Development 125, 2631-2639 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV2275

Two domains in vertebral development: antagonistic regulation by SHH and BMP4 proteins

Yuji Watanabe, Delphine Duprez, Anne-Hélène Monsoro-Burq, Christine Vincent and Nicole M. Le Douarin*

Institut d'Embryologie Cellulaire et Moléculaire du CNRS et Collège de France, 49 bis Avenue de la Belle Gabrielle, 94736 Nogent-sur-Marne Cedex, France
*Author for correspondence (e-mail: Nicole.le-douarin@infobiogen.fr)

Accepted 6 May; published on WWW 23 June 1998

SUMMARY

It has previously been shown that the notochord grafted laterally to the neural tube enhances the differentiation of the vertebral cartilage at the expense of the derivatives of the dermomyotome. In contrast, the dorsomedial graft of a notochord inhibits cartilage differentiation of the dorsal part of the vertebra carrying the spinous process. Cartilage differentiation is preceded by the expression of transcription factors of the *Pax* family (*Pax1/Pax9*) in the ventrolateral domain and of the *Msx* family in the dorsal domain. The proliferation and differentiation of *Msx*-expressing cells in the dorsal precartilaginous domain of the vertebra are stimulated by BMP4, which acts upstream of *Msx* genes. It has previously been shown that the SHH protein arising from the notochord (and floor plate) is necessary for the survival and further development of *Pax1/Pax9*-expressing sclerotomal cells. We show here that SHH acts antagonistically to BMP4. SHH-producing cells grafted dorsally to the neural tube at E2 inhibit expression of *Bmp4* and *Msx* genes and also inhibits the differentiation of the spinous process. We present a model that accounts for cartilage differentiation in the vertebra.

Key words: Vertebral development, Cartilage differentiation, Chick embryo, SHH, BMP4, *Msx*, *Pax1*, *Pax9*, *Pax6*, *HNF3β*

INTRODUCTION

Much attention is currently devoted to the molecular control of paraxial mesoderm development. It has been established that the onset of the dorsoventral polarity of the epithelial somite is accompanied by the expression of transcription factors of the *Pax* gene family: *Pax3* is expressed dorsally in the dermomyotomal part of the somite, which remains epithelial for a period, whereas the expression of *Pax1* and *Pax9* characterizes the ventral somitic moiety, which undergoes an epitheliomesenchymal transition and becomes the sclerotome (Deutsch et al., 1988; Goulding et al., 1994). The sclerotome generates the vertebrae and the intervertebral disks. Each vertebra is formed by a vertebral body that results from the chondrogenic differentiation of *Pax1/Pax9*-expressing sclerotomal cells migrating ventrally to the neural tube during vertebral morphogenesis and surrounding the notochord. *Pax1*-positive sclerotomal cells are also involved in the development of the neural arches of the vertebra that surround the neural tube. Vertebral chondrogenesis takes place according to a ventrodorsal gradient: it starts in the vertebral body and progresses dorsally in the neural arches so that the last part of the vertebra to be formed is that which ensures its dorsal closure and carries the spinous process.

It has been previously shown by this laboratory (1) that the overall survival of the paraxial mesoderm and somitic cells depends upon a signal emanating from the neural tube/notochord complex (Teillet and Le Douarin, 1983; Rong et al., 1992; Teillet et al., 1998); (2) that the graft of an extra-notochord dorsolaterally between neural tube and paraxial mesoderm at the level where it is still unsegmented enhances the differentiation of the sclerotomal and chondrogenic moiety of the somite (Pourquié et al., 1993); (3) that in strong contrast to this effect, if a notochord is implanted dorsomedially to the just-closed neural tube, the most dorsal part of the vertebral cartilage does not form. The mesenchymal cells, which start to accumulate between the superficial ectoderm and the roof plate from day 3 of incubation (E3) onwards, are reduced in number and subsequently fail to differentiate into cartilage. The vertebral arches thus remain open dorsally (Monsoro-Burq et al., 1994). A similar result was obtained when the neural tube was rotated through 180° at E2 at the level of the segmental plate, thus bringing the floor plate dorsally into contact with the mesenchyme fated to form the spinous process (Takahashi et al., 1992).

The gene activities that characterize the development of mediodorsal chondrogenic mesenchyme and its surrounding epithelia are different from those of the bulk of the sclerotome during the process of vertebral morphogenesis. The roof plate and the superficial dorsal ectoderm, and later on the dorsal mesenchyme located inbetween, are the sites of production of factors of the TGFβ family, specifically BMP4 (Liem et al.,

1995; Watanabe and Le Douarin, 1996; Monsoro-Burq et al., 1996). Moreover the latter tissues also express transcription factors of the *Msh* family, *Msx1* and *Msx2* (Takahashi and Le Douarin, 1990; Suzuki et al., 1991; Takahashi et al., 1992). The dorsal mesenchyme itself, in spite of its chondrogenic fate, does not express *Pax1* (A.-H. Monsoro-Burq, unpublished) and the introduction of either a notochord or a floor plate, in contact with this dorsal mesenchyme, suppresses the expression of *Msx1* and *Msx2* genes, together with cartilage differentiation (Takahashi et al., 1992; Monsoro-Burq et al., 1994, 1995). In contrast, grafts of BMP2- or BMP4-producing cells underneath the superficial ectoderm dorsally or laterally to the neural tube, induce *Msx* gene expression in mesenchymal cells and their further differentiation into subcutaneous pieces of cartilage (Watanabe and Le Douarin, 1996; Monsoro-Burq et al., 1996). If the same cells are grafted laterally between neural tube and somites they inhibit cartilage development from *Pax1*-expressing cells (Monsoro-Burq et al., 1996). It appeared therefore that the molecular pathways regulating the ventrolateral and the mediodorsal parts of the vertebra are radically different.

In view of the common effect of floor plate and notochord in the inhibition of spinous process development, we thought that the factor responsible for this effect might be the protein Sonic hedgehog (SHH), which is normally produced by both these structures. SHH and BMP4 have been shown to have opposite effects on the dorsoventral patterning of the neural tube (Liem et al., 1995), and on the mediolateral patterning of the somite (Hirsinger et al., 1997). We thus decided to compare the effect of dorsal and lateral grafts of SHH-producing cells with that of notochord grafts. We found that the dorsomedial graft of a notochord at E2 eliminates *Bmp4* expression as early as E3. Grafts of SHH-producing cells in the same situation similarly abolish expression of *Bmp4*, *Msx1* and *Msx2* genes in the dorsal neural tube and dorsal mesenchyme. The spinous process is then absent and the vertebrae remain open dorsally at the graft site. In addition, alterations of gene activities that indicate a partial ventralization of the neural tube have been recorded.

