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Ectodermal patterning in the avian embryo: epidermis versus neural plate

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SUMMARY

Ectodermal patterning of the chick embryo begins in the uterus and continues during gastrulation, when cells with a neural fate become restricted to the neural plate around the primitive streak, and cells fated to become the epidermis to the periphery. The prospective epidermis at early stages is characterized by the expression of the homeobox gene *DLX5*, which remains an epidermal marker during gastrulation and neurulation. Later, some *DLX5*-expressing cells become internalized into the ventral forebrain and the neural crest at the hindbrain level. We studied the mechanism of ectodermal patterning by transplantation of Hensen's nodes and prechordal plates. The *DLX5* marker indicates that not only a neural plate, but also a surrounding epidermis is induced in such operations. Similar effects can be obtained with neural plate grafts. These experiments demonstrate that the induction of a *DLX5*-positive epidermis is triggered by the midline, and the effect is transferred via the neural plate to the periphery. By repeated extirpations of the endoderm we suppressed the formation of an endoderm/mesoderm layer under the epiblast. This led to the generation of epidermis, and to the inhibition of neuroepithelium in the naked ectoderm. This suggests a signal necessary for neural, but inhibitory for epidermal development, normally coming from the lower layers. Finally, we demonstrate that BMP4, as well as BMP2, is capable of inducing epidermal fate by distorting the epidermis-neural plate boundary. This, however, does not happen independently within the neural plate or outside the normal *DLX5* domain. In the area opaca, the co-transplantation of a BMP4 bead with a node graft leads to the induction of *DLX5*, thus indicating the cooperation of two factors. We conclude that ectodermal patterning is achieved by signalling both from the midline and from the periphery, within the upper but also from the lower layers.

Key words: *DLX5*, BMP2, BMP4, Neural plate, Epidermis, Endoderm, Homeogenetic induction, Chick

INTRODUCTION

At the time of egg laying the chick embryo consists of a single layer of ectodermal cells, the epiblast. Although morphologically the epiblast appears largely as a homogeneous, single layer, it is already patterned along an axis of bilateral symmetry, which became established in the uterus under the combined influence of gravitational and mechanical forces (for a review, see Eyal-Giladi, 1997). The specific quality of the prospective posterior epiblast is evident in functional experiments and in the expression patterns of developmental control genes (for a review, see Lemaire and Kessel, 1997).

During gastrulation, the axis of bilaterality becomes morphologically distinct in the form of the primitive streak, the avian blastopore. The epiblast gives rise to cells of the endo- and mesodermal germ layers, which spread below the ectoderm, while the ectodermal patterning continues. A major distinction is now introduced through the induction of neuroectodermal cells around the tip of the primitive streak. The height of these ectodermal cells increases gradually towards the tip and the epithelium changes from a flat to a columnar, and finally to a pseudostratified type. Fate and specification maps identify the medial ectoderm as the neural plate, and the surrounding, peripheral ectoderm as the prospective epidermis (Garcia-Martinez et al., 1993; and references therein). However, neither epidermal nor neural fate is irreversibly fixed so early, and cells can revert their fate if transplanted into a different environment (Garcia-Martinez et al., 1997). Thus, the avian neural plate is evident before the first mesendodermal or axial mesodermal cells ingress, excluding the prechordal plate and the notochord as primary sources for neural induction (Pera and Kessel, 1997). In embryos with a fully elongated streak, the circular to pear-shaped neural plate can be identified by several molecular markers, including the homeobox gene *GSX* and the High Mobility Group (HMG) box gene *SOX2* (Lemaire et al., 1997; Rex et al., 1997).

The molecular mechanism of neural induction has been well studied in the amphibium *Xenopus laevis*, and was found to be tightly coupled to the establishment of the dorso-ventral axis (for reviews, see De Robertis and Sasai, 1996; Hemmati-

Brivanlou and Melton, 1997). In frogs, the prospective epidermal ectoderm is induced by secreted factors, the bone morphogenetic proteins (BMPs). A neural fate, on the other hand, requires the inactivation of BMPs, and is achieved by direct complex formation between BMPs and neural inducing factors such as chordin, noggin or follistatin (Piccolo et al., 1996; Zimmermann et al., 1996; Fainsod et al., 1997). The mechanism of action of a fourth neural inducing factor, Xnr3, is not known (Hansen et al., 1997). The amphibian model of neural induction cannot be directly applied to the situation in birds (for a review, see Kessel and Pera, 1998). Here, chordin does not induce neuroectoderm directly, but only secondarily after evocation of a primitive streak (Streit et al., 1998). In these experiments BMP4 appeared to interfere with primitive streak development, rather than with formation of the neural plate in general. Therefore, it seemed that further requirements for neural induction exist in the chick.

In this study we address the spatial aspects of neural induction and ectodermal patterning in the chick, using the homeobox gene *DLX5* as a marker. Homeobox genes related to the *Drosophila* gene *distal-less* are known from all vertebrates (for reviews, see Stein et al., 1996a; Stock et al., 1996). Mice seem to possess six *Dlx* genes, located in three tandem clusters on different chromosomes. The major expression domains described so far are the forebrain, branchial arches, otic vesicles, facial and limb primordia, and skeletal structures (for a review, see Stein et al., 1996a). The murine *Dlx-5* gene is expressed during basal ganglia differentiation in an overlapping, sequential pattern with *Dlx-1*, *Dlx-2* and *Dlx-6* (Liu et al., 1997). Its RNA was also found in skeletal precursors and during differentiation of osteoclasts (Simeone et al., 1994; Ryoo et al., 1997). The chicken *DLX5* gene was analyzed during limb bud development, where it is active in the apical ectodermal ridge and several mesodermal cell populations (Ferrari et al., 1995). We applied *DLX5* to study planar and vertical signalling during the patterning of the epiblast by transplantation of Hensen's node, the prechordal plate or the neuroectoderm, by explantation of the lower layer and by ectopic application of BMPs. Our findings indicate that ectodermal patterning is a consequence of signalling both from the midline and from the periphery. Midline signals originate from the tip of the primitive streak and are present in the upper (neural plate) and the lower layer (endoderm, mesendoderm, mesoderm). Peripheral signalling occurs by bone morphogenetic proteins, and can occur within the ectoderm (BMP4) and from below (BMP2). Surprisingly, peripheral patterning of the avian ectoderm can also be triggered from the midline.

MATERIALS AND METHODS

Embryos

Fertile chicken (White Leghorn) eggs were obtained from Lohmann Tierzucht, Cuxhaven, Germany, and incubated at 38°C. Embryos were staged according to Eyal-Giladi and Kochav (EK; Eyal-Giladi and Kochav, 1976) and Hamburger and Hamilton (HH; Hamburger and Hamilton, 1951).

