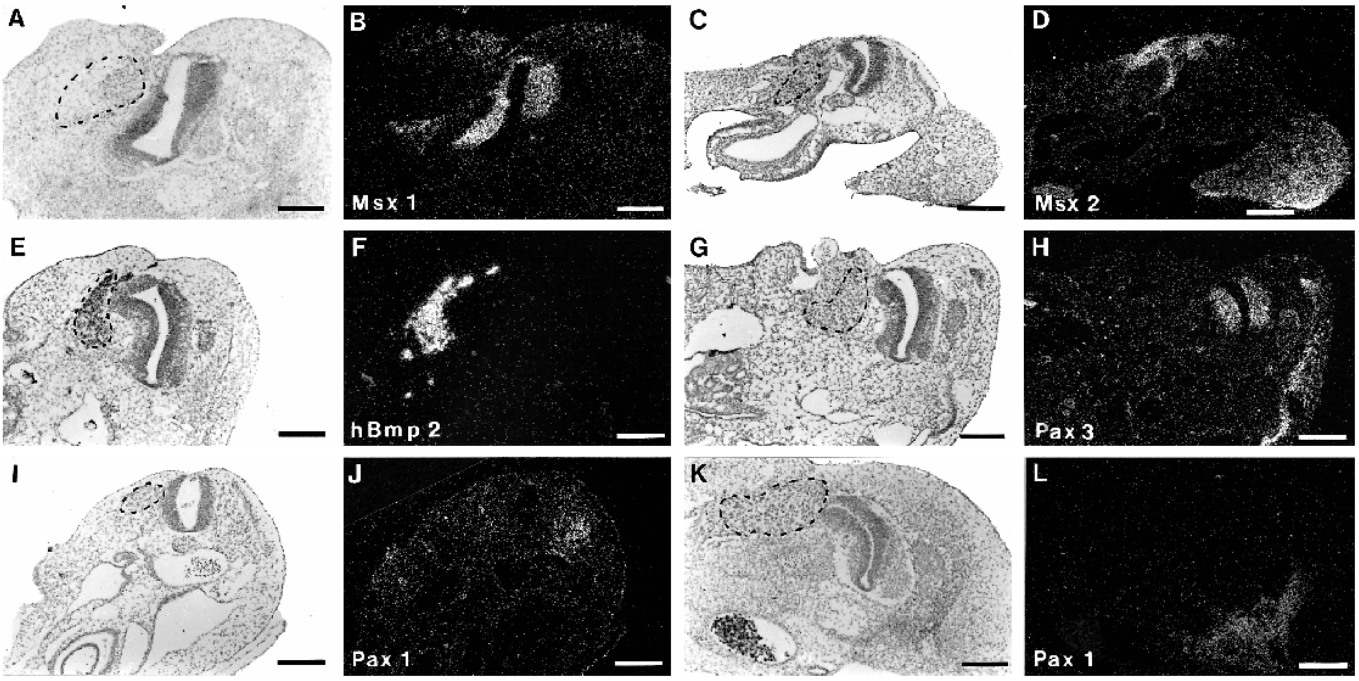

Fig. 7. Effects of lateral grafts of BMP-producing cells analysed at E3-E4. Transverse sections of E3-E4 embryos which have received lateral grafts (outlined with dotted lines) of hBMP2-producing cells show that *Msx1* (A,B) and *Msx2* (C,D) gene expression is induced around the grafted cells, in superficial (i.e. between the ectoderm and the graft) as well as in deep positions. Expression on the contralateral side was normal. The grafted cells did not express *Msx* genes but expressed the *hBMP2* gene strongly (E,F). Laterally to the graft, the dermomyotome is deeply perturbed and is completely absent at the most affected levels, whereas *Pax3* is expressed normally by the contralateral dermomyotome (G,H). The lateral graft also resulted in the absence of *Pax1* expression in the sclerotome on the grafted side as early as E3 (I,J) and this inhibition was still observed at E 4.5 (K,L). A,C,E,G,I,K: bright fields; B,D,F,H,J,L: dark fields. E,F and G,H: adjacent sections. Scale bars, 160 μm.