MATERIALS AND METHODS

Fertile chicken eggs from a commercial source (JA strain, Institut de Selection Animale, Lyon, France) were incubated at 38°C in a humidified atmosphere. Embryonic stages were determined according to the number of somites for young embryos and later to the developmental tables of Hamburger and Hamilton (HH; 1951).

Production of SHH-producing cells

A construct carrying the chick *Shh* gene coding region under the CMV promoter in pBK plasmid (Stratagen) was kindly provided by Hermann Rohrer (Germany). The construct was stably transfected into the quail QT6 cell line (SHH-QT6) and selected in G418 as described in Duprez et al. (1998). The SHH-QT6 or the control QT6 cells were trypsinized and cultured in bacterial Petri dishes to produce cell aggregates used for the grafting experiments.

Microsurgery

The notochord was dissected out from 15- to 20-somite stage (ss) quail embryos, at the level of unsegmented paraxial mesoderm, after treatment by 1.25% pancreatin (Gibco) in PBS. The notochord explants on the cell aggregates were inserted into a slit made in mediodorsal ectoderm (dorsal graft) or between somites and neural tube (lateral graft) of chick embryos at 15- to 20-ss (Fig. 1A,B). The level chosen was at the anterior area of the unsegmented paraxial mesoderm. The operated embryos examined at E3 were treated by non-radioactive whole-mount in situ hybridization or at E4 and E5 after radioactive in situ hybridization on sections.

In situ hybridization

The chick *Msx1* probe was a generous gift from Dr H. R. Suzuki (Suzuki et al., 1991) and the quail *Msx2* probe was cloned in our laboratory (Takahashi and Le Douarin, 1990). The *Msx1* and *2* probes were prepared for in situ hybridization as described by Monsoro-Burq et al. (1995). *Bmp4* probe was cloned by Francis et al. (1994). The probes of *Bmp4*, *Pax1*, *Pax6*, *Shh*, *Ptc* and *HNF3β* were kindly provided by Drs P. Brickell, R. Balling, P. Gruss, R. Riddle, C. Tabin and A. Ruiz i Altaba, respectively.

Serial paraffin sections of E4- and E5-operated embryos were hybridized with [^{35}S]dUTP-labeled RNA probes according to Wakamatsu and Kondoh (1990). The washing was performed in 0.1× SSC at 65°C for *Bmp4* or in 50% formamide-2× SSC-10 mM DTT at 52-60°C for the other probes.

Non-radioactive probes were labeled with digoxygenin-UTP according to the manufacturer's protocol (Promega). Hybridization on whole embryos was performed at E3 as described by Henrique et al. (1995). Stained embryos were embedded in gelatin/albumin and sectioned (50 μm) using a vibratome. The mounted sections were photographed with Nomarski optics (Leica).

Immunocytochemistry

The grafted embryos were fixed at E4 and E5 in Carnoy's fixative, dehydrated in ethanol, cleared in toluene and embedded in wax. 5 mm-thick serial sections were immunostained with the QCPN monoclonal antibody (mAb) (Hybridoma Bank), which recognizes an antigenic determinant common to all quail cells, including the QT6 quail cell line.

Skeletal staining

The operated embryos were treated at E10 with Alcian Blue and Alizarin Red, which stain cartilage and bone respectively, after KOH clearing of the non-skeletal tissues.

RESULTS

Mediodorsally grafted notochords repress the transcription of *Bmp4* gene

Notochords removed from 15- to 20-ss quail embryos were implanted mediodorsally beneath the dorsal ectoderm in chick embryos of equivalent stage (Fig. 1A) at the level of the unsegmented paraxial mesoderm. The operated embryos were fixed at E3 (stage HH19) and the expressions of *Msx2* and *Bmp4* genes were recorded (Fig. 2). The expression of *Msx2* was abolished in the roof plate and overlying ectoderm adjacent to the implanted notochord (Fig. 2A-C). In normal embryos, *Bmp4* is expressed in the same area as *Msx2* in roof plate and overlying ectoderm (Fig. 2E). This dorsal expression was also strongly downregulated by the notochord graft (Fig. 2F) along the entire level of the graft (Fig. 2D, boxed). Since both *Msx2* and *Bmp4* are expressed in the neural fold prior to the neural tube closure, these inhibitions are not due to the physical disruption of neural tube closure by grafted notochord (Monsoro-Burq et al., 1996).

These results confirm that a dorsal graft of the notochord inhibits the expression of dorsal-specific genes of the *Msx*

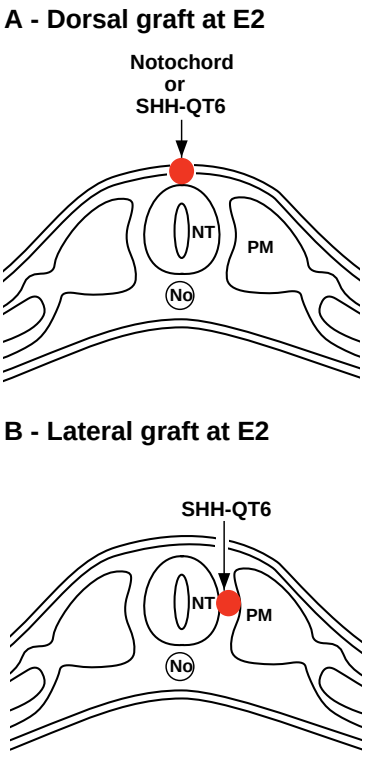

Fig. 1. Schema of dorsal or lateral graft of notochord or SHH-QT6 cell aggregates (red). (A) Notochord or SHH-QT6 cells are implanted through a slit made in the dorsal ectoderm. (B) SHH-QT6 cells are inserted between the neural tube and the non-segmented paraxial mesoderm. NT, neural tube; No, notochord; PM, paraxial mesoderm.

family (Monsoro et al., 1994, 1995) and show that this inhibition also concerns the *Bmp4* gene.

The effect of mediodorsal grafts of SHH-producing cells on dorsal gene expression

The possibility that the molecule produced by the notochord and responsible for the inhibition of dorsal-specific genes might be Sonic hedgehog (SHH) was tested. The QT6 cell line producing chicken SHH was used as a source of this protein in the following in vivo assay: aggregates of cells were implanted into E2 chick embryos mediodorsally, as represented in Fig. 1A. The grafted embryos were fixed at E3-5 and treated for in situ hybridization. In E3 embryos, 1 day after the operation, the grafted cells had remained in their position and strongly expressed *Shh* (Fig. 3A). No ectopic *Shh* expression was detected in the neural tube. *HNF3β* is normally expressed in the floor plate and was not induced in the roof plate adjacent to the graft (Fig. 3B). Ectopic expression of these two genes was not detected in E4 embryos, i.e. 2 days after the operation (Fig. 3C,D), whereas *Bmp4* expression was already inhibited adjacent to the graft (Fig. 3E-H).