BMP sources

Human BMP2 was produced in the quail cell line Q2bn transfected with CRNCM retrovirus DNA as described (Duprez et al., 1996). Cell aggregates were prepared for transplantation as described (Pera and Kessel, 1997). Heparin beads (Sigma) were loaded with human recombinant BMP2 and BMP4 protein (Genetics Institute Inc.). They were incubated in phosphate-buffered saline (PBS) containing 100 μg BMP/ml with 0.1% bovine serum albumin (BSA) for at least 1 hour at 4°C. Control beads were soaked in 0.1% BSA/PBS. BMP-containing and control beads were washed in PBS before implantation.

Transplantations

Pieces of primitive streak were excised from HH3-4 donor embryos in Pannett-Compton saline (PCS) using a sharpened tungsten wire as described by Lemaire et al. (1997). Prechordal plate tissue (HH5-6) was prepared with 0.2% (w/v) Dispase (Boehringer Mannheim)/PCS, using a sharpened tungsten wire and a bent insect needle (Pera and Kessel, 1997). Similarly, pieces of the endoderm, the neural plate and epidermis were obtained from HH3-4 embryos and freed of adhering tissue. Dispase-treated grafts were washed before implantation. Labeling of grafts with the fixative-stable, lipophilic marker DiI (1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate; CellTracker CM-DiI, Molecular Probes, Oregon) was performed as described (Pera and Kessel, 1997). Tissue grafts, cell aggregates or beads were implanted into small pockets between the epiblast and the lower layer of the host embryos (HH3-4). In some cases the anterior and posterior part of the primitive streak, the neural plate and epidermis, BMP-sources and their respective controls were inserted into contralateral sides at equivalent levels of the host axis. In the explantation experiments, the lower layer, i.e. the hypoblast and endoderm, was removed unilaterally from the anterior part of HH3-4 embryos. The excision was repeated every 1.5-2 hours up to five times. Both transplantation and explantation experiments were performed in a modified New culture (Stern, 1993).

Isolation and characterization of the *DLX5* cDNA

A *CNOT1* homeobox fragment (Stein and Kessel, 1995) was labeled by random priming and used to screen a HH17 chick cDNA library generated in Lambda ZAPII under low stringency conditions. A 1450 bp *DLX5* clone encompassing the entire open reading frame was isolated and sequenced.

Probes and in situ analysis

Antisense riboprobes labelled with Digoxigenin (Boehringer Mannheim) were synthesized from the following chicken DNAs: 1.4 kb *DLX5* cDNA (see above), 1.6 kb *GSX* genomic DNA (Lemaire et al., 1997), 1 kb *BMP4* cDNA (Francis et al., 1994), 1.3 kb *SOX2* cDNA (Rex et al., 1997). Whole-mount in situ hybridization, histology and photography were performed as described (Pera and Kessel, 1997; Stein and Kessel, 1995).

RESULTS

DLX5: An early marker for prospective epidermis

We isolated a cDNA clone related to the *Drosophila* homeobox gene *Distal-less* by screening an HH17 (Hamburger and Hamilton, 1951) chick cDNA library with a *CNOT1* homeobox probe under low stringency conditions (see Materials and methods). The isolated homeobox gene is identical to *DLX5*, as described by Ferrari et al. (1995). We analyzed the expression of *DLX5* by whole-mount in situ hybridization in chick embryos incubated for 0-84 hours.

In the non-incubated blastoderm, expression was detectable in the marginal zone of the area pellucida, excluding the posterior zone (stage EK X (Fig. 1A,B; Eyal-Giladi and Kochav, 1976). Histological sections revealed transcripts not only in the epiblast, but also in lower layers

(Fig. 2A). With the formation of the hypoblast (EK XII), *DLX5* expression expanded into the area pellucida, and the negative posterior domain broadened (Fig. 1C,D). Transcripts were now localized exclusively in the epiblast (Fig. 2B). At the onset of gastrulation (stage HH2), *DLX5* expression formed a crescent-shaped domain distal to the tip of the primitive streak (Fig. 1E). Its intensity increased while the streak was elongating so that a demarcation line was drawn onto the embryo by the gene expression pattern, which is morphologically not evident (HH3$^+$; Figs 1F, 2C). After the streak had reached its full length, additional transcripts were detected in the outgrowing posterior part of the embryo (HH5; Fig. 1G). The whole extent of the neural plate was now flanked by *DLX5*-positive cells with a maximal concentration at the anterior margin (Fig. 2D). Towards the periphery, expression declined gradually. Thus, *DLX5* expression demarcated ectodermal cells that abut the border of the neural plate, which is itself absolutely negative. With elongation of the embryo, the expression of *DLX5* segregated into an anterior and a posterior (prospective mesodermal) domain (HH7; Fig. 1H). The anterior domain then split and characterized the epidermis around the forebrain vesicle and the prospective neural crest at the hindbrain level (HH10; Figs 1I, 2E). By HH17, *DLX*5 expression was found in the olfactory placodes, the otic placodes and vesicles, the distal mesectoderm of the branchial arches, the caudal body folds and the tail bud (Fig. 1K). These domains and their derivatives also remained positive in more advanced stages (HH21, Fig. 1L). We also then detected transcripts in the CNS, the basal telencephalon and ventral diencephalon. The domains in the apical ectodermal ridge and distinct mesodermal parts of the outgrowing limb buds have already been described elsewhere (Ferrari et al., 1995).