ing cells gave the more reproducible and pronounced effects. This can be accounted for by the fact that the amount of protein secreted is particularly high with this construct where the *Bmp2* gene is under the control of the strong CMV promoter (de la Pompa and Zeller, 1993). Although *Bmp2* is not expressed in the mediodorsal region of the embryo, we consider that the observa-

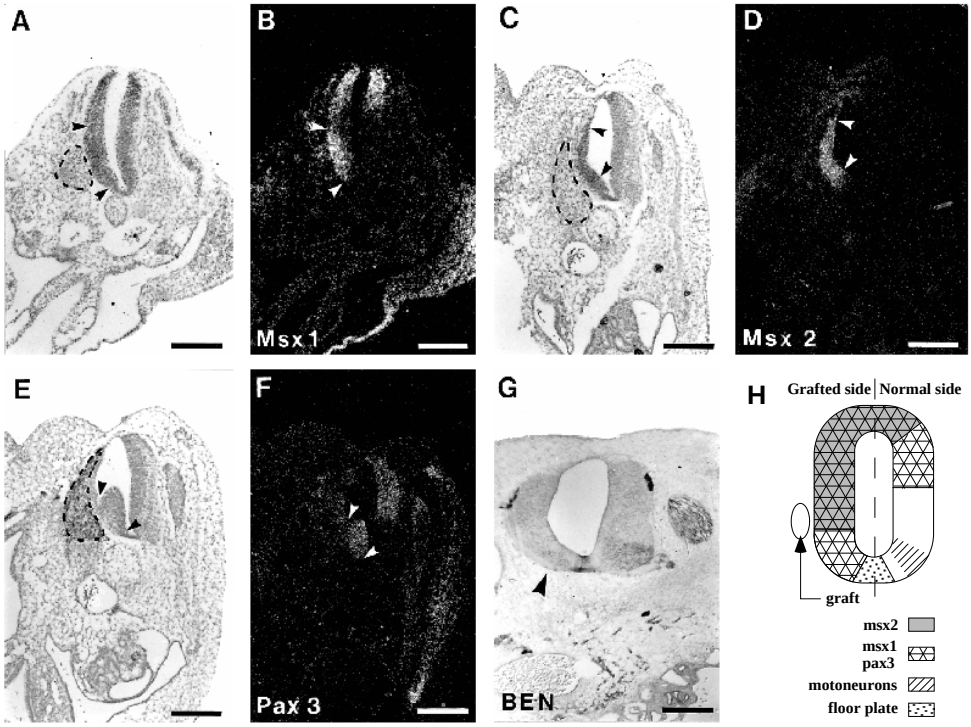

Fig. 8. Effects of lateral grafts of BMP-producing cells on neural tube patterning. Lateral grafts of BMP2-producing cells induced the expression of dorsal markers in the lateral and ventral parts of the neuroepithelium. *Msx1* (A,B), *Msx2* (C,D) and *Pax3* (E,F) are expressed in the alar and basal plates whereas BEN-positive motoneurons are absent in the basal plate on the grafted side (G, arrowhead). The dorsalization of the neural tube is schematized in H: the dorsal marker *Msx2* is upregulated in most of the basal plates, *Msx1* and *Pax3* were observed in the entire basal plate, but not in the floor plate. (A,C,E,G: bright fields; the grafted cells are indicated by dots; B,D,F: dark fields; the ectopic expression domains are delimited by pairs of arrowheads). Scale bars, 160 μm.

tions made on the effect of hBMP2 in this series of experiments are physiologically relevant owing to the close structural relationships existing between BMP2 and 4 and the fact that they bind to the same types of receptors (Estevez et al.,1993; Koenig et al., 1994; Yamaji et al., 1994; Mishina et al., 1995).