The grafted embryos fixed at E5 (Fig. 4C,D,G,H,K,L, right panels) showed obvious morphological abnormalities that were not seen in embryos grafted with QT6 cells (Fig. 4A,B,E,F,I,J, left panels). In SHH-QT6-grafted embryos, the neural tube was reduced in size along the dorsoventral axis; moreover, the dorsal ventricular zone had a round shape, different from the normal shape of the roof plate. At E5 an ectopic expression of *Shh* was induced in the dorsal ventricular zone of the neural tube at the site where the roof plate was transformed, proximal to the SHH-producing cells (Fig. 4D). *Pax6* expression, which is normally essentially present in the ventrolateral part of the neural tube (Fig. 4J), extended dorsally to the alar plates and dorsal neural tube areas (Fig. 4L). These results indicate that the dorsal part of the neural tube is able to express ventral markers following a prolonged exposure to SHH signal. However, no *HNF3β* was induced in dorsal neural tube where ectopic *Shh* was detected (Fig. 3H).

Effect of SHH on dorsal mesenchyme

In operated embryos examined at E5, the mesenchymal cells located dorsally to the neural tube were reduced in number. This resulted in the formation of a mediodorsal groove, the bottom of which was occupied by SHH-QT6 cells, suggesting that they inhibited the normal proliferation of these dorsal

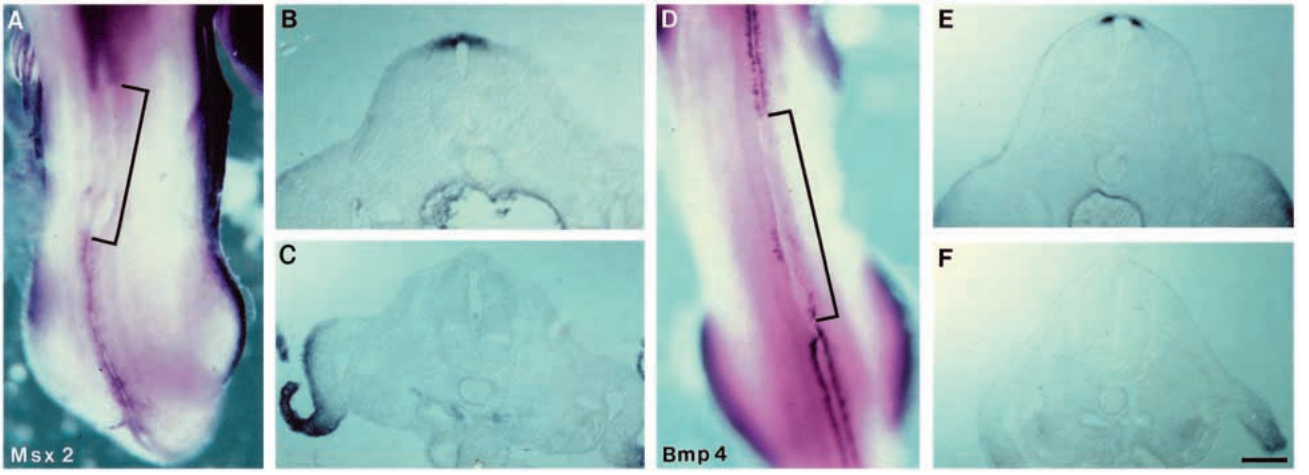

Fig. 2. Expression of *Msx2* (A) and *Bmp4* (D) in the operated embryos where notochord was grafted mediodorsally to the neural tube (boxed area) observed at stage 19 HH. In transverse sections, dorsal-specific expressions of *Msx2* and *Bmp4* (B,E) in normal embryos have disappeared adjacent to the notochord graft (C,F). Bar, 160 μm (A,D), 120 μm (B,C,E,F).

mesenchymal cells (Figs 4C,G, 5D). Notable is the fact that such an inhibition does not occur when control QT6 cells were placed in the same position (Figs 4A,E,I, 5A). The effect of SHH-QT6 cells is strikingly different from that of cells secreting BMP2 placed in a similar location: the dorsal graft of BMP-producing cells induced a large hypertrophy of the dorsal mesenchyme (Monsoro-Burq et al., 1996).

In normal E5 embryos, as well as in embryos into which C-QT6 cells were grafted dorsally at E2 (Fig. 5A-C), *Msx1* was expressed in the dorsal part of the neural tube (Fig. 5B) and is hardly detectable in the dorsal mesenchyme of E5 embryos, although strongly expressed at E4 as described previously (Monsoro-Burq et al., 1995, 1996). *Msx2* is expressed in the roof plate, the dorsal mesenchyme and the dorsal ectoderm (Fig. 5C). *Bmp4* was detected in the roof plate, the dorsal mesenchyme and ectoderm at E4 (Fig. 3F) and E5 (data not shown). Expression of these genes was totally abolished by dorsal grafts of SHH-QT6 cells, and also by notochord grafts (Figs 3H, 5D-F).

Lateral grafts of SHH-producing cells induce an extension of the *Pax1* expression domain

In contrast to the dorsal grafts of notochord, which inhibit *Bmp4* expression at E3 (this study) and result in the loss of the dorsal part of vertebra (Monsoro-Burq et al., 1994), lateral grafts of notochord produce the expansion of the *Pax1*-expressing domain followed by the hypertrophy of the lateral vertebral arch and of the vertebral body (Pourquié et al., 1993; Brand-Saberi et al., 1993). We thus examined the effects of the lateral implantation of SHH-producing cells on sclerotome development. The SHH-QT6 cell aggregates were grafted between neural tube and paraxial mesoderm (Fig. 1B) at the anterior part of the unsegmented region and *Pax1* expression was subsequently recorded in E4 embryos (Fig. 6). Lateral and dorsal grafts of control QT6 cells did not modify the normal expression pattern of *Pax1*, which occupies the primordium of the vertebral body at this stage (Fig. 6A,B,E,F; Ebensperger et al., 1995). In strong contrast, lateral grafts of SHH-QT6 cells caused the enlargement of the *Pax1*-positive area in the region of the sclerotome adjacent to the graft (Fig. 6G,H). Dorsal grafts of SHH-QT6 cells also resulted in the extension of the *Pax1*-positive region, but to a much lesser extent than lateral grafts. Owing to the position of the source of SHH this extension concerned the most dorsally located lateral sclerotomal cells (Fig. 6C,D). These results indicate that SHH produced by the SHH-QT6 cells is able to mimic the effect not only of the dorsal but also of the lateral grafts of the notochord.

Medio-dorsal and lateral SHH-QT6 grafts affect vertebral development differently

Since dorsal and lateral grafts of SHH-QT6 cells affect the expression of genes correlated with the development of the dorsal and ventral vertebral components in an opposite manner, we examined the effects of both types of graft on vertebral morphogenesis in E10 embryos.

