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 somatopleural mesoderm 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 µm³), 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 µm³) (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.,
The embryonic somatopleure can be divided by the rostrocaudal axis into two ~150 μm parts that behave differently (Altaf 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 dermal formation. The juxtaposition of the ventral pteryla and the midventral apertium 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 apertium (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 apertium. 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 apertium. 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 Wnt3α 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 proximal mesoderm taken between the levels of 20th and 25th somites was 2002). The embryonic somatopleure can be divided by the rostrocaudal axis into two ~150 μm parts that behave differently (Altaf 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.

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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 mediadorsal dermis in mouse embryos (Houzelstein et al., 2000). As the formation of ventral pteryla 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

Iisa 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 somatopleural mesoderm taken between the levels of 20th and 25th somites was
Feather-forming dermis from somatopleure

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Feather-forming dermis from somatopleure 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 apertium, but can be induced to form a pteryla when transferred in a proximal location.

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,
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 pteryla 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 pteryla (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

<table>
<thead>
<tr>
<th>Grafts</th>
<th>Total number of cases</th>
<th>With a supplementary pteryla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>In midventral apterium</td>
</tr>
<tr>
<td>Whole 2-day-old neural tube</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Ventral neural tube</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Dorsal neural tube</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Shh/QT6 cells</td>
<td>87</td>
<td>9</td>
</tr>
<tr>
<td>QT6 (control) cells</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>Noggin/CHO cells</td>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td>CHO/DHFR- (control) cells</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>BMP2/Rat-B1a cells</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>Wnt1/Rat-B1a cells</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Wnt3a/Rat-B1a cells</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>
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 pteryla 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.
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 aperium. 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.
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.
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.
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 pteryla

The dermis of the induced ectopic pteryla, 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 pteryla 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 pteryla 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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References


Lauer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C.