The main result obtained in this series of studies is that when BMPs are overexpressed dorsally to the neural tube from E2 onward, the mesenchyme which is normally recruited from the somites to occupy a superficial position to the neural tube is significantly overdeveloped and strongly expresses *Msx* (especially *Msx2*) genes, as compared to stage-matched control embryos observed at the same anterioposterior level. This hyperdevelopment of dorsal mesenchyme is followed by the differentiation of hypertrophied dorsal vertebral cartilage which, if the hBMP2-producing implants are grafted in E3 embryos, forms an enlarged spinous-like process (Fig. 3G-J). The effect of similar grafts on the lateroventral part of the vertebra is strikingly different, since depletions of vertebral cartilage are induced by the local hyperproduction of BMPs. The latter effect is preceded in the sclerotomal moiety of the somite, by an inhibition of *Pax1* expression which has been related to the determination of the ventral somitic cells toward the cartilage differentiation pathway (Ebensperger et al., 1995).

In certain embryos in which the hBMP2-producing cells were in contact with both the dorsal and the lateral somitic mesenchyme, both effects (i.e. hypertrophy of the dorsal vertebral cartilage and aplasia of the vertebral arches and body) coexisted.

This supports the hypothesis put forward in a previous study (Monsoro-Burq et al., 1994) that the molecular mechanisms controlling the formation of the vertebra are different for its lateroventral and dorsomedial parts. Differentiation of cartilage for the former takes place from ventral somitic mesenchyme, expressing *Pax1* at least in its ventralmost part, whereas the latter arises from superficial mesenchyme, which in its precartilaginous state, expresses *Msx1* and *2* genes. *Pax1* expression and cartilage differentiation of the sclerotome are induced by a notochord-floor plate-derived signal (Pourquié et al., 1993) in which *shh* is likely to play a role (Fan and Tessier-Lavigne, 1994). In contrast, cartilage differentiation of the superficial mesenchyme yielding the vertebral spinous process is inhibited by the notochord-floor plate signal while being induced by BMPs. Physiologically, BMP4 plays this role, but it can be replaced experimentally by the closely related protein BMP2. The fact that BMP4 is transiently expressed by the dorsolateral ectoderm overlying the dermatome at a stage just preceding the migration of mesenchymal cells between the dorsal neural tube and the ectoderm suggests that this region might be the source of the cells migrating dorsomedially to yield the vertebral spinous process. Quail-chick grafting experiments are presently in progress to test this hypothesis.

Taken together with other results reported recently from this laboratory (Pourquié et al., 1995, 1996) the present experiments show that BMP4 is an important signalling molecule for somitic patterning and differentiation. BMP4 expressed in the lateral plate ectoderm and mesoderm controls the dorsolateral organization of the paraxial mesoderm by maintaining *Pax3* and inducing *cSim1* gene expression in the lateral half of the somites (Pourquié et al., 1996) which is fated to give rise to the hypaxial muscles (Ordahl and Le Douarin, 1992; Williams and Ordahl, 1994; Goulding et al., 1994). At the same time a signal of unknown nature arises from the neural tube to repress *Pax3* while inducing *MyoD* and *Myf5* gene expression in the myotomes (Pourquié et al., 1995, 1996). We show here, that at a later stage, BMP4 produced in the dorsal ectoderm and neural tube exerts a positive effect on the recruitment of somitic cells to the dorsal midline, and on their commitment toward the subcutaneous cartilage differentiation pathway which also seems to involve the activity of *Msx 1* and *2* genes.

These experiments also show that, as already demonstrated in vitro, BMPs can exert a strong effect on gene expression and patterning of the neural tube in vivo. When an ectopic source of hBMP2 protein is applied laterally to the neural tube at E2, the entire alar plate and the basal plate as well are induced to express the dorsal-type genes *Msx1,2* and *Pax3*. This results in the absence of motoneuron differentiation in the basal plate, thus showing that even in close proximity to the floor plate, a source of ventralizing signals, the dorsalizing effect of BMP2 can be dominant.

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