# Signaling dynamics of feather tract formation from the chick somatopleure

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## Summary

**In the chick, most feathers are restricted to specific areas of the skin, the feather tracts or pterylae, while other areas, such as the apteria, remain bare. In the embryo, the expansion and closure of the somatopleure leads to the juxtaposition of the ventral pteryla, midventral apterium and amnion. The embryonic proximal somatopleural mesoderm is determined to form a feather-forming dermis at 2 days of incubation (E2), while the embryonic distal and the extra-embryonic somatopleure remain open to determination. We found a progressive, lateral expression of *Noggin* in the embryonic area, and downregulation of *Msx1*, a BMP4 target gene, with *Msx1* expression being ultimately restricted to the most distal embryonic and extra-embryonic somatopleural mesoderm. *Msx1* downregulation thus correlates with the formation of the pterylae, and its maintenance to that of the apterium. Suspecting that the inhibition of BMP4 signaling might be linked to the determination of a feather-forming dermis, we grafted Noggin-expressing cells in the distal somatopleure at E2. This elicited the formation of a supplementary pteryla in the midventral apterium. Endogenous Noggin, which is secreted by the intermediate mesoderm at E2, then by the proximal somatopleure at E4, could be sufficient to suppress BMP4 signaling in the proximal somatopleural mesoderm and then in part of the distal somatopleure, thus in turn allowing the formation of the dense dermis of the future pterylae. The same result was obtained with the graft of Shh-producing cells, but Noggin and Shh are both required in order to change the future amnion into a feather-bearing skin. A possible synergistic role of endogenous Shh from the embryonic endoderm remains to be confirmed.**

Key words: Amnion, Bmp4, Chick, Dermis, Dorsoventral axis, Feather, Msx1, Noggin, Skin, Shh, Somatopleure

## Introduction

One of the significant steps of skin morphogenesis is the establishment of the cutaneous appendage fields. In the avian embryo, the different feather tracts, or pterylae, arise sequentially following a dorsolateral and a lateroventral morphogenic wave (Mayerson and Fallon, 1985). The way in which the distinct pterylae are laid out is called the feather macropattern (Jung and Chuong, 1998; Sengel, 1976). In chick, the trunk feather macropattern is composed of the spinal and scapular pterylae on the dorsal side, and the pectoral and ventral pterylae on the ventral side. In addition to its location and time of appearance, each tract is characterized by its contour, size, number and distribution (micropattern) of feathers. Moreover, the different pterylae are separated from each other by semi-apteria, which are characterized by unorganized and scarce feathers. The only sizeable true featherless region is the midventral apterium – the extreme ventral region on each side of the midventral closure. This apterium is surrounded by the ventral pteryla and is contiguous with the amnion via the umbilical cord. The first morphological indication of the formation of a pteryla versus a semi-apterium is the early densification of the predermal mesenchyme to form a dense dermis (2.6 nuclei/1000 $\mu m^3$), and the subsequent differentiation of its overlying ectoderm into an epidermis (Wessells, 1965). This dermal densification occurs by day 6 in the spinal and ventral pterylae, but occurs several days later in the semi-apteria. In the midventral apterium, by contrast, the dermal fibroblasts remain loose (1.98 nuclei/1000 $\mu m^3$) (Sengel et al., 1969).

Two questions thus arise: what are the origins of and how are the dermal progenitors of a pteryla specified? Numerous experiments have been conducted using heterotopic mesoderm transplantations (Mauger, 1972) and dermal-epidermal recombinations (Dhouailly, 1977) that have shown that the information relative to the feather macropattern and micropattern resides first in the mesoderm and then in the dermis. In avian embryos, the origin of the dermis from different body regions has been investigated by the heterospecific chick/quail marking technique. In the trunk, the ventral dermis is derived from the somatopleure, whereas the dorsal dermis derives from the somite dermomyotome (Mauger, 1972). More precisely, the dermis of the spinal pteryla derives from the medial part of the dermomyotome, whereas its lateral part probably gives rise to the dermis of the lateral semi-apterium, which forms the frontier with the ventral side (Olivera-Martinez et al., 2000; Olivera-Martinez et al.,

2002). The embryonic somatopleure can be divided by the rostrocaudal axis into two ~150 μm parts that behave differently (Altabef et al., 1997; Michaud et al., 1997). The area that is the closest to the somites corresponds to the proximal somatopleure and the other to the distal somatopleure. The aim of our study was to understand how these two somatopleural mesoderm areas interact to form the ventral feather macropattern, and what molecular factors are involved.

Until recently, the specification of pterylae formation has been poorly documented at the molecular level. With respect to the dorsal pteryla, results have shown that a dorsal neural tube signal, which can be substituted by Wnt1, causes the commitment of median dermomyotomal cells into dermal progenitors (Olivera-Martinez et al., 2002; Olivera-Martinez et al., 2001), whereas nothing was known about the signaling involved in ventral dermis formation. The juxtaposition of the ventral pteryla and the midventral apterium provides a unique opportunity to understand the mechanisms that allow the specification of these two different types of skin.

Interestingly, experimental manipulation of the chick distal somatopleure at E2 can lead to the induction of a supplementary pteryla in the midventral apterium (Sengel and Kieny, 1967a; Sengel and Kieny, 1967b). This has been achieved by implanting either a living piece of neural tube or agar implants impregnated with brain extract into the presumptive territory of the midventral apterium. In preliminary experiments, we were able to reproduce these results by grafting the ventral part of stage HH 13 anterior neural tube into the same region. By contrast, the graft of the dorsal half of the same fragment of neural tube did not change the fate of the apterium. In the past ten years, many different diffusible signaling factors synthesized by the neural tube have been identified (Capdevila and Johnson, 1998; Echelard et al., 1993; Krauss et al., 1993; Parr et al., 1993; Pourquié et al., 1996; Riddle et al., 1993; Sela-Donenfeld and Kalcheim, 2000; Watanabe and Le Douarin, 1996). The anterior neural tube, between somites 15 and 19, expresses, in particular, Wnt1 and Wnt3a in its dorsal half and principally Noggin and Shh in its ventral half. *Noggin* has been shown to be dorsally expressed, but only in the posterior part of the neural tube (Sela-Donenfeld and Kalcheim, 2000). Among the diffusible signaling factors produced by the anterior ventral neural tube at stage HH 13 that can inhibit Bmp4 signaling, two are also produced in the vicinity of the somatopleure: Noggin in the intermediate mesoderm (Sela-Donenfeld and Kalcheim, 2000) and Sonic Hedgehog in the endoderm (Watanabe et al., 1998). Moreover, fusions between the splanchnic and somatic tissues have previously been shown to be correlated with the formation of ectopic pterylae (Sengel and Kieny, 1967a; Sengel and Kieny, 1967b; Dhouailly, 1978). Noggin and Shh thus seemed plausible candidates to play a role in pterylae induction from the somatopleure.

Noggin is known to operate by binding to Bmp2, Bmp4 and Bmp7, and preventing their interaction with their cognate receptors (Hirsinger et al., 1997; Zimmerman et al., 1996). The relationship between Shh and Bmps appears to be variable in vertebrate organogenesis. In the limb bud, expression of *Bmp2*, but not *Bmp4*, can be induced by ectopically expressed *Shh* and, at least partially, mediates the polarizing activities of Shh (Drossopoulou et al., 2000; Duprez et al., 1996; Laufer et al., 1994), whereas ectopic expression of *Shh* in the dorsal neural tube abolishes *Bmp4* expression (Watanabe et al., 1998). In the case of the somatopleure, the expression of *Bmp4* (Hirsinger et al., 1997; Sela-Donenfeld and Kalcheim, 2000) does not necessarily lead to *Bmp4* signaling. Therefore, it was of interest to analyze the pattern of expression of target genes of Bmp4 signaling, in order to distinguish between the presence of Bmp4 transcription and a signaling effect. *Msx1* is one of the target genes for Bmp signaling (Alvarez Martinez et al., 2002) but we had another reason to be interested in it: its expression is thought to be involved in delaying differentiation events (Houzelstein et al., 1999; Woloshin et al., 1995) and has previously been shown to be implicated in the delayed formation of the mediodorsal dermis in mouse embryos (Houzelstein et al., 2000). As the formation of ventral pterylae occurs in a wave from the flank to the medioventral line, we formed the hypothesis that Msx1 might play a similar role during the establishment of the chick ventral feather macropattern.

In this paper, we report that a wave of *Noggin* expression occurs in somatopleural mesoderm, and is involved in the formation of the ventral pteryla, whereas the more distal somatopleural mesoderm remains labile and expresses *Msx1*. However, whereas ectopic expressions of either *noggin* or *Shh* in the distal embryonic lateral plate is sufficient to induce the formation of a supplementary pteryla, they are both required in the extra-embryonic area.