In summary, *DLX5* demarcates the prospective non-neural

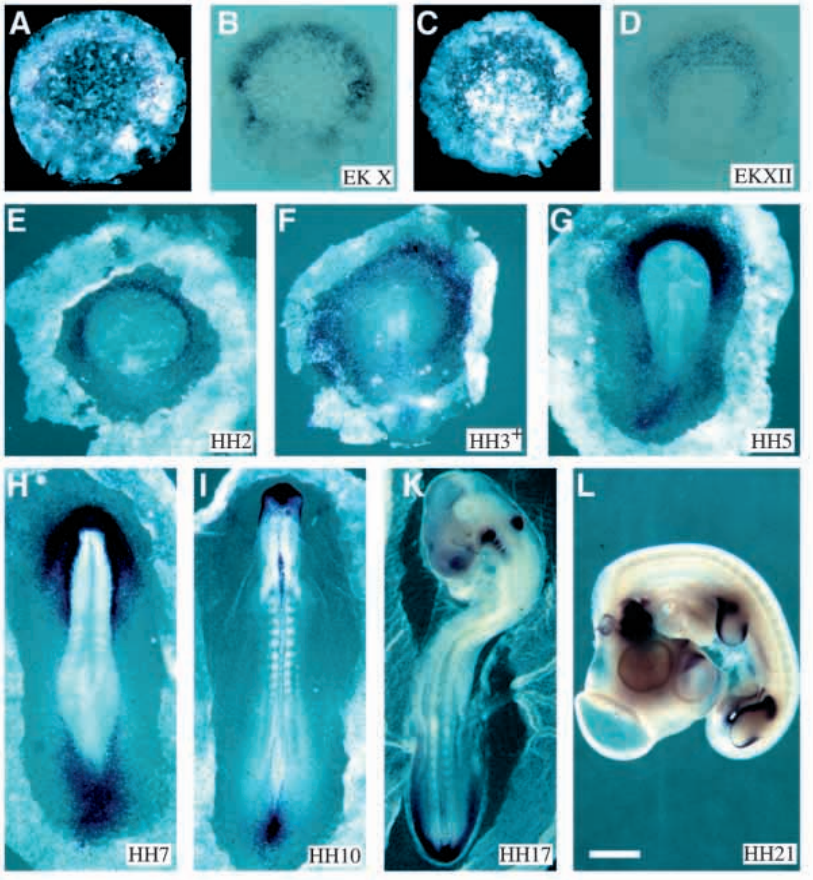

Fig. 1. Expression of *DLX5* during early chick embryogenesis. A detailed description of the patterns is given in the text. (A,B) Stage EK X. Note the absence of a secondary hypoblast in the dark field exposure. (C,D) Stage EK XII. Note the secondary hypoblast covering half of the area pellucida in the dark field exposure. (E-G) Stages HH2, HH3$^+$, HH5. Note the formation of the *DLX5* epidermal territory surrounding the developing neural plate. (H,I) Stages HH7, HH10. Note the segregation of the *DLX5* domains to the anterior and posterior poles, and the *DLX5* domain at the level of the dorsal hindbrain, the only position where *DLX5* cells become internalized as neural crest. (K). Stage HH17. Note expression in the forebrain, the otic vesicle, the neural crest of the branchial arches, the posterior body folds and the tailbud. (L) Stage HH21. Note expression in the limbs, the forebrain and the branchial arches. Bar, 880 μm (A-D), 500 μm (E,F), 700 μm (G-I), 850 μm (K) or 1000 μm (L).

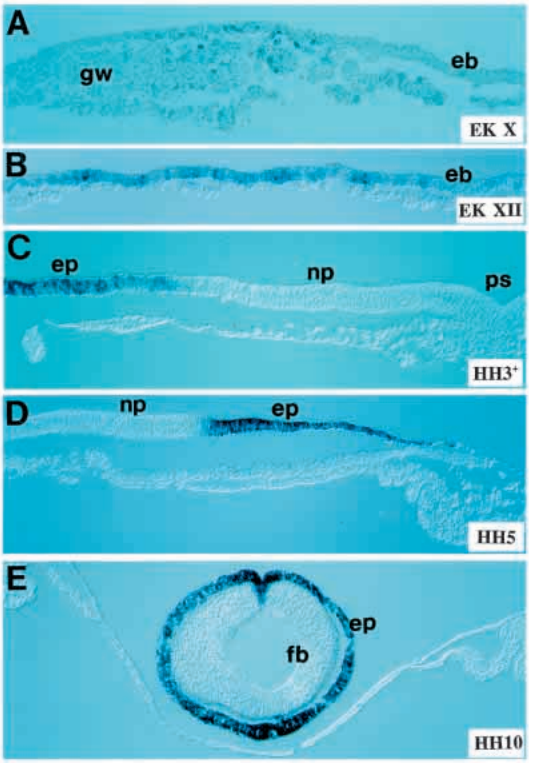

Fig. 2. Histology of unmanipulated chick embryos focusing on *DLX5* expression domains.
(A) Midsaggital section through an EK X embryo showing the anterior germ wall (gw). Note *DLX5*-positive cells in the epiblast (eb) and in the lower, yolk-rich layer. (B) Midsaggital section through an EK XII embryo showing the restriction of *DLX5*-positive cells to the epiblast (eb). (C) At HH3$^+$ note the gradually increasing height of the epithelium towards the primitive streak (ps). The epidermis (ep) is *DLX5* positive, the neural plate (np) completely negative. (D) At HH5, the discrimination between *DLX5*-positive epidermis and neural plate is even more pronounced, and now also becomes morphologically evident. (E) At HH10, note the restriction of *DLX5* expression to the surface ectoderm, completely excluding the forebrain (fb) neuroepithelium.

epidermis from early on. Exceptions occurred relatively late in development, such as the branchial arch neural crest and the ventral forebrain. We found *DLX5* expression in positions distal to the embryonic midline, away from the organizer (Koller's sickle, tip of the primitive streak, Hensen's node) or organizer-derived structures (endoderm, prechordal plate, notochord).

Hensen's node and the prechordal plate trigger not only neural, but also peripheral, patterning of the ectoderm

The early expression of *DLX5* adjacent to the border of the neural plate makes it a useful marker for the study of morphoregulatory processes. In order to provide an ectopic origin for ectodermal patterning we transplanted Hensen's nodes (HH3$^+$/4) or prechordal plates into the periphery of HH3-4 host embryos in New cultures (Fig. 3F). Such transplants are known to be powerful inducers of neuroectoderm (Waddington, 1932; Hara, 1961, 1978; Dias and Schoenwolf, 1990; Storey et al., 1992; Lemaire et al., 1997; Pera and Kessel, 1997). It was shown that only a very limited contribution of grafted node cells occurs to the neuroepithelium (Dias and Schoenwolf, 1990; Storey et al., 1992). Primitive streak grafts do not contribute cells to the induced upper layer (Lemaire et al., 1997). After incubation at 38°C for 2-10 hours the embryos were processed for whole-mount in situ hybridization with a *DLX5* riboprobe (*n*=32).

The endogenous *DLX5* expression was locally extinguished by underlying node transplants (Fig. 3A,D). In a systematic analysis of the dynamics, this repression was already observable after an incubation period of 2 hours (not shown). The decrease of *DLX5* expression preceded a thickening of the ectoderm, i.e. the indication for ectopic neural plate formation. Upon implantation into the anterior area pellucida and incubation for 7-10 hours, ectopic DLX5 expression was detected surrounding an induced neural plate (Fig. 3B). The ectopic *DLX5* expression was particularly intense on the side closest to the endogenous axis, whereas it was weak towards the area opaca, and virtually absent within the area opaca (Fig. 3A,B). In none of the specimens did we observe an ectopic *DLX5* domain without a *DLX5*-free neural plate in its center.

We have shown recently that a derivative of Hensen's node, the prechordal plate, has the ability to induce anterior neural ectoderm (Hara, 1961, 1978; Pera and Kessel, 1997, but also see Foley et al., 1997). Transplantation of a prechordal plate (HH5; *n*=4) into the anterior area pellucida of host embryos (HH3-4) resulted in ectopic neural plates with surrounding *DLX5* domains in a similar, though slightly less extensive, manner to grafted nodes (Fig. 3C). The induced neuroectoderm overlying the graft was locally thickened by a factor of five, so that the height of the epithelium equalled that of the primary neural plate (Fig. 3G,H).