Dorsal grafts of control cells did not disturb normal vertebral development (Fig. 7A). In these embryos the implanted C-QT6 cells remained at their dorsal position (as revealed by the QCPN-mAb staining, not shown) without disturbing the development of the spinous process. In embryos in which SHH-QT6 cells were grafted dorsally, the vertebrae were open at the level of the graft and the spinous process were missing (Fig. 7B), as when a notochord was grafted in the same position (Monsoro-Burq et al., 1994). In addition, dorsal grafts of SHH-QT6-producing cells induced the enlargement and, in some

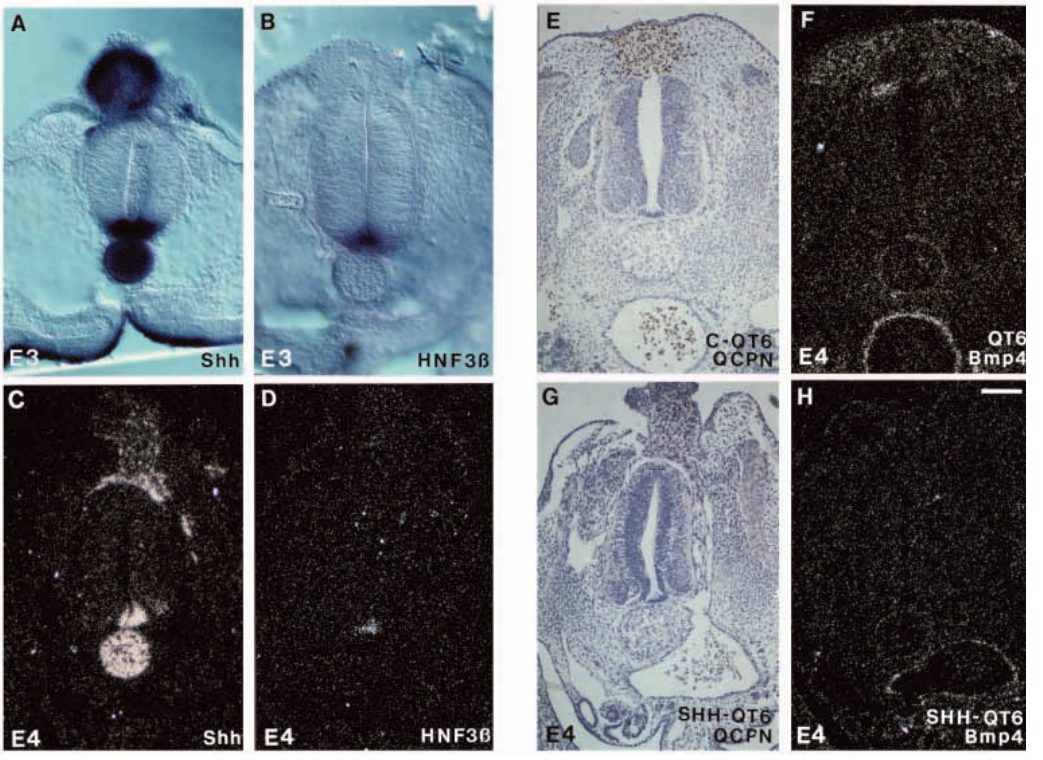

Fig. 3. Transverse sections of chick embryos into which intact C-QT6 cells (E,F) or SHH-QT6 cells (A-D,G,H) were grafted mediodorsally at E2. The expression of *Shh* and *HNF3β* at E3 (A,B) and E4 (C,D) are shown. *Bmp4* expression at E4 is shown in (F,H) with QCPN-mAb staining of their adjacent sections (E,G). (A) At E3, the endogenous expression of *Shh* was detected in the floor plate, notochord and endoderm. The grafted SHH-QT6 cells strongly express *Shh* but do not induce ectopic *Shh* expression in neural tube. (B) *HNF3β* is detected in the floor plate but is not induced in the roof plate. (C) *Shh* and (D)*HNF3β* were not induced ectopically at E4. (E,G) Dorsally grafted C-QT6 quail cells or SHH-QT6 quail cells are shown by staining with the QCPN-mAb. (F) *Bmp4* expression is not affected by a C-QT6 cell graft but is abolished by the SHH-QT6 cell graft in (H). C,D,G and H are serial sections of the same embryo. E,G: bright fields; C,D,F,H: dark fields. Bar, 60 μm (A,B), 120 μm (C-H).