Our results allow us to propose a model to explain the formation of the ventral skin feather macropattern, according to which the ventral pteryla is induced at E2 by endogenous Noggin, with a possible synergistic effect of Shh, which progressively downregulates Bmp4 signaling in the proximal somatopleure.

## Materials and methods

### In ovo DNA electroporation procedure

Isa Brown chick eggs were incubated at 38°C and windowed on day 2 of incubation (E2). For electroporation at E2, we used the pCAGGS-GFP (Momose et al., 1999) or the EF1-myc-cyto-GFP (Invitrogen) with the GFP-coding sequence under the control of the chick actin promoter and elongated factor 1 (EF1) promoter, respectively. The plasmid DNA were injected (6 μg/ml) in the right coelom, between the 20th and 25th somites. Electrodes were placed on both sides of the embryo axis, with the positive electrode on the left side of the embryo for the electroporation of the proximal somatopleure. Six pulses of 40 V, lasting 60 mseconds were applied. At E3, the plasmids were injected in the coelom at the flank level, close to the dorsoventral boundary. The electrodes were placed on both sides of the injection point with the cathode being on the left, neural tube side. For GFP visualization, embryos were fixed in 4% formaldehyde in PBS and flattened between a hollow glass slide and a thick coverslip. The GFP fluorescence was evaluated by comparing it with the right half of the neural tube and the presumptive extra-embryonic boundary.

### Microsurgical procedures

Chick and quail embryos were staged, respectively, according the Hamburger and Hamilton (Hamburger and Hamilton, 1951), and Zacchei table (Zacchei, 1961). Ectopic grafts of the proximal and distal somatic chick mesoderm, rotated 90°, were performed under the ectoderm as described by Saunders and Reus (Saunders and Reus, 1974). Quail eggs were incubated at 38°C until they reached their chick counterpart stage. Quail proximal or distal somatopeural mesoderm taken between the levels of 20th and 25th somites was

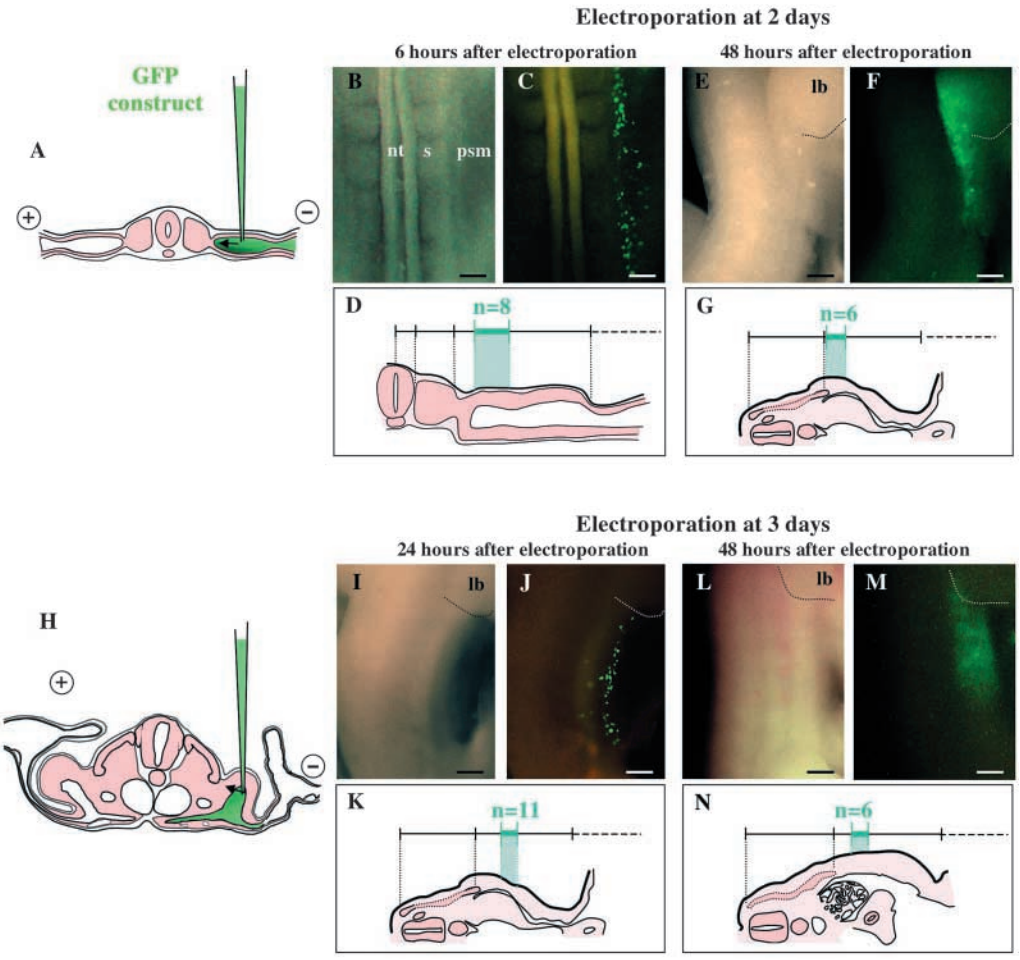


**Fig. 1.** Behavior of chick proximal somatopleural cells after in ovo electroporation of a GFP plasmid at E2 (A-G) and E3 (H-N). (A) Experimental procedure at E2. (B-D) Six hours after electroporation, GFP-expressing cells are located in the proximal somatopleural mesoderm (psm). (E-G) Forty-eight hours after electroporation at E2, GFP-labeled cells are located in the dorsal part of the limbs (lb) and/or in the lateral part of the flank. (H) Experimental procedure at E3. (I-K) Twenty-four hours after electroporation at E3, GFP-expressing cells are located in a lateral compartment of the flank. (L-N) Forty-eight hours after electroporation at E3, GFP-labeled compartment has expanded slightly towards ventral direction. *n*, the number of embryos analyzed. (B,E,I,L) Light microscopy; (C,F,J,M) fluorescence microscopy; (D,G,K,N) schematic localization of fluorescent cells. nt, neural tube; s, somite. Scale bars: 60 μm in B,C; 250 μm in E,F,I,J; 280 μm in L,M.

grafted in chick in an orthotopic manner. The grafts in the presumptive territory of the midventral apterium consisted of axial organs as described by Kieny and Sengel (Kieny and Sengel, 1964). Cell aggregates of various transformed cell lines producing diffusible factors were grafted between the ectoderm and the mesoderm at the limit or at the exterior of the embryonic area, posteriorly to the level of the 20th somite.

### Cyclopamine treatment

Cyclopamine (BIOMOL) was resuspended in 95% ethanol as previously described (Sukegawa et al., 2000) at a concentration of 10 mM. Embryos were treated at E2 with 1 μl of cyclopamine suspension. Control embryos were treated with an equivalent volume of 95% ethanol.

### Cell lines and obtention of cell aggregates

The Noggin-producing CHO cell line (CHO.B3A4) and the parent cell line (CHO DHFR-) were supplied by Dr R. Harland and Dr J. M. de Jesus (Lamb et al., 1993). The QT6 cell line, which expresses chick *Shh* under the influence of the CMV promoter in the pBK plasmid, and the corresponding control-QT6 cells, as well as the Bmp2/QT6 cell line were provided by Dr Duprez (Duprez et al., 1996). A Wnt1-producing fibroblast Rat-B1 cell line and the control cells were a gift from Dr Nusse. A Wnt3a-producing Rat-B1 cell-line was provided by Dr Kitajewski. The cells were plated on uncoated bacteriological Petri dishes to form aggregates.

### In situ hybridization, immunohistochemistry and histology

Whole-mount in situ hybridization was carried out as described by Wilkinson (Wilkinson, 1995). The cDNA templates for chicken *Bmp2*, *Bmp4* (Francis et al., 1994) and *Shh* (Riddle et al., 1993) were generated by RT-PCR, while the *Noggin* and *follistatin* probes were provided by Dr Hurle, and the *Msx1* probe by Dr B. Robert. The stained embryos were processed for cryosections (30 μm) following inclusion in gelatin/sucrose. For immunohistochemistry, embryos were fixed in Carnoy's fluid, embedded in paraffin wax and sectioned at 7 μm. In order to distinguish quail cells from chick cells, we used the monoclonal antibody QCPN (Developmental Studies Hybridoma Bank) as described (Catala et al., 1996); sections were counterstained with Hematoxylin.