The posterior part of the epiblast is not competent to develop ectopic neural epithelium upon transplantation of organizer tissue (Woodside, 1937; Gallera, 1971; Storey et al., 1992; Streit et al., 1997). Node transplants were also capable of *DLX5* repression in this area (*n*=3; Fig. 3A,E), where normally a prominent *DLX5* domain develops after extended streak stages (Fig. 1G,H). Grafts of the middle part of the primitive streak are not capable of neural induction (Lemaire et al., 1997). Such grafts induced a small *DLX5* domain without a *DLX*-negative, neural center in the overlying ectoderm (*n*=4; Fig. 3A,B). Similar results were obtained with

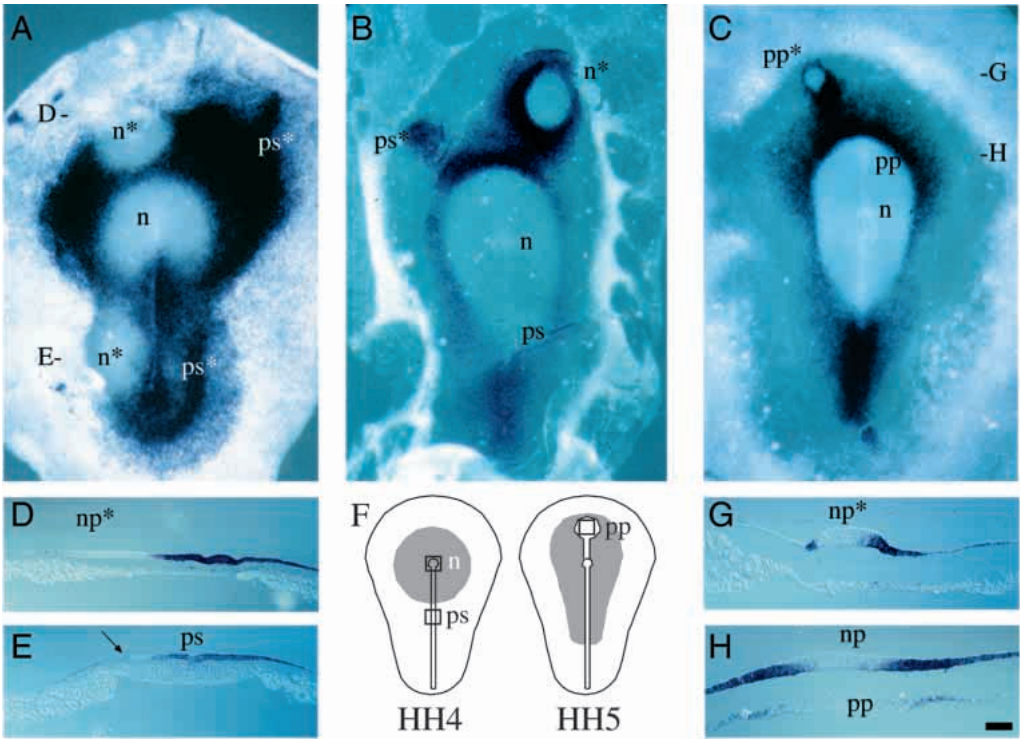

Fig. 3. Neural induction by Hensen's node and prechordal plate grafts. Indicated are the levels of sectioning (D,E and G,H), the primary node (n), the primary neural plate (np), the primary prechordal plate (pp) and the primitive streak (ps); all specimens were probed with *DLX5*. The three different grafts are indicated schematically in (F). Further details are explained in the text. (A,D,E) Transplanted nodes (n*) abolished the endogenous *DLX5* expression domains. The anterior graft has induced an ectopic neural plate (np*). Note the absence of *DLX5* expression from the area opaca. Primitive streak grafts (ps*) induced a small increase of *DLX5* expression. (B) A transplanted node (n*) or primitive streak graft (ps*) induces ectopic *DLX5* expression, with or without a central neural plate, respectively. Note the absence of *DLX5* expression from the area opaca. (C,G,H) A transplanted prechordal plate (pp*) induces an ectopic neural plate (np*), with a thickness comparable to the primary neural plate, and a surrounding *DLX5*-positive epidermis.

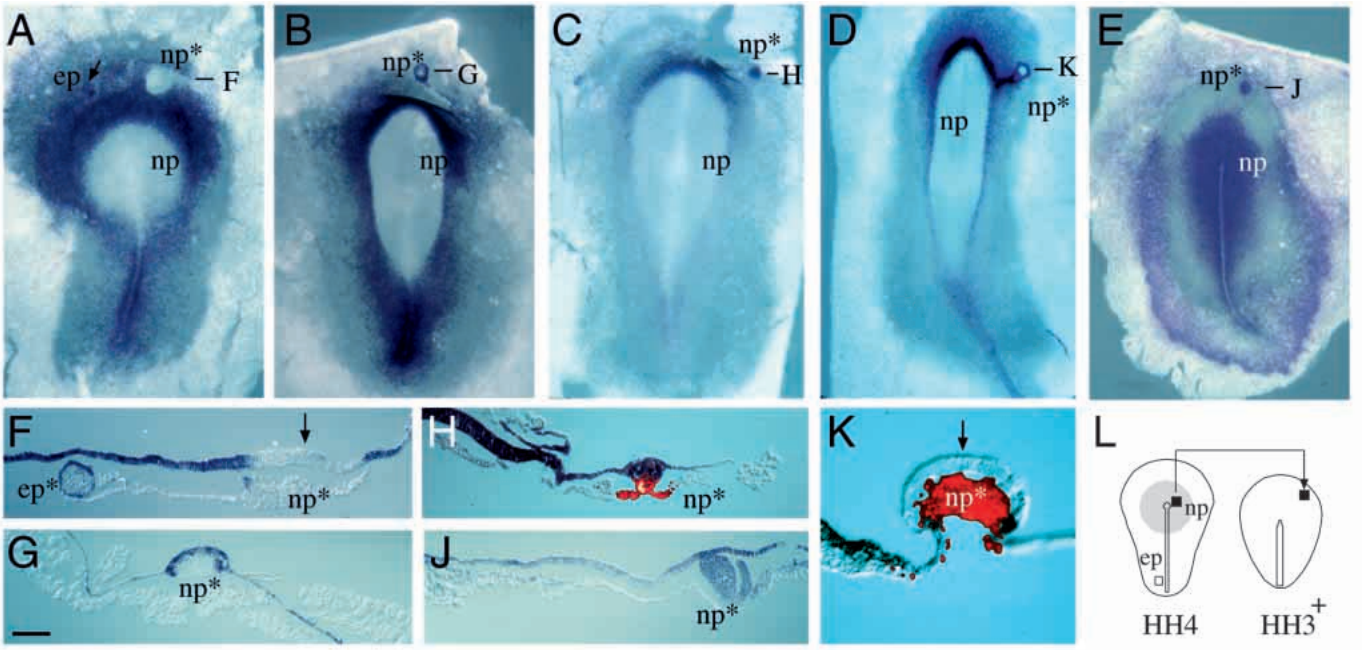

Fig. 4. Induction of neuroectoderm and *DLX5* epidermis by neural plate grafts. Indicated are the levels of sectioning (F-H,K,J in A-E, respectively), the primary neural plate (np), the neural plate grafts (np*), and the epidermis graft (ep*). Specimens in (A-D,F-H,K) were probed with *DLX5* and in (E,J) with *GSX*. The bright field (*DLX5* in situ analysis) and dark field (DiI staining) data in (H,K) were electronically superimposed (Photoshop). Details are explained in the text. (A,F) Note the homeogenetic induction of a neural plate (arrow in F), whereas the grafted epidermis formed a vesicle and gave no response. (B,G) Note the induction of a *DLX5*-positive epithelial thickening, with almost no formation of a non-expressing central area. (C,H) The neural plate graft was labelled with DiI (red colour); most labelled cells remained restricted to the lower layer. Note the induction of a *DLX5*-positive epithelial thickening. (D,K) Induction of an ectopic neural plate (arrow in K), with surrounding *DLX5* expression by a DiI-labeled neural plate graft. Note that the induced neural plate folds upward from the plane of the epiblast. No grafted cells appeared in the induced upper layer. (E,J) Note the induction of an ectopic *GSX* domain. (L) Transplantation scheme. Bar, 540 μm (A-D), 50 μm (E-H).

old prechordal plates (HH5^{+}-6; *n*=3; not shown), which are known to have a significantly reduced capacity for neural induction (Pera and Kessel, 1997). These findings demonstrated that *DLX5* repression and induction can be separated from neural induction.