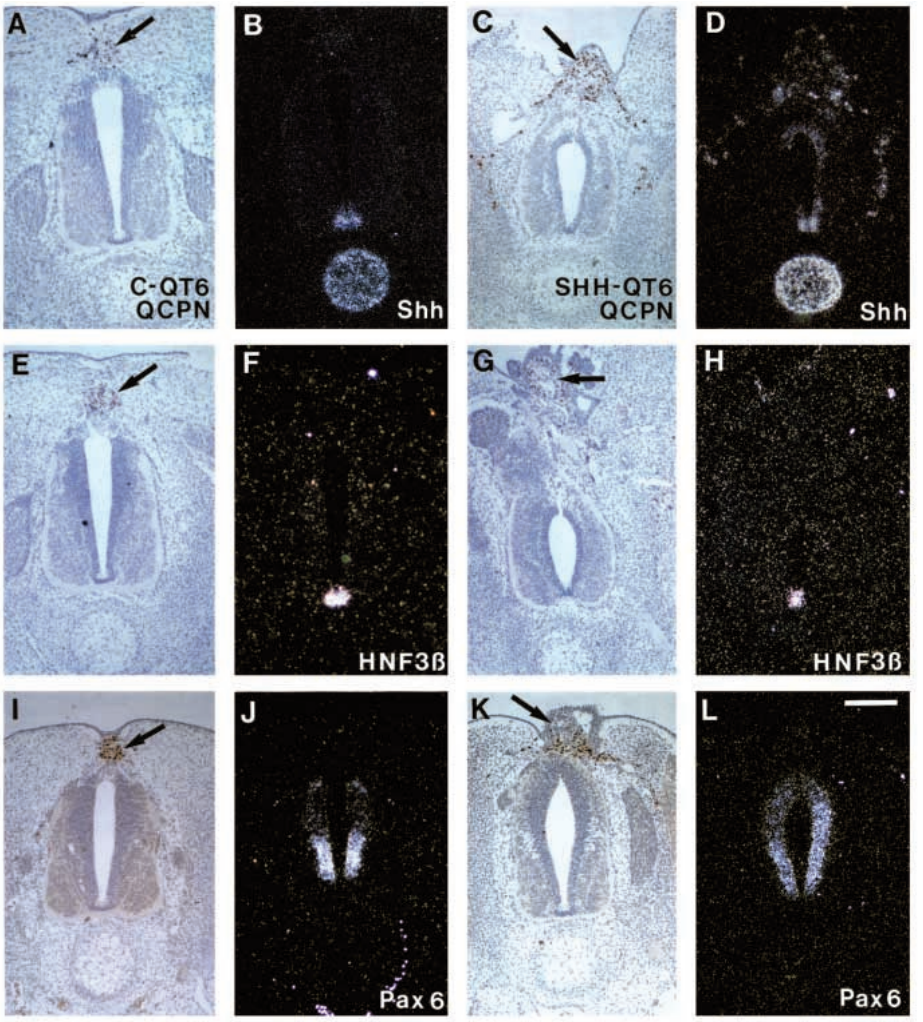

Fig. 4. Effects of dorsal grafts of SHH-QT6 cells in E5 embryos. Transverse sections where C-QT6 cells (A,B,E,F,I,J) or SHH-QT6 cells (C,D,G,H,K,L) were implanted dorsomedially. The grafted quail cells are labelled by QCPN mAb (arrows in A,C,E,G,I,K). In SHH-QT6 cell grafted embryos, ectopic expression of *Shh* and *Pax6* is induced in the roof plate and alar plate, respectively (D,L). *HNF3β* is not induced in the neural tube (H). A,C,E,G,I,K: bright fields; B,D,F,H,J,L: dark fields. Bar, 120 μm.

cases, the fusion of the dorsal extremities of the neural arches that were in contact with the graft (Fig. 7B). This effect was much more pronounced when SHH-QT6 cells were grafted laterally (Fig. 7C). The effect observed on the neural arches was expected since it was preceded by a dorsal extension of the *Pax1*-expressing domain (Fig. 6C,D). Such an enlargement of the neural arches was not seen in the case of dorsal grafts of the notochord (see Fig. 5 in Monsoro-Burq et al., 1994). This may be due to the quantitative differences of SHH protein production between the grafted pellet of SHH-QT6 cells and the transplated notochord.

Dorsal tissues directly respond to ectopic SHH protein by expressing the *Patched* gene

Since mediodorsal grafts of SHH-QT6 cells strongly affect the expression of the dorsal genes in the ectoderm, the dorsal mesenchyme and the neural tube (Figs 3, 4, 5), we wanted to see whether the SHH protein directly affects these tissues. As *Patched (ptc)* is known to be a downstream target gene and receptor of Sonic hedgehog (Marigo et al., 1996; Goodrich et al., 1996; Marigo and Tabin, 1996), we analysed the expression of chick *ptc* in response to the ectopic SHH protein. Aggregates of SHH-QT6 cells were grafted dorsally or dorsolaterally to the neural tube at the non-segmented level in E2 chick embryos (15-ss). The operated embryos were fixed at E3 to analyze the *Ptc* expression pattern. Fig. 8 shows in situ hybridization with the chick *Ptc* probe of an operated embryo. In the non-operated area *Ptc* expression is detectable in the luminal part of neural tube and in the medial part of myotome (Fig. 8B,D). In contrast, at the level of the graft, *Ptc* expression is significantly up-regulated in the dorsal neural tube, the ectoderm, and in the mesoderm located in contact with the graft (Fig. 8C, right). On the control side, no up-regulation was observed (Fig. 8C, left). This result indicates that, as soon as 24 hours after the operation, the ectopically administrated SHH affects directly all the tissues in the dorsal region of the embryo visualized by *Ptc* overexpression.

DISCUSSION

In spite of the fact that vertebrae are formed by a single cell type, cartilage, their development involves different molecular pathways according to the vertebral region considered. The ventrolateral part of the vertebra (i.e. vertebral body and neural arches) develops from the ventral sclerotomal cells that express the transcription factor *Pax1* before the onset of chondrogenesis (Deutsch et al., 1988). Previous work has

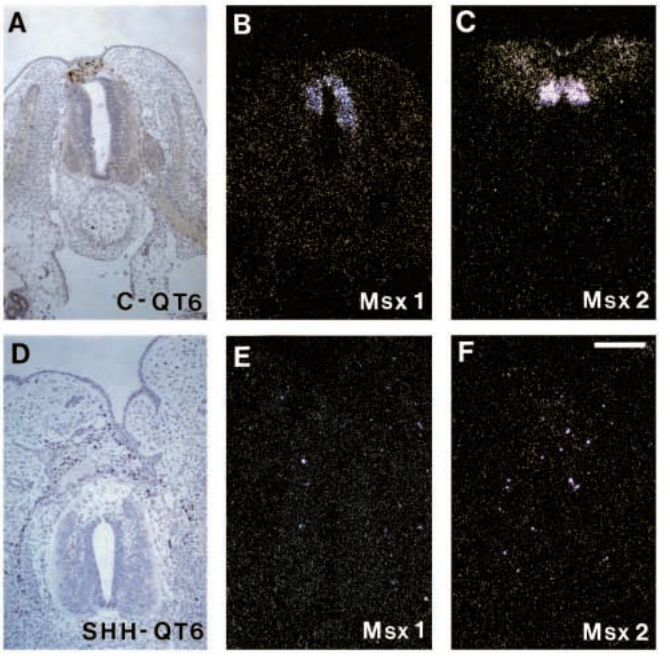

Fig. 5. Effects of dorsal grafts of SHH-QT6 cells on *Msx* genes at E5. The dorsal implantation of C-QT6 (A) does not modify the normal E5 pattern of expression of *Msx1* (B) and *Msx2* (C). In contrast, SHH-QT6 cells (D) abolish both *Msx1* (E) and *Msx2* (F) expression in the roof plate, the ectoderm and the dorsal mesenchyme. A,D: bright fields; B,C,E,F: dark fields. Bar, 120 μm.