## Results

### Behavior of proximal somatopleural cells

Although it is evident that there is an expansion of the lateral embryonic somatopleure during the first few days of chick development, it is not known whether the cellular expansion was by coordinated groups or individual cells. We therefore used plasmids expressing the GFP protein under the control of a ubiquitously active promoter as a transient cell lineage marker. The proximal part of the somatopleural mesoderm was electroporated at E2 (Fig. 1A). Six hours afterwards (Fig. 1B-D), the GFP-expressing cells were located in a narrow stripe in the most proximal part of the lateral plate (Fig. 1C). Because the plasmid was injected in the coelom, the stained stripe extended along the anteroposterior axis, usually as far as the

level of the limbs. Forty-eight hours after electroporation, the GFP-expressing cells made a thicker stripe in most of the embryos (Fig. 1E-G) in the dorsal part of the limbs or in the lateral part of the flank (Fig. 1F). Embryos electroporated at 3 days in the proximal mesoderm of the flank (Fig. 1H) were harvested after 24 and 48 hours. At E4, the GFP-expressing cells have migrated in a slightly ventral direction (Fig. 1I-K), when compared with the marked cells in embryos electroporated at E2 (Fig. 1G). This could be due to the difficulty of reaching the most lateral part of the somatopleural mesoderm at E3. Forty-eight hours after the electroporation at E3, the GFP-labeled cells were located slightly more ventrally than at 24 hours before (Fig. 1L-N). Thus, labeled cells from the proximal part do not diffuse throughout the whole somatopleure, but remain in a defined compartment, which expands from stage to stage in the direction of the midventral line.

### The proximal somatopleural mesoderm gives rise to the ventral pteryla, while the distal somatopleural mesoderm stays labile

In order to find out which region of the somatopleure is responsible for the establishment of the ventral skin pattern, we first grafted chick proximal somatopleural mesoderm rotated 90° under the ectoderm of the chick presumptive ventral region at stage HH13 (Fig. 2A). After 8 days of incubation, a supplementary pteryla was formed in 6 out of 12 cases, crossing the ventral pteryla, and extending over the midventral apterium (Fig. 2B). In the six remaining cases, we had no way of knowing whether or not the graft fitted well. In a second series of experiments, we took advantage of the difference between the chick (Fig. 2C) and the quail (Fig. 2D) ventral feather macropattern. The quail midventral apterium is almost imperceptible, and there is no true semi-apterium between the pectoral and ventral pterylae. The graft of the quail proximal somatopleural mesoderm under the right proximal ectoderm of stage HH13 chick embryo (Fig. 2E) resulted (in 12 out of 25 cases) in the formation of a quail-type ventral feather macropattern on the right side of the embryo (Fig. 2F). In the 13 cases in which the ventral pattern was of chick type, quail dermal cells were not found upon histological analysis, leading us to conclude that the graft was lost.

The next experiments involved homospecific chick grafts of distal somatopleural mesoderm, in place of the proximal mesoderm ($n$=25) or rotated 90° ($n$=24). These resulted in the formation of a normal ventral skin feather macropattern in all cases (data not shown). Thus, the proximal somatopleural mesoderm at E2 is responsible for the formation of the ventral pteryla, whereas the distal somatopleural mesoderm is not yet committed. Its more distal part gives rise to an apterium, but can be induced to form a pteryla when transferred in a proximal location.

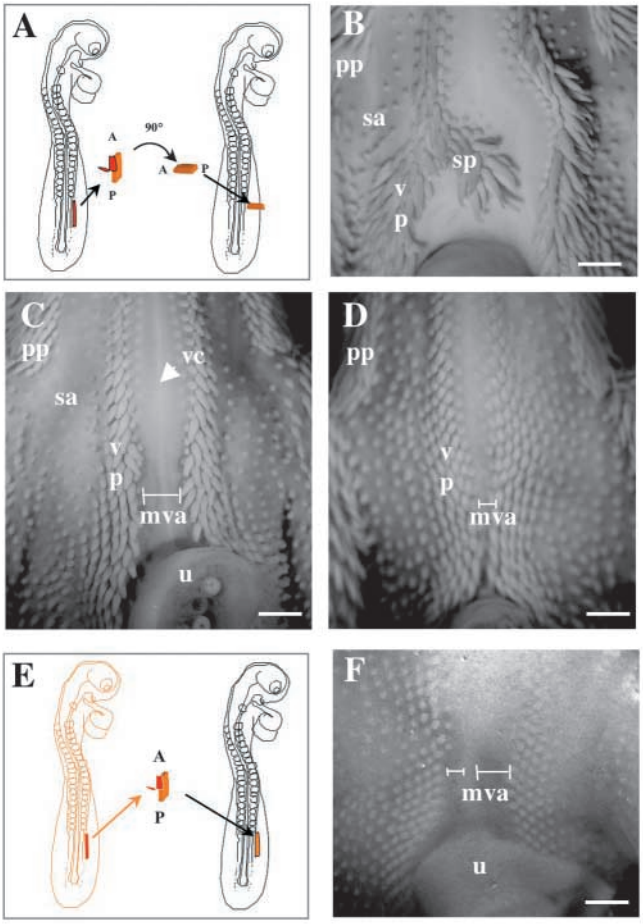


**Fig. 2.** The proximal somatopleural mesoderm is determined to form the ventral pteryla at stage HH13. (A) Diagram of the microsurgical procedure in chick embryo. The proximal somatopleural mesoderm (after peeling off the ectoderm) was grafted with a 90° rotation under the ectoderm of a host at the same stage. (B) At day 11: a supernumerary pteryla (sp) across the midventral apterium, which is rotated almost 90° compared with the endogenous ventral pteryla (vp), is formed. (C) Ventral feather macropattern of a E10 chick embryo: the pectoral (pp) and ventral pterylae (vp) are separated by a semi-apterium (sa). The midventral apterium (mva) is delineated on each side of the midventral closure (vc). (D) Ventral feather macropattern of a E10 quail embryo: there is no semi-apterium between the pectoral and ventral pterylae, and the midventral apterium is almost imperceptible. (E) The microsurgical procedure: a fragment of the right proximal somatopleure is removed from a HH13 quail embryo, the ectoderm is peeled off and the mesoderm is grafted orthotopically under the ectoderm of a HH13 chick embryo. (F) Ventral view at E10: the operated side presents a quail type feather macropattern, characterized by no distinguishable semi-apterium and a very narrow midventral apterium. ant, anterior; post, posterior; u, umbilical cord. Scale bar: 1.3 mm in B,C,D,F.

### Noggin- or Shh-expressing cells can induce the formation of a supplementary pteryla in the embryonic midventral apterium

In order to identify what signals might be responsible for the formation of the somatopleural derived feather tracts, we investigated the molecular mechanisms of the induction of a supplementary pteryla in the midventral apterium. First, we grafted (Fig. 3A) either fragments of stage HH13 anterior whole neural tube associated with chord, the dorsal half of the neural tube or the ventral half plus chord under the ectoderm of the presumptive midventral apterium. Second, we grafted (Fig. 3A) aggregates of cells engineered to produce diffusible signaling factors, including Wnt1/Rat-B1a, Wnt3a/Rat-B1a, Shh/QT6 cells, Noggin/CHO cells and control cells (Table 1). Only grafting the ventral half of the neural tube plus chord (Fig. 3B), Shh- (Fig. 3C) or Noggin-producing cells (Fig. 3D) led,