In summary, the experiments provide evidence for two separate activities originating from the organizer, the adjacent repression and the distant activation of *DLX5* expression.

Induction of neuroectoderm and *DLX5* positive epidermis by neural plate grafts

How does a signal from the midline reach the periphery of the neural plate? We investigated if the neural plate itself could become the source for *DLX5*-inducing signals. According to Waddington (1933) and Rasilo and Leikola (1976), neuroectoderm can induce ectopic neuroectoderm in the avian epiblast. We transplanted pieces of the neural plate derived from HH4/5 embryos. Such pieces would develop into neural tissue if explanted and cultivated on the chorioallantoic membrane (Rao, 1968). However, transplants into the non-neural ectoderm would revert to epidermal fate (Garcia-Martinez et al., 1997). We excised the neuroectoderm either anteriorly or laterally from Hensen's node and implanted the tissue below the non-neural epiblast of host embryos (HH3/4^{-}; *n*=38; Fig. 4L). In the same way we grafted control pieces of prospective epidermis from regions adjacent to the base of the streak (HH4/5). The grafts were cultured in close contact with the host epiblast for 10 hours. They finally became located within the area pellucida, in varying distances to the primary neural plate.

Neural plate grafts located below the *DLX5* domain led to a local depletion of *DLX5*, and a thickening of the overlying epithelium (*n*=9; Fig. 4A,F). Epidermal control grafts neither reduced the *DLX5* expression nor increased the thickness of the overlying ectoderm (Fig. 4A,F). Explants located close to the anterolateral margin of the area pellucida induced an independent *DLX5* domain above the implant, whereas a central *DLX5*-free area was not always obvious (*n*=21; Fig. 4B-D,G,H,K). To confirm the neural plate identity of the induced epithelium we performed whole-mount in situ hybridizations with probes of the homeobox gene *GSX* (*n*=4; Fig. 4E,J) and the HMG box gene *SOX2* (*n*=4, not shown). Both probes remained positive in the graft during the relatively short incubation period (8 hours) and were ectopically activated in the adjacent ectoderm. After labeling the grafted neural plates with DiI (*n*=19) we detected virtually no, or only very few, grafted cells in the induced upper layer (Fig. 4C,D,H,K). The induced epithelial thickening, the downregulation of *DLX5* in its primary domain, the upregulation of GSX and the ectopic activation of *DLX5* indicate that neuroectoderm can induce homeogenetically in the area pellucida of early chick embryos.

In summary, we found evidence that both activities that are triggered by node and prechordal plate grafts, namely *DLX5* and neural induction, can also be elicited by the neural plate.

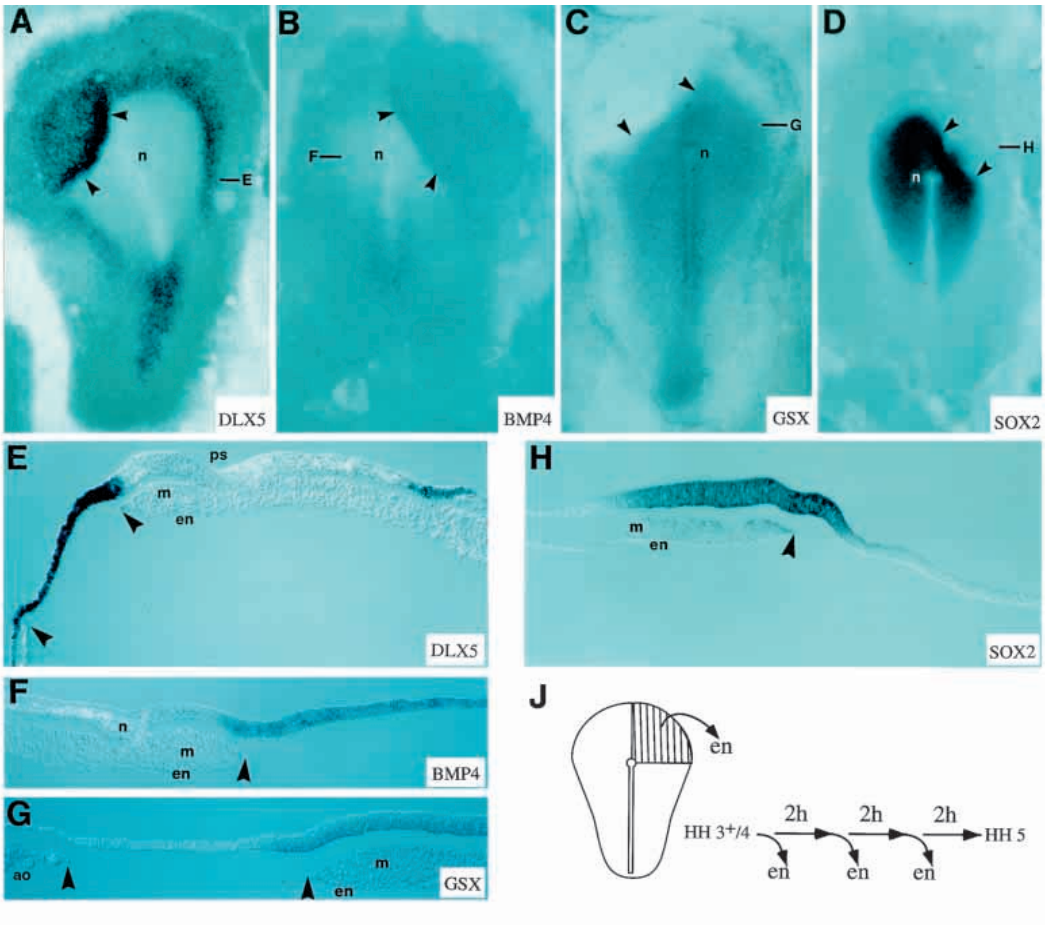

Fig. 5. Development of epidermis instead of neural plate in the absence of the lower layers. Indicated are the node (n), the levels of sectioning (E-H in A-D, respectively), the site of endoderm/mesoderm absence (arrowheads), mesoderm (m), endoderm (en), primitive streak (ps), and area opaca (ao). The operation scheme to remove the endoderm (3-5×) is indicated in (J); further details are given in the text. Specimens were probed with (A,E) *DLX5*, (B,F) *BMP4*, (C,G) *GSX* and (D,H) *SOX2*. Note the correspondence between the absence of a lower layer (endoderm and mesoderm), the widening of the epidermal field and the restriction of the neural plate.