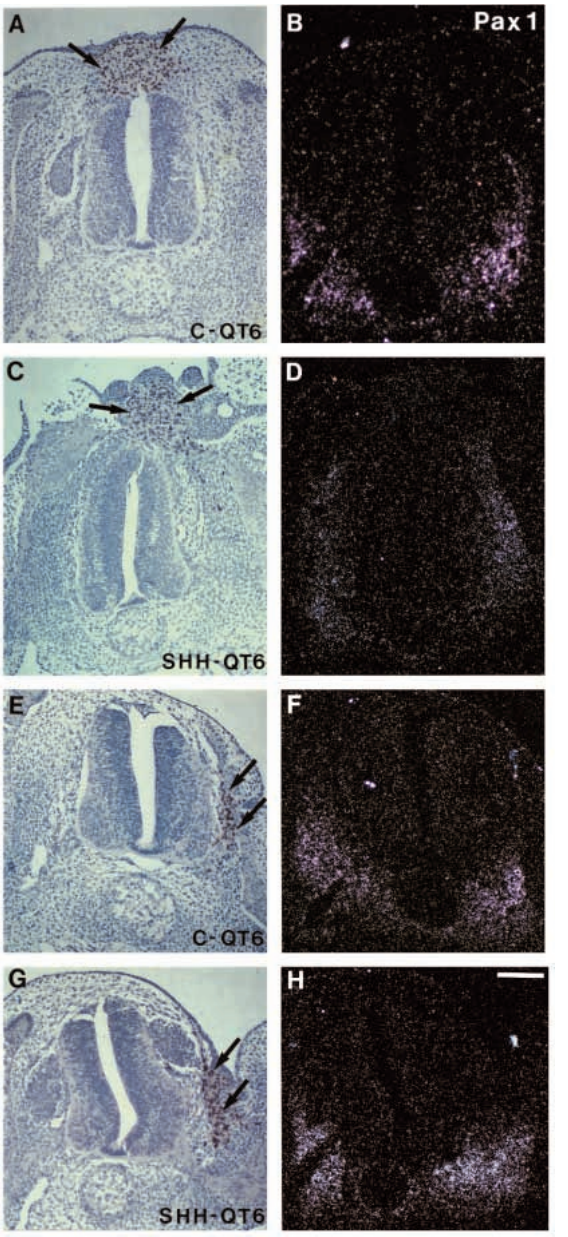

Fig. 6. Effects of dorsal and lateral grafts of SHH-QT6 cells on *Pax1* expression in E4 embryos. Control QT6 (C-QT6) cells or SHH-QT6 cells were grafted dorsally (A-D) and laterally (E-H). The grafted quail cells are labelled by the QCPN mAb (A,C,E,G, arrows). While the C-QT6 cell grafts do not alter the *Pax1* expression (B,F), the *Pax1* expression domain is extended dorsally (D) or laterally (H) in the proximity of the SHH-QT6 cell graft. A,C,E,G: bright fields; B,D,F,H: dark fields. Bar, 120 μm.

shown that chondrogenesis of the ventrolateral part of the vertebra takes place under the influence of the notochord: a supernumerary notochord grafted dorsomedially to the somite extends the *Pax1*-expressing somitic domain dorsally, and subsequently its differentiation into cartilage to the point that the development of the dorsal somitic derivatives (i.e. the dermomyotome) can be totally suppressed (Pourquié et al., 1993; Brand-Saberi et al., 1993).

The most dorsal part of the vertebra that closes the vertebral arch differentiates between two ectodermal layers, the superficial ectoderm and the roof plate, from mesenchymal cells of somitic origin. Thus, the unilateral graft of quail somites into chick embryos results in the formation of chimeric vertebrae with a hemivertebral body and hemispinous process, and a neural arch made up of donor cells on the operated side and of host cells on the intact side. The limit between the host's and donor's territories corresponds strikingly to the sagittal plane of the embryo (A.-H. Monsoro-Burq and N. M. Le Douarin, unpublished). Therefore, somitic cells with a chondrogenic fate must migrate medially in order to surround the neural tube and form the vertebral body ventrally and the spinous process dorsally. The cells that migrate dorsally from E3 onward fail to express *Pax1* (A.-H. Monsoro-Burq and N. M. Le Douarin, unpublished) but start to express *Msx1* and *Msx2* as they become positioned between the superficial ectoderm and the roof plate, which produces BMP4 (Watanabe and Le Douarin, 1996; Monsoro-Burq et al., 1996). Moreover, the lateral graft of a roof plate or of cells producing BMP4 induces ectopic expression of *Msx* genes in the host somitic mesenchyme (Takahashi et al., 1992; Watanabe and Le Douarin, 1996; Monsoro-Burq et al., 1996). Such an induction, however, can occur only if the inducer (e.g. the roof plate) is placed in close proximity to the superficial ectoderm (Takahashi et al., 1992; Monsoro-Burq et al., 1994). This supports the contention that bone formation in the subcutaneous site, where the spinous process is formed, is under the control of BMP4, and that *Msx* genes are involved in the pathway leading to chondrogenesis (see Monsoro-Burq et al., 1994, 1996 and discussions therein). This view was

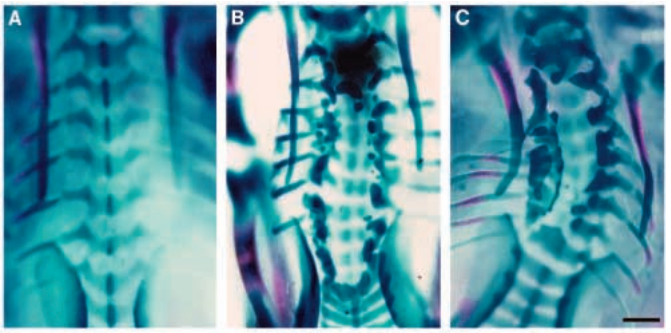

Fig. 7. Dorsal view of skeletal staining of E10 operated embryos in which C-QT6 cells were grafted dorsally (A) or SHH-QT6 cells were grafted dorsally (B) or dorso-laterally (C). The dorsal part of the vertebrae is missing at the level of the graft while the vertebral arches show fusion and hypertrophy adjacent to the graft (arrow). Bar, 1 mm.

confirmed by the fact that overexpression of BMP4 (or of the closely related compound BMP2) dorsally to the neural tube results in the expansion of the *Msx1-2*-positive mesenchymal territory and subsequently in the enlargement of the spinous process (Monsoro-Burq et al., 1996). Duality in vertebral chondrogenesis was further underlined by the opposite effect of BMPs on the development of the ventrolateral part of the vertebra. Chondrogenesis was strongly inhibited by the graft of BMP2/4-producing cells in a ventrolateral position with respect to the neural tube (Monsoro-Burq et al., 1996).

These observations raised the question of the nature of the factor of notochord/floor plate origin that is responsible for chondrogenesis in the ventrolateral domain of the vertebra. The most obvious candidate was the protein SHH. Lateral grafts of SHH-producing cells do indeed enhance *Pax1* expression in sclerotomal cells and induce the over-development of cartilage laterally at the level of the neural arches. The positive influence of SHH protein on *Pax1* expression by somitic cells has already been demonstrated by in vitro experiments (Fan and Tessier-Lavigne, 1994) and in vivo by the use of retroviral vectors controlling *shh* gene expression (Johnson et al., 1994). Here we demonstrate that enhancement of the number of *Pax1*-expressing cells by SHH is followed in vivo by the increase in size of the ventrolateral part of the vertebral cartilage.