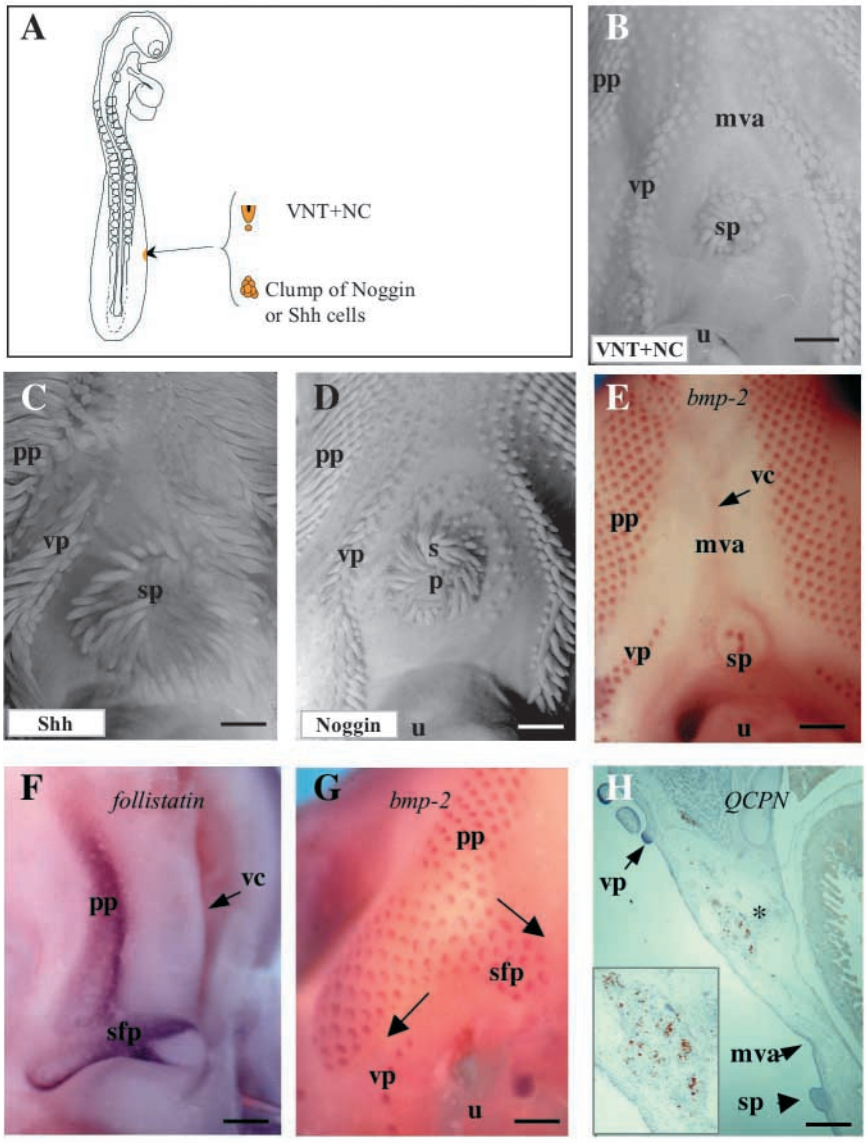


**Fig. 3.** Induction and analysis of supplementary pterylae (sp) in the midventral apterium (mva). (A) Microsurgical procedure: fragments of 2-day-old ventral neural tube plus chord, or aggregates of cells expressing Shh or Noggin were grafted under the ectoderm of HH13 embryo in the presumptive territory of the midventral apterium, posterior to the level of the 20th somite. (B-D) Supplementary pteryla obtained 8 days after the graft of (B) ventral neural tube (VNT) plus chord (NC) fragment, (C) aggregate of Shh cells and (D) aggregate of Noggin cells. Compare with the midventral apterium of an unoperated embryo in Fig. 2C. (E) *Bmp2* expression 6 days after the graft of Shh cells. A midventral stripe forms a fork around the supplementary pteryla. (F) *follistatin* expression 6 days after the graft of Noggin cells. A supplementary fused pteryla (sfp) appears distinct from the pectoral pteryla (pp). (G) *Bmp2* expression at E8 of an embryo grafted with Noggin cells and presenting a supplementary pteryla fused to the pectoral pteryla. *Bmp2* transcripts are localized in the rostral part of the placodes, which allows us to distinguish the different orientations (arrows) of the ventral (vp) and supplementary fused pteryla. (H) Section at the level of a supplementary pteryla obtained with Shh cells, labeled with QCPN antibody and revealed with the peroxidase reaction (brown). The Shh-implanted cells are found (asterisk and enlargement) far from the feather buds of the supplementary pteryla. u, umbilical cord; vc, ventral closure. (E-G) In situ hybridization. Scale bars: 1.3 mm in B,D,F; 1.2 mm in C; 2 mm in E; 1.5 mm in G; 400 μm in H.

in about 27% of cases, to the formation of a supplementary pteryla (13 independent and 20 fused ones out of 121 cases in total). The graft of control QT6, CHO and Rat-B1a cells, and of Wnt1- and Wnt3a-producing cells (182 cases in total) never caused the formation of a supplementary pteryla.

The supplementary pterylae obtained within the midventral apterium have several characteristics (Fig. 3B-E). They are demarcated from the normal feather tracts by a semi-apterium. Their feather buds appear concomitantly with the feathers of the ventral pteryla. They arise in circular waves from a central point, whereas the feather buds normally appear in successive rows. Moreover, the feather orientation is not correlated with the rostrocaudal axis of the embryo, but is at a tangent to the circular wave. In more than half of the cases, the supernumerary pteryla fused with the neighboring feather field (Fig. 3F,G). We used *follistatin* and *Bmp2* expression patterns (Jung and Chuong, 1998; Patel et al., 1999) as markers. At E7, *follistatin* expression demarcates the future pterylae (Fig. 3F), and at E8 *Bmp2* labels the anterior part of the feather buds (Fig. 3G) and the location of the midventral apterium. When the supplementary pteryla is located in the midventral apterium, the midventral stripe of *Bmp2* expression forks around it (Fig. 3E). When the ectopic pteryla is fused to the ventral pteryla, the *follistatin* and *Bmp2* expression patterns showed that two distinct feather tracts were induced at the beginning: the feather buds arose from two single rows extending in divergent directions (Fig. 3F), with their orientation following the extended axis of the pteryla (Fig. 3G).

**Table 1. Formation or lack of formation of a supplementary pteryla as a function of the type of grafted tissue or engineered cells**

| Grafts | Total number of cases | With a supplementary pteryla | |
|---|---|---|---|
| | | In midventral apterium | Fused with ventral pteryla |
| Whole 2-day-old neural tube | 9 | 2 | 2 |
| Ventral neural tube | 8 | 1 | 2 |
| Dorsal neural tube | 8 | 0 | 0 |
| Shh/QT6 cells | 87 | 9 | 13 |
| QT6 (control) cells | 41 | 0 | 0 |
| Noggin/CHO cells | 34 | 4 | 7 |
| CHO/DHFR- (control) cells | 14 | 0 | 0 |
| BMP2/Rat-B1a cells | 56 | 0 | 0 |
| Wnt1/Rat-B1a cells | 60 | 0 | 0 |
| Wnt3a/Rat-B1a cells | 11 | 0 | 0 |

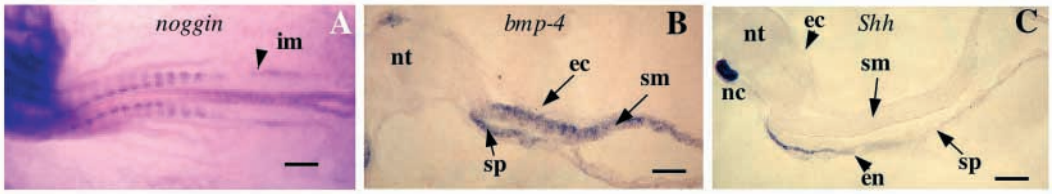


**Fig. 4.** Expression patterns of *Noggin* (A), *Bmp4* (B) and *Shh* (C) at late stage HH13 in chick embryo. (A) *Noggin* expression, dorsal view. Posteriorly to the 20th somite, the transcripts are localized in the dorsal neural tube and in the intermediate mesoderm (im). (B) *Bmp4* expression at the same level in transverse section. The transcripts are localized in the dorsal neural tube (nt), the entire somatopleural mesoderm (sm) and in proximal splanchnopleural mesoderm (sp). (C) *Shh* expression at the same level in transverse section. The transcripts are localized in the chord (nc) and in the proximal endoderm (en). ec, ectoderm. Scale bars: 200 μm in A; 60 μm. in B,C