Vertical signals from the lower layer are necessary for the establishment of the neural plate

During early gastrulation cells invaginate through the tip of the growing streak and spread radially to form the definitive endoderm (Vakaet, 1970; Rosenquist, 1972). Size and shape of the concentrically growing endoderm layer resemble at least roughly the neural plate dimensions in the overlying ectoderm. The ability of Hensen's node to induce neural tissue correlates with the presence of the prospective endodermal or mesendodermal cells in the node (Dias and Schoenwolf, 1990). However, no neural inducing capacity could be demonstrated for the endoderm once it is organized as a layer (Gallera and Nicolet, 1969). To study whether signals from the deep layer are involved in the regulation of *DLX5* gene activity we performed transplantation and extirpation experiments. Pieces of HH3 endoderm grafted below the non-neural epiblast of midstreak embryos had no obvious effects, in particular no neuralization could be observed (n=13; not shown). After removal of a small endodermal patch the cell layer was regenerated very fast and neither morphological defects nor changes of gene expression followed. Therefore, we excised large pieces, about one quadrant, of the HH3 endoderm plus the adjacent hypoblast, and repeated this operation in 1.5-2 hour intervals 3-5 times (n=26; Fig. 5J). Thus, we not only kept the ectoderm free of the underlying endoderm, but also prohibited a subsequent repair and invasion of the mesoderm, until the streak had reached its full length. Embryos were fixed about 1 hour after the final explantation (HH4-5), when only a small region close to the node was populated by regenerated endoderm and mesoderm. Ectopic *DLX5* transcripts appeared in the whole part of the ectoderm devoid of endoderm (n=6; Fig. 5A,E). Similar results were obtained with another epidermal marker, *BMP4*. When the endoderm/mesoderm was removed as described above, *BMP4* was expressed in the overlying ectoderm, again more strongly towards the midline (n=8; Fig. 5B,F). We also studied the neural plate markers *GSX* (n=5; Fig. 5C,G) and *SOX2* (n=3; Fig. 5D,H). At HH4-5, no transcripts of these genes were detectable in the manipulated area. Histological sections at the head process stage revealed that the naked ectoderm was relatively thin, further supporting the conclusion that the formation of the neural plate was impaired (Fig. 5E-H).

Thus, in the absence of the lower germ layers, the epidermis expanded into the region that normally forms the neural plate. Vertical signals from the deeper layer are apparently necessary for the establishment of the neural plate.

BMP2 and BMP4 signaling in the periphery establishes the epidermal ectoderm

The expression patterns of *BMP2* and *BMP4* resemble the *DLX5* pattern in some aspects (Watanabe and Le Douarin, 1996; Schultheiss et al., 1997; Andrée et al., 1998; Streit et al., 1998). Both BMPs are transcribed in the marginal zone at the pre-streak stage and, as gastrulation proceeds, they are localized in the posterior primitive streak and adjacent to the

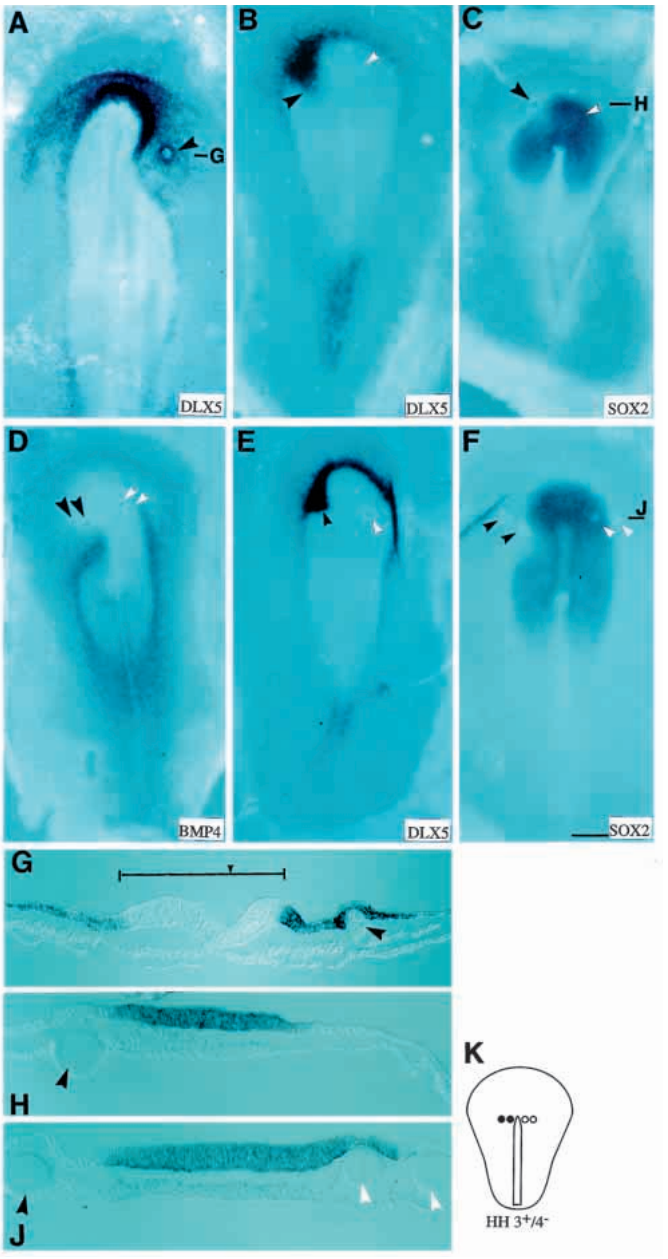

Fig. 6. BMP influence on the formation of the neural plate-epidermis boundary. BMP2-expressing cells (A,G), BMP2-containing beads (B-D,H), and BMP4-coated beads (E,F,J) were implanted into the vicinity of the node as indicated in (K). Black arrowheads point to BMP-positive sources, white arrowheads to negative controls, the levels of sectioning (G,H and J in A,C and F, respectively) and the probes used for whole-mount analysis are indicated. Note the distortion of the neural plate that occurs when a bead becomes located close to the boundary, but no independent, ectopic activation of the genetic markers.