In contrast, dorso-medial grafts of notochord and of SHH-QT6 cells inhibit the expression of the *Bmp4* gene in dorsal ectoderm, dorsal mesenchyme and roof plate. Since *Msx* gene expression has been shown to be controlled by BMP signaling in several induction systems (e.g. Monsoro-Burq et al., 1996; Tonegawa et al., 1997), it is probable that, under the experimental conditions described here, the inhibition of *Bmp4* expression is primarily responsible for that of *Msx1* and *Msx2* and for the failure of chondrogenesis in the dorsal part of the vertebra.

This leads to the identification of two molecular pathways in bone development. They concern cartilage and bone formation in 'deep' and 'subectodermal' positions, respectively. Ectoderm has previously been shown to reduce or

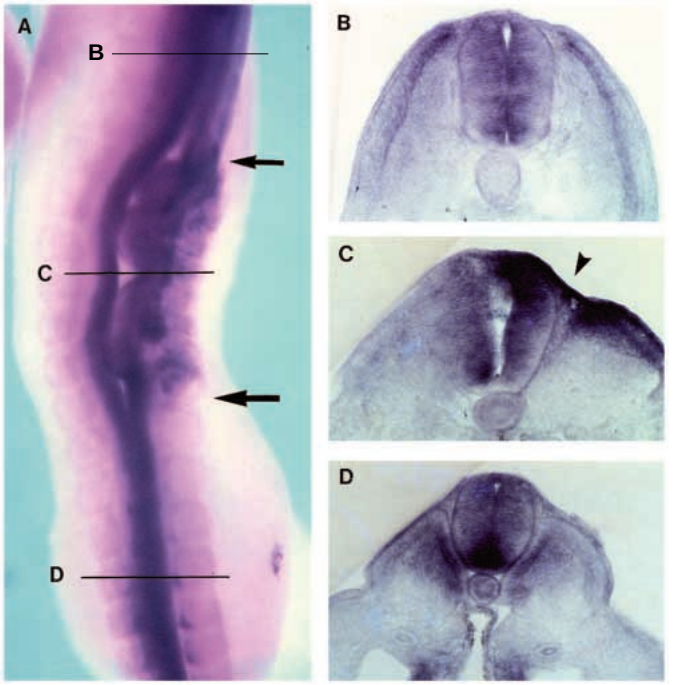

Fig. 8. Up-regulation of *ptc* expression in E3 embryo after a dorsolateral graft of SHH-QT6 cells. Dorsal aspect of the grafted region is shown in (A). The level of the graft is indicated by two arrows. The embryo was sectioned at 3 levels (B, line B in A; C and D, at lines C and D, respectively, in A). SHH-QT6 cells are marked by and arrowhead in C.

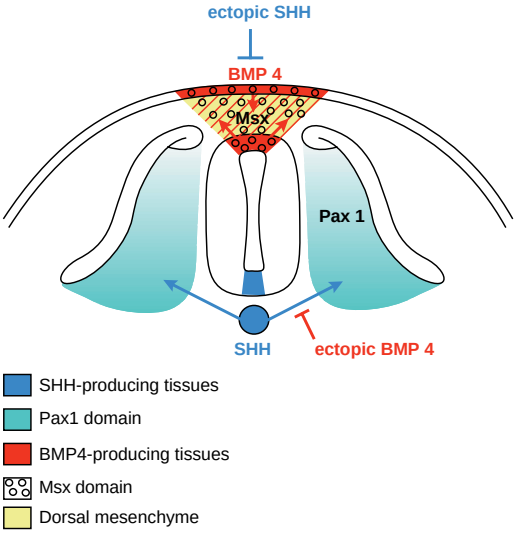

Fig. 9. Proposed model of molecular interactions during vertebral development. In the ventral area, SHH (dark blue) induces *Pax-1* expression (light blue) in the sclerotome; this is followed by the formation of the cartilage of the vertebral body and the ventral parts of the neural arches. Ectopic implantation of BMPs inhibits *Pax1* expression and, later on, cartilage differentiation in this domain. Dorsally, BMP4 (red) is expressed in the ectoderm, the roof plate of the neural tube and in the dorsal somite-derived mesenchyme. BMP4 induces *Msx* gene expression at early stages, followed by the differentiation of cartilage under the ectoderm, in spite of the inhibitory effect of this latter tissue on chondrogenesis recorded previously (Kenny-Mobbs and Thorogood, 1987). Ectopic SHH blocks this dorsal cartilage differentiation, by inhibiting *Bmp4* expression; *Msx* genes are then repressed and dorsal cartilage is absent. The experiments reported here show that antagonistic interactions exist between the ventral and dorsal chondrogenesis pathways during formation of the vertebra.

inhibit chondrogenesis in somitic explant cultures (Kenny-Mobbs and Thorogood, 1987). We have proposed in a previous study that such an inhibition is relieved by the local production of BMP4 by the dorsal ectoderm and neural tube, thus allowing the formation of superficial bony structures from mesodermal (or mesectodermal) mesenchyme to take place (Monsoro-Burq et al., 1996). The deep vertebral cartilage that develops at a distance from the ectoderm and surrounds the notochord and the ventrolateral part of the neural tube requires SHH signalling to differentiate from the sclerotome.

We show here that the ectopic release of SHH in the mediodorsal domain of the somitic mesenchyme inhibits the expression of *Bmp4* and therefore disrupts the molecular pathway leading to subcutaneous cartilage differentiation. In addition, our experiments show that the factors necessary for cartilage differentiation in the ventrolateral and the dorsal domains of the vertebra act antagonistically. Ectopic BMP4 abolishes the positive effect of SHH on deep cartilage development in the ventrolateral part of the vertebra (Monsoro-Burq et al., 1996; Tonegawa et al., 1997), whereas ectopic SHH antagonizes the effect of BMP4 on chondrogenesis in its dorsal part (Fig. 9).

An additional observation made during this work deals with the capacity of SHH to induce its own expression in the neuroepithelium. Although such an induction has already been reported to take place in chicken neuroepithelium cultured in a medium supplemented with SHH protein (Marti et al., 1995), such an effect has not previously been described in vivo. Here, we show that SHH-QT6 cells grafted medio-dorsally at E2 induce *Shh* gene activity 3 days after the beginning of exposure of the neuroepithelium to the SHH factor, although *Ptc* gene expression is activated 24 hours after grafting. It is interesting to note that *Shh* starts to be expressed ectopically in the dorsal neural tube 2 days after expression of *Bmp4* is extinguished in the roof plate and dorsal neural tube. Secondly, the autoactivation of the *Shh* gene in the neuroepithelium is not accompanied by that of *HNF3β*. This result is at odds with the model according to which induction of floor plate or of a floor plate-like structure in the neural tube results from the effect of an SHH signal arising from the notochord and inducing first the neural cells to switch on the *HNF3β* gene and then the *Shh* gene (Echelard et al., 1993).