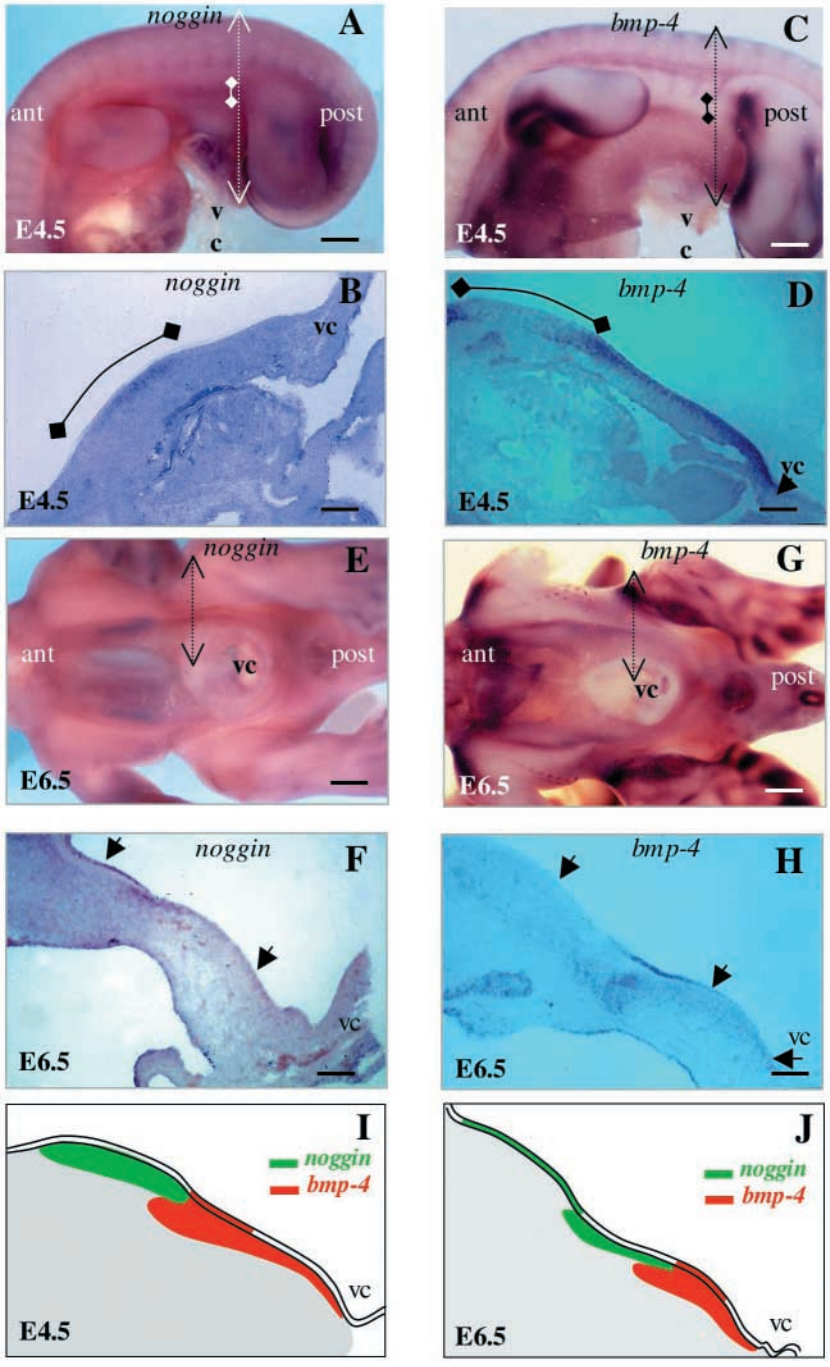


**Fig. 5.** Expression patterns of *noggin* and *Bmp4* in E4.5 and E6.5 chick embryo. (A-D) E4.5; lateral view (A,B) and transversal sections (C,D) showing the expression of *Noggin* (A,B) and *Bmp4* (C,D). The diamond-bounded line delimits a similar area in A-D. (E-H) E6.5; ventral view (E,G) and transversal section (F,H) showing the expression of *noggin* (E,F) and *Bmp4* (G,H). Arrows delimit a similar area in F and H. Broken lines in A,C and E,G show the level of sections in B,D and F,H, respectively. (I,J) Diagram of transversal sections on one side of the ventral closure (vc) showing the exclusive expression domains of *noggin* and *Bmp4* at E4.5 (I) and E6.5 (J). ant, anterior; post, posterior. Scale bars: 500 μm in A,C; 80 μm in B,D; 700 μm in E,G; 120 μm in F,H.

Previous experiments have shown that overexpression of *Noggin* and *Shh* at the time of feather primordia causes the formation of supplementary primordia (Jiang et al., 1999; Noramly and Morgan, 1998; Morgan et al., 1998; Ting-Berreth and Chuong, 1996). In order to determine whether or not the supernumerary pterylae could result from such late effects of Noggin or Shh in our experiments, the grafted cells (quail cells for the Shh-producing cells) were localized at the time of feather formation (E9/E10). In five out of five analyzed cases, they were detected in the deep mesenchyme, at the level of the future semi-apteria, or under the ventral pteryla, but always a clear distance away from the supplementary pteryla (Fig. 3H). This result rules out the possibility that the supplementary pteryla could result from a late effect of grafted cells on feather initiation. The induction of a supplementary pteryla is thus an effect of the signaling factors at the time of grafting (E2) that leads to an autonomous differentiation of the distal embryonic somatopleural mesoderm into dermal progenitors, which are able to trigger the formation of feather primordia several days later.

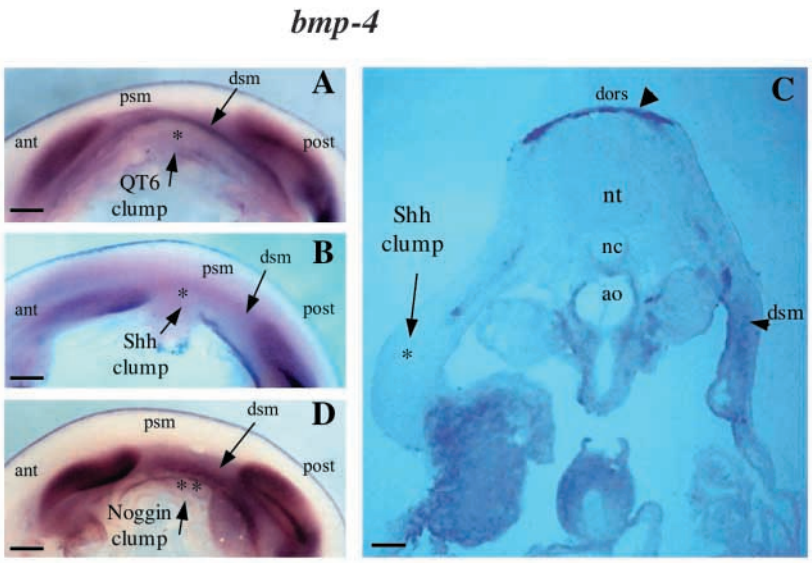


**Fig. 6.** Effect of grafting Shh and Noggin expressing cells on *Bmp4* expression in the distal somatopleure (dsm). (A) At E3, 24 hours after the graft of QT6 control cells (asterisk), the embryo shows a normal extent of *Bmp4* expression. (B) At E3, 24 hours after the graft of Shh cells at the embryonic boundary, *Bmp4* expression is disrupted around the graft. This was also observed (C) 48 hours after the graft. On this transversal section, there is *Bmp4* expression in the dorsal (dors) mesenchyme (short arrow). (D) By contrast, no change in *Bmp4* expression was seen around the two adjacent aggregates at E3, 24 hours after the graft of Noggin cells at the extra-embryonic limit. ant, anterior; ao, aorte; nc, chord; dsm, distal somatopleure; nt, neural tube; post, posterior; psm, proximal somatopleure. Scale bars:150 μm in A,B,D; 80 μm in C.

### Distribution of *Noggin, Bmp4* and *Shh* transcripts during ventral skin morphogenesis, as well as after the graft of Noggin- or Shh-producing cells

In order to determine which factors might be implicated in the induction of the ventral pteryla, we followed the distribution of the transcripts for *Noggin*, *Bmp4* and *Shh* in the body wall region from E2 to E10. Previous studies (Hirsinger et al., 1997; Sela-Donenfeld and Kalcheim, 2000) have already described *Noggin* and *Bmp4* expression patterns in the somatopleure at stage HH13. At this stage, *Noggin* is expressed in the dorsal neural tube and the intermediate and lateral plate mesoderm beside the unsegmented presomitic mesoderm (Fig. 4A), *Bmp4* is expressed throughout all the somatopleural mesoderm and the proximal part of the splanchnopleural mesoderm (Fig. 4B), and *Shh* is expressed in the chord and part of the endoderm beneath the proximal lateral plate mesoderm (Fig. 4C).

At E4.5, *Noggin* expression shifts to a restricted area (Fig. 5A,B) that corresponds to the proximal part of the presumptive ventral pteryla, while *Bmp4* transcripts are detected on serial sections more distally in the somatopleural mesoderm in a field that excludes *Noggin*-expressing cells (Fig. 5C,D). *Bmp4* is expressed in the ectoderm at this stage, just above its mesenchymal localization (Fig. 5D). At E6.5, *Noggin* expression is detected in the ectoderm of the prospective ventral pteryla, and its mesenchymal expression shifts laterally towards a more distal region (Fig. 5E,F), and the same for *Bmp4* ectodermal and mesenchymal detected transcripts (Fig. 5G,H). The location of *Noggin* and *Bmp4* transcripts at E4.5 (Fig. 5I) and E6.5 (Fig. 5J) are depicted schematically to emphasize their exclusive pattern. From E4.5, *Shh* transcripts are no longer detectable in the endoderm (data not shown), and by E8.5 they are localized in the epidermis of the feather primordia (Morgan et al., 1998; Ting-Berreth and Chuong, 1996). At E8.5, *Noggin* is no longer expressed in ventral skin, while *Bmp4* is transiently expressed in the ectoderm throughout the entire field of the pteryla in the anterior mesoderm of the feather primordia (Patel et al., 1999; Noramly and Morgan, 1998; Jung et al., 1998).