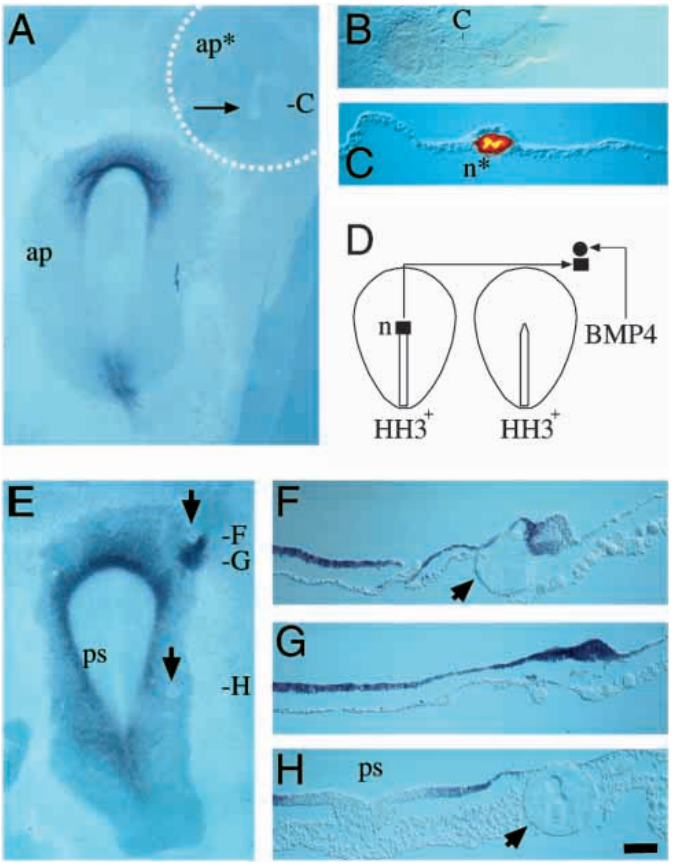

Fig. 7. Co-transplantation of Hensen's node with BMP4-loaded beads into the area opaca. Indicated are the primitive streak (ps), the primary area pellucida (ap), the induced secondary area pellucida (ap*, dashed white circle), the DiI-labelled node graft (n*) the levels of sectioning (C in A and B; F,G and H in E), and the position of BMP4-loaded beads (short arrows). Embryos were probed with a *DLX5* probe; the bright field and dark field (DiI staining) data in (C) were electronically superimposed (Photoshop). (A-C) Node transplantation into the area opaca. Note the absence of staining in the induced structure (long arrow), which is more clearly shown with Nomarski optics in (B). Sectioning through the induced structure reveals an epithelial thickening and the absence of grafted cells from the upper layer (C). (D) Schematic representation of the operation. (E-H) Co-transplantation of a node and a BMP4-loaded bead into the area opaca. Note the localized epithelial thickening induced adjacent to the BMP4-loaded bead on the side of the node graft, but not towards the opposing side (F,G). Note the absence of a thickening associated with the more posterior located BMP4-loaded bead in the absence of a node graft (H).

neural plate. *BMP2* RNA is confined to the lower layer, first the endodermal and then the mesodermal layer, while *BMP4* RNA is restricted to the ectoderm. Thus, BMP2 and BMP4 proteins can be expected to be present at the right time and place to affect *DLX5* expression. To test this, we transplanted sources of these factors below the epiblast of HH3-4 host embryos. We used either retrovirus-transfected cells or beads loaded with recombinant protein and placed them below the ectoderm in the area pellucida. As negative controls we grafted non-producing cells or beads loaded only with BSA, often within the same specimen (Fig. 6K). BMP sources remaining close to the tip of the growing streak caused inhibition of further development, as was recently described by Streit et al. (1998). BMP sources grafted slightly further away from the streak were finally positioned under the neural or epidermal epiblast, and did not interfere with further growth during the relatively short incubation times of 5-12 hours.

When BMP2 or BMP4 sources became localized close to

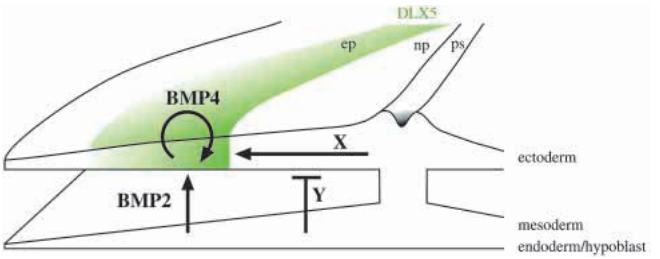

Fig. 8. A model for ectodermal patterning in the chick. The drawing shows the posterior part of a HH3$^+$ embryo, cut transversely behind the node. The direction of view is anterior to posterior. The mesoderm/endoderm layer and the ectoderm are shown separated for clarity. Epidermis (ep), neural plate (np) and primitive streak (ps) are indicated; the *DLX5* expression domain is shown in green. Note the positive effects on epidermis formation as well as *DLX5* expression by BMPs, either within the ectoderm (BMP4) or from the lower layers (BMP2). The *DLX5* induction triggered by the midline, transmitted via the neural plate, is marked 'X'. This effect was concluded from node, prechordal plate, streak and neural plate transplantations. The *DLX5*-inhibiting, neural plate-permitting, effect from the lower layers is marked by 'Y'. It was detected in the endoderm extirpation experiments.

the border of the neural plate, the *DLX5* expression domain expanded into the neural plate (*n*=21; Fig. 6A,B,E,G). However, BMP grafts positioned under the neural plate or under the normally *DLX5*-free epiblast did not induce independent *DLX5* expression domains (Fig. 6B,E). Similar results were obtained when a *BMP4* probe was applied (*n*=7, Fig. 6D). Furthermore, the BMP effects on the shape of the neural plate could also be directly demonstrated with the neural markers *SOX2* (*n*=8; Fig. 6 C,F,H) and GSX (*n*=9; not shown), which became downregulated.

So far, our experiments have demonstrated a role of midline tissues (Hensens's node, neural plate) and of BMP2/4 in *DLX5* induction. In order to analyze the induction phenomena outside the influence of the primary embryo, we performed transplantations into the area opaca (Fig. 7D). Here, grafted nodes did not induce *DLX5* expression after up to 10 hours of incubation (*n*=3), although a first morphological response was detectable above the DiI-labeled node graft (Fig. 7A-C). This had been indicated already by the absence of staining in the area opaca observed in the experiments depicted in Fig. 3A,B. Weak, ectopic expression in the area opaca was observed, when a morphologically significant neuroectoderm was induced after longer incubation times (18-20 hours, *n*=2). We then co-transplanted nodes together with BMP4-loaded beads (*n*=5). Clearly, in these experiments no neural plates formed, due to either slower induction kinetics, the inhibitory effects of BMP4, or both (see also Streit et al., 1998). These experiments resulted in a strong *DLX5* expression adjacent to the implanted BMP4-loaded beads on the side of the node graft, whereas no response occured on the opposite side (Fig. 7E-H).

We conclude that factors from the node in collaboration with BMPs are responsible for the observed induction events. BMPs are factors involved in the formation of the boundary between the neural and epidermal ectoderm, and restrict the extension of the neural plate by establishing an epidermal identity.