Another important point is that the antagonistic effects of BMP4/2 and SHH are not restricted to the somitic mesoderm but also concern the neuroepithelium, since SHH protein induces (albeit with a long delay) *Shh* gene activity in the dorsal neural tube while repressing *Bmp-4* at the transcriptional level. Conversely, BMP4/2-producing cells grafted ventrally in close contact to the neural tube inhibit motoneurone differentiation in the basal plate while inducing the expression of dorsal genes such as *Pax3*, *Msx1* and *Msx2* (Monsoro-Burq et al., 1996).

These observations thus indicate that similar genetic controls are responsible for the establishment of the dorsoventral polarity in the neural tube and the paraxial mesoderm.

The authors are grateful to Drs P. Brickell, R. Balling, P. Gruss, R. Ruddle, C. Tabin and A. Ruiz i Altaba for providing them with the probes for *Bmp4*, *Pax1*, *Pax6*, *Shh*, *Ptc* and *HNF3β*, respectively. The SHH-QT6 cells were made in P. Brickell's laboratory. QCPN antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, USA and the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA, under contract N01-HD-6-2915 from NICHD. We thank F. Viala and F. Beaujean for photography, and C. Guilloteau and S. Gournet for preparing the manuscript and figures. This work was supported by the CNRS and grants from the following Foundations: LFRM, ARC and FRMF. Y. W. was supported by JSPS Postdoctoral Fellowship for Research Abroad.

REFERENCES

Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R. and Christ, B. (1993). The ventralizing effect of the notochord on somite differentiation in chick embryos. *Anat. Embryol.* **188**, 239-245.

Deutsch, U., Dressler, G. R. and Gruss, P. (1988). *Pax-1*, a member of a paired box homologous murine gene family, is expressed segmenting structures during development. *Cell* **53**, 617-625.

Duprez, D., Fournier-Thibault, C. and Le Douarin, N. M. (1998). *Sonic hedgehog* induces proliferation of committed skeletal muscle cells in the chick limb. *Development* **125**, 495-505.

Ebensperger, C., Wilting, J., Brand-Saberi, B., Mizutani, Y., Christ, B., Balling, R. and Koseki, H. (1995). *Pax-1*, a regulator of sclerotome development is induced by notochord and floor plate signals in avian embryos. *Anat. Embryol.* **191**, 297-310.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Fan, C. M. and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.

Francis, P. H., Richardson, M. K., Brickell, P. M. and Tickle, C. (1994). Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb. *Development* **120**, 209-218.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the *hedgehog/patched* signaling pathway from flies to mice: induction of a mouse patched gene by *Hedgehog*. *Genes Dev.* **10**, 301-312.

Goulding, M., Lumsden, A. and Paquette, A. J. (1994). Regulation of *Pax-3* expression in the dermomyotome and its role in muscle development. *Development* **120**, 957-971.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J. and Pourquié, O. (1997). Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. *Development* **124**, 4605-4614.

Johnson, R. L., Laufer, E., Riddle, R. D. and Tabin, C. (1994). Ectopic expression of *Sonic hedgehog* alters dorsal-ventral patterning of somites. *Cell* **79**, 1165-1173.

Kenny-Mobbs, T. and Thorogood, P. (1987). Autonomy of differentiation in avian brachial somites and the influence of adjacent tissues. *Development* **100**, 449-162.

Liem, K. F., Tremml, G., Roelink, H. and Jessel, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Marigo, V. and Tabin, C. (1996). Regulation of *Patched* by Sonic hedgehog in the developing neural tube. *Proc. Nat. Acad. Sci. USA* **93**, 9346-9351.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. (1996). Biochemical evidence that Patched is the Hedgehog receptor. *Nature* **384**, 176-179.

Marti, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

Monsoro-Burq, A. H., Bontoux, M., Teillet, M. A. and Le Douarin, N. M. (1994). Heterogeneity in the development of the vertebra. *Proc. Nat. Acad. Sci. USA* **91**, 10435-10439.

Monsoro-Burq, A. H., Bontoux, M., Vincent, C. and Le Douarin, N. M. (1995). The developmental relationships of the neural tube and the notochord: short and long term effects of the notochord on the dorsal spinal cord. *Mech. Dev.* **53**, 157-170.

Monsoro-Burq, A. H., Duprez, D., Watanabe, Y., Bontoux, M., Vincent,

C., Brickell, P. and Le Douarin, N. M. (1996). The role of bone morphogenetic proteins in vertebral development. *Development* **122**, 3607-3616.

Pourquié, O., Coltey, M., Teillet, M. A., Ordahl, C. and Le Douarin, N. M. (1993). Control of dorsoventral patterning of somite derivatives by notochord and floor plate. *Proc. Nat. Acad. Sci. USA* **90**, 5242-5246.

Rong, P. M., Teillet, M. A., Ziller, C. and Le Douarin, N. M. (1992). The neural tube/notochord complex is necessary for vertebral but not limb and boby wall striated muscle differentiation. *Development* **115**, 657-672.

Suzuki, H. R., Padanilam, B. J., Vitale, E., Ramierez, F. and Solursh, M. (1991). Repeating developmental expression of G-Hox, a novel homeobox-containing gene in the chicken. *Dev. Biol.* **148**, 375-388.

Takahashi, Y. and Le Douarin, N. M. (1990). cDNA cloning of a quail homeobox gene and its expression in neural crest-derived mesenchyme and lateral plate mesoderm. *Proc. Nat. Acad. Sci. USA* **87**, 7482-7486.

Takahashi, Y., Monsoro-Burq, A. H., Bontoux, M. and Le Douarin, N. M. (1992). A role for *Quox-8* in the establishment of the dorsoventral pattern during vertebrate development. *Proc. Nat. Acad. Sci. USA* **89**, 10237-10241.

Teillet, M. A. and Le Douarin, N. M. (1983). Consequences of neural tube and notochord excision on the development of the peripheral nervous system in the chick embryo. *Dev. Biol.* **98**, 192-211.

Teillet, M. A., Watanabé, Y., Jeffs P., Duprez, D., Lapointe, F., and Le Douarin N. M. (1998). Sonic Hedgehog is required for survival of both myogenic and chondrogenic somitic lineages. *Development* (in press).

Tonegawa, A., Funayama, N., Ueno, N. and Takahashi, Y. (1997). Mesodermal subdivision along the mediolateral axis in chicken controlled by different concentrations of BMP-4. *Development* **124**, 1975-1984.

Wakamatsu, Y. and Kondoh, H. (1990). Conditions for detection of embryonic N-mye expression by *in situ* hybridization. *Acta Histochem. Cytochem.* **23**, 367-374.

Watanabe, Y. and Le Douarin, N. M. (1996). A role for BMP-4 in the development of subcutaneous cartilage. *Mech. Dev.* **57**, 69-78.