In order to help define the early effects of ectopic *Shh* or *Noggin* expression in the somatopleural mesoderm, we followed the expression of *Shh*, *Noggin* and *Bmp4*, 24 and 48 hours after grafting of Shh- or Noggin-producing cells. In comparison with the control (Fig. 6A) a downregulation of *Bmp4* expression occurs after grafting of Shh cells within the lateral plate at 24 hours ($n$=5/14) (Fig. 6B) and 48 hours ($n$=5/12) (Fig. 6C), whereas *Bmp4* expression remains unaffected by the graft of Noggin cells ($n$=15) (Fig. 6D). The graft of Shh or Noggin cells has no effect on endogenous *noggin* and *Shh* expression, respectively (five cases for each).

### Distribution of *Msx1* transcripts during ventral skin morphogenesis is altered after the graft of Noggin- or Shh-producing cells or cyclopamine treatment

At late stage HH13, *Msx1* is expressed in the distal somatopleural mesoderm (Fig. 7A,B), and its expression becomes more and more restricted ventrally until E8.5 (Fig. 7C-F), when it is limited to the scarce mesenchyme of the midventral apterium. The grafts of aggregates of Noggin cells ($n$=2/5) in the distal somatopleural mesoderm led to the downregulation of *Msx1* expression after 24 hours (Fig. 7G), which still persisted after 72 hours (Fig. 7H). A similar effect is obtained after the graft of Shh cells ($n$=2/5) (Fig. 7I). By contrast, the grafts of CHO and QT6 control cells had no effect on *Msx1* expression (five cases for each) (data not shown). In the reverse case, when embryos were treated at E2 with cyclopamine, which is known to block Shh signaling (e.g. Chen et al., 2002), at E4.5 the *Msx1* territory was maintained in four cases out of ten surviving embryos (Fig. 7J-L), by comparison with the restricted *Msx1* territory of a control embryo (Fig. 7M-N). Altogether, these results suggest that endogenous Noggin and Shh can block Bmp4 signaling. Moreover, the loss of *Msx1* expression after grafts of both types of cells and, by contrast, the persistence of *Msx1* expression when Shh signaling is blocked by the cyclopamine suggests a direct relationship between Bmp4 signaling and the expression of *Msx1*.

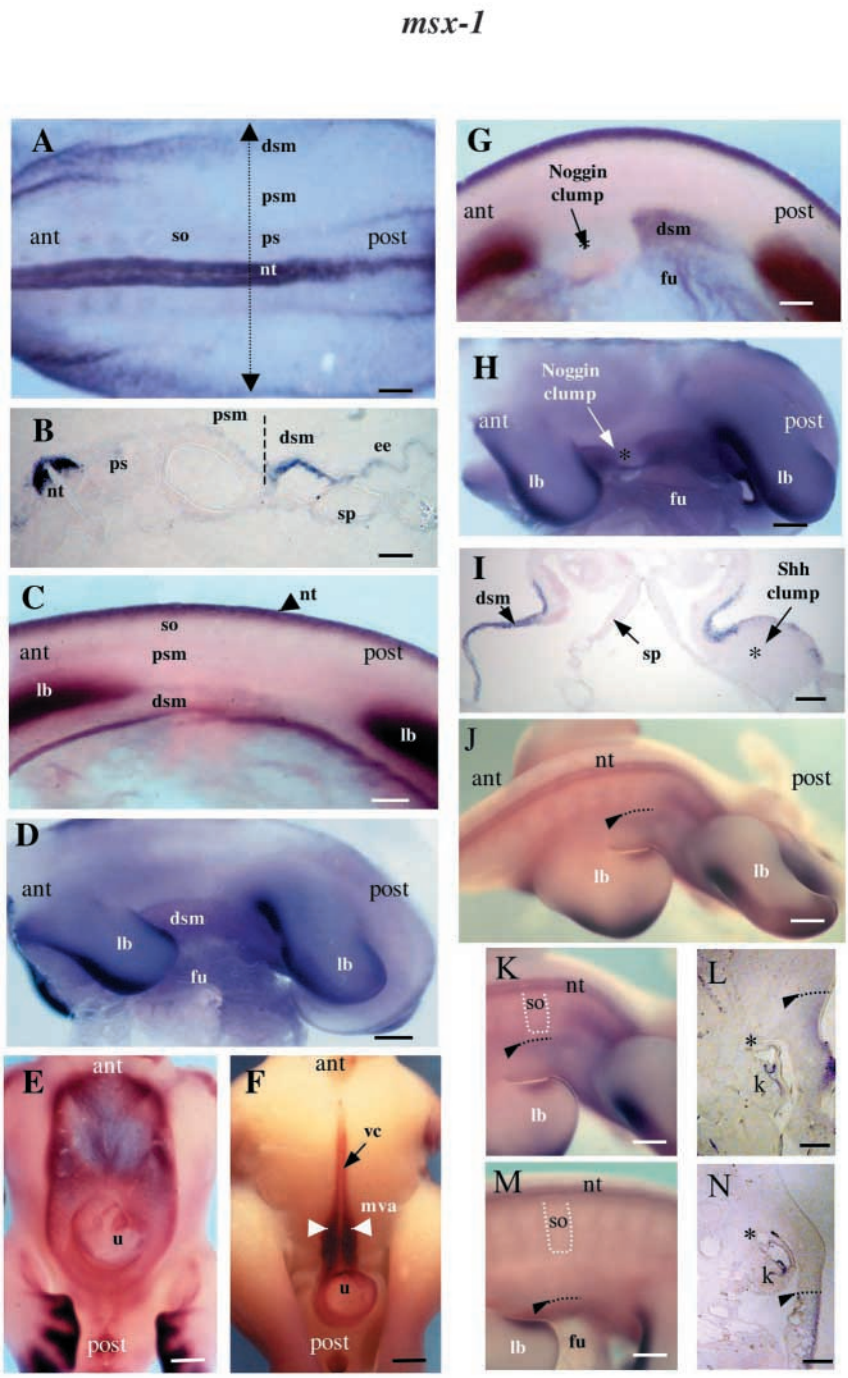


**Fig. 7.** *Msx1* expression at different stages of chick development from E2 to E8.5 (A-F), and its regulation after the graft of Noggin (G,H) or Shh (I) cell aggregates, and after treatment with cyclopamine (J-N). (A,B) Dorsal view (A) and transversal section (B) at late stage HH13 posterior to the level of the last somite formed (double arrow in A). *Msx1* is expressed in the dorsal neural tube (nt), in the distal somatopleural mesoderm (dsm) and weakly in the extra-embryonic area (ee). (C-F) *Msx1* expression on the lateral side at E3 (C) and E4.5 (D), and on the ventral side at E6.5 (E) and E8.5 (F). There is progressive restriction of the domain of *Msx1* expression from E4.5 to E8.5 to the midventral apterium (mva), as well as expression in the amnion forming the umbilical cord (fu) at early stages, or later, the umbilical cord wall (u). (G,H) *Msx1* expression is downregulated 24 hours (G) and 72 hours (H) after grafting of Noggin cells (asterisk). (I) Transverse section at E3 of an embryo showing that *Msx1* expression is downregulated over the Shh cell aggregate (asterisk). (J-N) The proximal limit of the *Msx1* expression domain (arrowhead, dotted line) is extended in the proximal somatopleure 72 hours (J-L) after the inhibition of Shh signaling mediated by a treatment with cyclopamine, in contrast to a control (M,N) embryo. (K) Close-up of the embryo shown in J. The domain of expression is larger in the treated embryo (K,L) than in the control (M,N). The extent of *Msx1* expression can be determined by reference to the kidney (k) and to the extremity of the coelom (*). ant, anterior; lb, limb bud; post, posterior; ps, presomitic mesoderm; psm, proximal somatopleural mesoderm; so (white dotted line), somite; sp, splanchnopleure. Scale bars: 100 μm in A,I; 60 μm in B; 75 μm in C,G; 500 μm in D,H,J; 700 μm in E; 750 μm in F; 110 μm in K,L; 100 μm in M,N.

### Noggin and Shh work synergistically to promote feather forming skin formation from the extra-embryonic somatopleure

We grafted (Fig. 8A) either Shh (*n*=25) or Noggin (*n*=11) cells under the E2 extra-embryonic ectoderm. Eleven days afterwards, no pterylae formed among the 36 cases recovered, but in some of the Shh cases only minute protrusions formed from the umbilical cord or from the amnion (data not shown). Only by grafting Shh and Noggin cells together (forming a mixed clump) in nine cases out of 25 (36%) a supplementary pteryla was obtained on the extra-embryonic amnion (Fig. 8B). At E14, a section through this ectopic pteryla, which displays numerous feather filaments, shows a dense dermis, exclusively formed by chick cells, overlaid by a multilayered epidermis. By contrast, the featherless surrounding amnion contains very few mesodermal cells, overlaid by a thin ectoderm. No quail cells (Shh-producing cells) were found in the vicinity of the ectopic pterylae (data not shown). In order to be able to find the grafted cells, we recovered the embryos after a shorter time, at E11. At this stage, the feather primordia allows the localization of the ectopic pteryla. In 100% of cases (*n*=6) histological analysis showed that the ectopic pteryla is constituted exclusively of chick cells (Fig. 8C,D). By analyzing the sections that were proximal to the embryo, the Shh cells (Fig. 8E,F) were found at about 750 μm from the ectopic pteryla. These results lead to two conclusions. First, *Shh* and *noggin* expression appear to be required simultaneously to induce the formation of a feather forming skin from the extra-embryonic somatopleure. Moreover, localized host somatopleural mesoderm cells are induced to become a feather-forming dermis by the grafted cells during a short time window, and are subsequently dragged distally by the general extension of the extra-embryonic area.

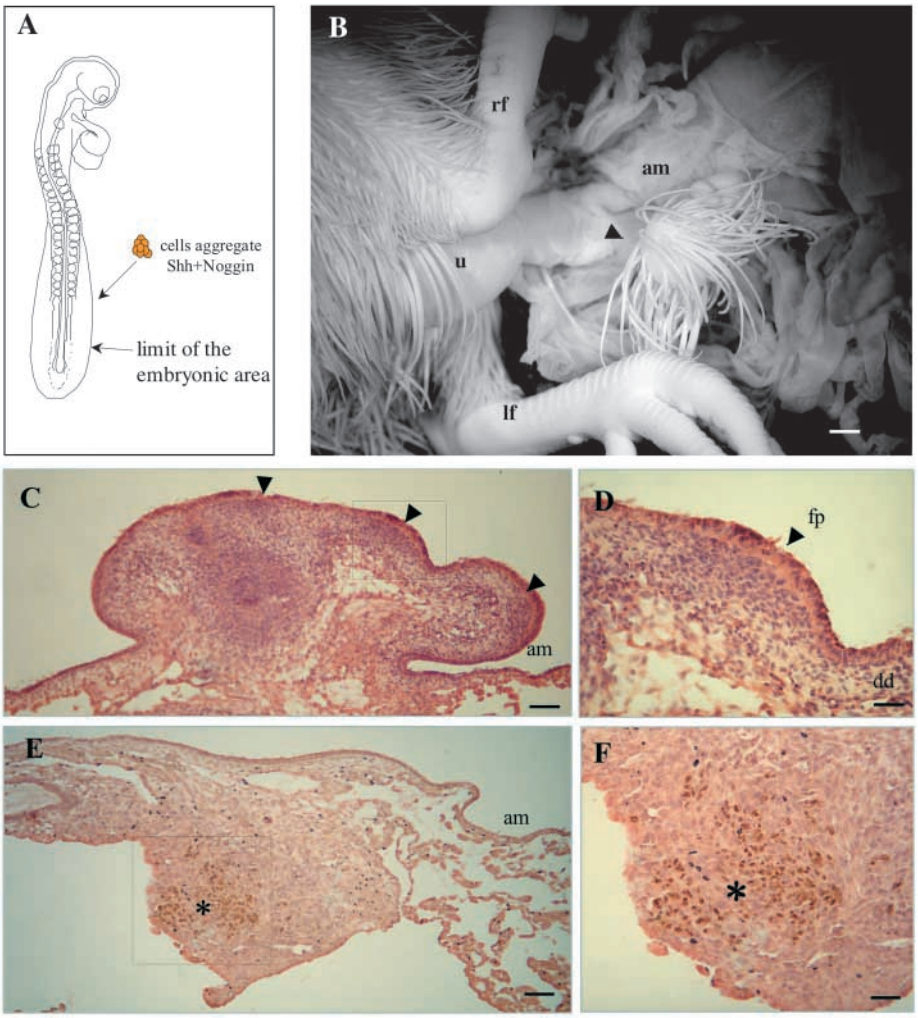


**Fig. 8.** Production of an ectopic feathered skin in the chick amnion. (A) Microsurgical procedure: aggregates of Shh cells together with Noggin cells were grafted under the ectoderm of a HH13 embryo at the exterior of the embryonic boundary, under the level of the 20th somite. (B) At E14, the embryo shows a supplementary pteryla (arrow) in the amnion (am). (C-F) Histological sections at E11 of a supplementary pteryla and surrounding amnion. (C,D) Ectopic pteryla showing dense dermis (dd) and feather primordia (fp and arrowheads) but no Shh-producing cells. (E,F) Shh quail cells (asterisks) are found on a section located 750 μm away from the ectopic pteryla as labeled with QCPN antibody and revealed with the peroxidase reaction (brown). lf, left foot; u, umbilical cord; rf, right foot. Scale bars: 1.3 mm in B; 60 μm in C,E; 220 μm in D; 150 μm in F.

## Discussion

### Ventral versus dorsal dermis formation

A primary difference between dorsal and ventral dermis formation in birds concerns the behavior of committed mesodermal cells. Dorsally, the dermal cell progenitors, on leaving the somite dermomyotome, migrate independently from one another to reach their final location under the ectoderm (Olivera-Martinez et al., 2002). Ventrally, the formation of a dense dermis by the upper part of the somatopleure is concomitant with the ventral expansion of this layer. The molecular mechanisms required to bring about the determination of a feather forming dermis appear also to differ. Indeed, the fate of an ectopic graft of labile distal somatopleural mesoderm depends on the location of the graft: when it replaces the somites, it leads to the formation of a patch of bare skin (Mauger, 1972), whereas when it replaces the proximal somatopleural mesoderm it leads to the formation of a feathered skin. Before the specification of the dorsal dermal progenitors, Wnt1 induces *noggin* expression in the medial part of the somite, which counteracts the effect of Bmp4 signaling originating from the somatopleural mesoderm (Hirsinger et al., 1997), and allows the further specification of the medial dermomyotome by Wnt1, to form the dermal progenitors of the spinal pteryla (Olivera-Martinez et al., 2001; Olivera-Martinez et al., 2002). By contrast, Wnt1 signal does not lead to the formation of a feathered skin in the presumptive midventral apterium, rather this is obtained by Noggin or Shh. Another difference that cannot be currently explained concerns the role of Msx1. During the expansion of the ventral somatopleure, *Msx1* expression appears to be at least correlated with delaying of dermal specification. By contrast, Bmp2 application in a dorsal future semi-apteria leads to ectopic *Msx1* expression, followed by the formation of an ectopic pteryla (Scaal et al., 2002).

### The early commitment of a ventral feather-forming dermis requires Noggin and possibly Shh

The grafts of quail proximal or distal somatopleural mesoderm or chick proximal somatopleural mesoderm rotated 90° showed that, in contrast to the distal somatopleure, the proximal somatopleural mesoderm is committed to the formation of a feather-forming dermis at E2. At this stage, *Bmp4* transcripts are detected throughout the whole somatopleural mesoderm, whereas *Msx1* transcripts are located only in its distal part, suggesting that Bmp4 is active only in the distal somatopleure. The inhibition of Bmp4 signaling in the proximal part is therefore correlated to its determination as a feather tract. Likewise, at the time of feather formation (Jung et al., 1998), BMPs signaling is blocked by their antagonists (Chuong, 1998), and forced expression of *Bmp4* leads to the formation of patches of bare skin (Noramly and Morgan, 1998).

A supplementary pteryla was obtained by the grafts of Noggin or Shh cells in the prospective midventral apterium. No change of *Bmp4* expression was detected after the graft of Noggin cells, but the expression of *Msx1* was downregulated.

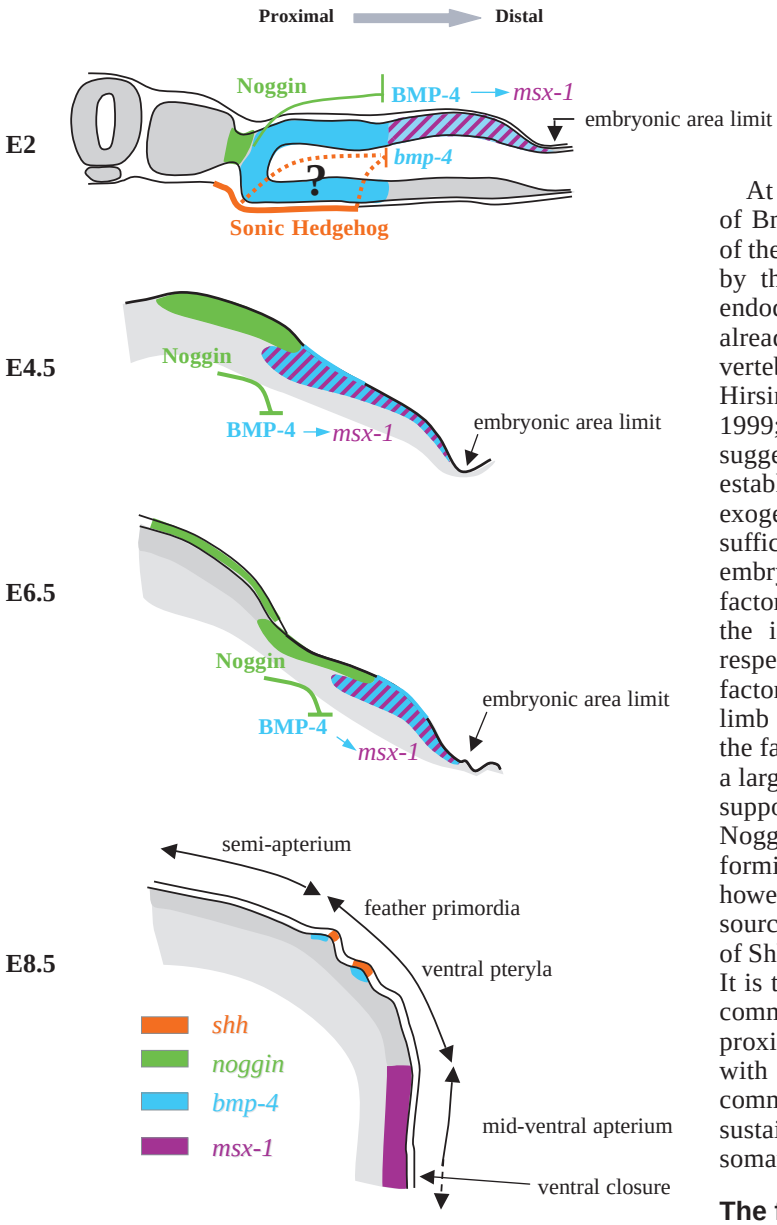


**Fig. 9.** Proposed model for the formation of the ventral pteryla versus the mid-ventral apterium in chick embryo showing *noggin*, *Shh*, *Bmp4* and *Msx1* expression from E2 to E8.5 in the body wall region.

Our data suggest that Noggin inhibits Bmp4 signaling directly, as it has been shown to in *Xenopus* (Piccolo et al., 1996; Zimmerman et al., 1996). By contrast, *Bmp4* was rapidly downregulated in the distal mesoderm after the graft of Shh cells, showing that in our model, Shh is able to act, either directly or indirectly, on *Bmp4* transcription levels. This hypothesis is supported by the use of cyclopamine, an inhibitor of Shh signaling (e.g. Chen et al., 2002). When chick embryos were treated at E2, significant expression of *Msx1* persisted at E4.5. Unfortunately, the embryos did not survive until day 11, precluding the analysis of pteryla and apteria formation. The toxicity of cyclopamine at an early stage might suggest effects beyond those on Shh activity, given that the *Shh*-null mouse survives until late stages of embryogenesis (Chiang et al., 1996). Moreover, we cannot exclude a possible role of other Bmps, such as Bmp2 or Bmp7, that were not analyzed in our study.

At the onset of somatopleural development, the inhibition of Bmp4 signaling, which correlates with the determination of the ventral pteryla, may be due to the production of Noggin by the intermediate mesoderm and, possibly, Shh by the endoderm. Interactions between Shh and Bmp4 have been already observed during the morphogenesis of several vertebrate organs and tissues (Capdevila et al., 1999; Hirsinger et al., 1997; Merino et al., 1999; Schilling et al., 1999; Watanabe et al., 1998; Zhang and Yang, 2001), suggesting that this may act as a common mechanism to establish and maintain distinct compartments. In our system, exogenous expression of either *noggin* or *Shh* alone was sufficient to produce a supplementary pteryla in the distal embryonic somatopleure. The potential complementary factor, either Shh or Noggin, is not far away, diffusing from the intermediate mesoderm and the proximal endoderm, respectively, and may act in synergy with the ectopic grafted factor. The Shh signal is known to act at a long-range during limb bud polarization (Drossopoulou et al., 2000). Besides the fact that inhibiting Shh signaling with cyclopamine led to a larger Bmp4-signaling domain, there is another observation supporting the hypothesis that Shh acts synergistically with Noggin: both are required simultaneously to induce a feather-forming skin in the extra-embryonic somatopleure. This can, however, be explained either by the fact that both endogenous source are too far away, or, alternatively, by a potential effect of Shh on mesodermal cell proliferation (Duprez et al., 1998). It is therefore conceivable that, in the case of ventral pteryla commitment, Shh might diffuse from the endoderm to the proximal somatopleural mesoderm, and hence acts together with Noggin to inhibit Bmp4 signaling to initiate commitment of the ventral pteryla, which will be later sustained by the lateral extension of *noggin* expression in the somatopleure.

### The feather field morphogenetic wave is a consequence of the lateral extension of *noggin* expression

As the embryo develops, the mesodermal expression of *noggin* shifts more distally, and so pushes back the activity of Bmp4, i.e. the *Msx1* expression in the midventral region and amnion. The ventral shift of *noggin* expression appears to reflect the ventral expansion of mesodermal cells, which is illustrated by the behavior of GFP-labeled cells. Depending to the stage of injection of the GFP plasmid in the proximal somatopleure, the labeled cells were later found in a compartment corresponding to the expression domain of *noggin*, or in the most distal half of the *noggin* expression domain, respectively. These cells then shifted slightly more ventrally, concurrent with the *noggin* expression domain. The expression of *Msx1*, which shifts progressively towards the midventral line, might correlate with the delay, and finally, end of the formation of the ventral dense dermis. This confirms that the formation of the midventral apterium is due to the lack of differentiation of a true dermis (Sengel et al., 1969).

### Autonomous somatopleural formation of supplementary pterylae

The dermis of the induced ectopic pterylae, in either the embryonic or extra-embryonic somatopleure is comprised exclusively of chick host somatopleural cells. This is clearly shown by the use of quail *Shh*-producing cells, and has already been shown by using mouse tissues for the inducer (Dhouailly, 1978). The fact that the inducing cells are always found at a distance and proximally to the ectopic pterylae suggests that there is a narrow window of induction, corresponding to the short-lived contact between the cell clump and the distal somatopleural mesoderm. This is also confirmed by the fact that the formation of the independent ectopic pteryla starts from one central point, expands slightly in a circular wave and ends. The lack of rostral-caudal orientation in the supernumerary circular pterylae show that they are not integrated with endogenous positional information. This contrasts with the usual appearance of feathers in rows more or less parallel to the rostrocaudal axis. The formation of the fused pteryla might result from a slightly different mechanism, as they also start independently, but as a row. Such a difference is probably linked to the location of the cell clump at the time of grafting, closer to the proximal somatopleure.

### Ventral skin feather macropattern formation

Integrating all our results, we propose a model (Fig. 9) for the formation of the ventral pteryla versus the mid-ventral apterium. Noggin, which is produced by the intermediate mesoderm, and possibly Shh, which is secreted by the proximal endoderm at E2, act to commit the proximal somatopleural mesoderm to differentiate into a dense feather-forming dermis by inhibiting Bmp4 signaling. During the expansion of the somatopleure, proximal somatopleural mesodermal cells expressing *noggin* shift distally and push the zone of distal mesodermal cells expressing *Msx1* under Bmp4 signaling action back towards the ventral closure. *Msx1* expressing cells might delay the commitment to form a feather-forming dense dermis and, finally, give rise to the loose dermal cells of the midventral apterium.

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