DISCUSSION

Prospective epidermal and neuroectodermal territories in the early chick epiblast

During the uterine period a state of eccentricity is created in the chick blastoderm by mechanical and gravitational forces (Callebaut, 1994). Cells fated to become neuroectoderm or epidermis, respectively, are already located in distinct territories, although their fate is not yet committed (Hatada and Stern, 1994; Callebaut et al., 1996; Garcia-Martinez et al., 1997). An idea about the size and position of these territories at the time of egg-laying can be obtained from Fig. 1B,D.

The prospective neuroectoderm lies towards the area, which pointed upwards during the intrauterine egg rotations (Eyal-Giladi and Fabian, 1980; Eyal-Giladi et al., 1992; Callebaut et al., 1996; for a review, see Eyal-Giladi, 1997). Around the time of egg-laying, expression of many early genetic markers is found here, such as the homeobox genes *GSC*, *GSX*, *CNOT1*, *CNOT2* and *CMIX*, but also other genes such as *brachyury* and *chordin* (Izpisúa-Belmonte et al., 1993; Stein and Kessel, 1995; Stein et al., 1996b, 1998; Knezevic et al., 1997; Lemaire et al., 1997; Streit et al., 1998). Their activation is closely associated with the development of all germ layers and axial levels, including the dorsal, anterior and posterior ectoderm, the dorsal and ventral mesoderm, as well as the dorsal and ventral endoderm. The elaboration of the axial patterns occurs during gastrulation, when all these proximal tissues will be internalized.

The prospective epidermis lies in the area of the uterine embryo, which points downwards during the egg rotations (for a review, see Eyal-Giladi, 1997). With the *DLX5* gene we have a marker for this area, which demarcates the epidermal fate from pre-gastrulation to neurulation stages. After gastrulation, the only non-internalized tissue will be the epidermis, which then encloses the endoderm, mesoderm and the neuroectoderm. The early non-neural, epidermal *DLX* expression is also found for the *Xenopus X-dll3* gene (T. Hollemann, personal communication), the ortholog of chicken *DLX5* (Papalopulu and Kintner, 1993). It is furthermore reminiscent of the pattern detected for *AmphiDll*, the putatively single *distal-less* homeobox gene of *Amphioxus*, which was proposed to represent a prototype gene for the six genes found in birds and mammals (Holland et al., 1996). *AmphiDll* also possesses a later expression phase in the anterior nervous system, corresponding well to the prosencephalic domains found for chicken *DLX5*, *Xenopus X-dll3* and other *Dlx* genes.

Re-patterning of the ectoderm

The classical method to restart ectodermal patterning is the grafting of organizer tissue to competent sites (Spemann and Mangold, 1924). Our investigation has indicated that it is not only neural induction that occurs in the vicinity of a grafted node or a prechordal plate, but also that the epidermis surrounding the ectopic neural plate changes, as evident by the induction of *DLX5* expression. How can an organizer graft trigger a repatterning of the ectoderm at such a distance? The most likely mechanism would involve a *DLX5*-inducing signal derived from the induced neural plate. This hypothesis is strengthened by the observation that transplanted nodes always induce a neural plate first, and that an initial and direct

induction of the *DLX5* epidermis did not seem to occur. When we used neural plate grafts to analyze this question, we observed a homeogenetic induction of the neuroepithelial tissue, an effect that was originally described in urodeles (Mangold and Spemann, 1927) and further recognized in birds (Waddington, 1933; Rasilo and Leikola, 1976). In our experiments, homeogenetic induction was most obvious near the primary neural plate. More laterally grafted neural plates induced mainly *DLX5*-positive ectoderm, suggesting a *DLX5*-inducing signal from the neural plate (Fig. 4F). This could either be a direct signal, or an intermediate activating *BMP4* expression, or a combination of a direct signal and induced BMP4. We will argue below in favour of such a combination.

Establishment of the neural plate

It is widely accepted that the induction of a neural plate in the chick embryo must involve signals derived from the tip of the primitive streak from HH3 onwards (Dias and Schoenwolf, 1990, and references therein; Storey et al., 1992; Lemaire et al., 1997). We have now demonstrated that a vertical signal from the lower germ layers is also involved in the establishment of the neural plate. In its absence, neural markers (*GSX, SOX2*) are lost and epidermis develops, as evident by ectopic *DLX5* and *BMP4* transcripts. Due to the complexity of such an experiment, it is impossible to attribute this effect to either the endoderm or the mesoderm. Although we repeatedly removed the endoderm, we prohibited at the same time the formation of a mesodermal layer beneath the ectoderm. Our experiments suggest a permissive nature of the signal from the lower germ layers. We conclude this from the fact that neither the definitive endoderm nor the non-axial mesoderm appears to be capable of neural induction in the chick embryo (Gallera and Nicolet, 1969; our unpublished observations). Could the signal be one of the known BMP antagonizing molecules? Chick *chordin*, *noggin* and *follistatin* RNAs are expressed in the node, and not in the endoderm, nor in the more lateral mesoderm, but the distribution of the respective proteins is not known (Connolly et al., 1995, 1997; Streit et al., 1998). Directly applied, these proteins are not able to elicit neural induction in competent chick epiblasts. However, a possible permissive role has not yet been investigated or ruled out.

In conclusion, there is evidence for a signal from the lower layers necessary for neural development, and inhibitory for epidermal development.

BMPs at the epidermis-neural plate boundary

BMPs play a highly conserved role in dorsoventral patterning, as was shown in the invertebrate *Drosophila melanogaster*, the amphibium *Xenopus laevis* and the zebrafish *Danio rerio* (for reviews, see De Robertis and Sasai, 1996; Neave et al., 1997; Schulte-Merker et al., 1997; Kishimoto et al., 1997; Nguyen et al., 1998). The co-appearance of *DLX5* and *BMP4* expression during normal but also during manipulated embryogenesis suggests a regulatory relationship. Grafting of BMP sources indicated an inductive pathway from BMP2/4 to *DLX5*. The effects of BMP2/4 were limited to distortions of the neural plate, while independent, ectopic inductions of *DLX5* domains in the neural plate, the lateral margin of the area pellucida, or in the area opaca were not obtained. These findings either indicate the presence of an inhibitory factor or the insufficiency of BMP2/4 to act alone. An obvious explanation would be a second factor, which would have to overlap with BMP2/4 at the neural plate boundary. This interpretation is supported by the induction of *DLX5* in the area opaca after co-transplantation of Hensen's node and Bmp4 beads (Fig. 7). A candidate for a node or neural plate derived factor is the Anti-Dorsalizing Morphogenetic Protein (ADMP), a BMP3-related protein that has been identified in *Xenopus* (Moos et al., 1995). While the ventralizing activities of ADMP resemble those of BMP2/4, its expression in dorsal structures such as the organizer, the prechordal plate and the neural plate represents a paradox. In any case, our investigations point to a role of a signal originating from the midline, which in combination with BMP2/4 generates a major demarcation line of the early embryo, the boundary between the prospective epidermis and the neural plate.

Signals in ectodermal patterning: a model

Summarizing our information on the various inductive activities affecting ectodermal patterning in the early chick embryo, we find evidence for planar and vertical interactions coming from the midline as well as the periphery (Fig. 8). We provide evidence for a role of BMP2 and BMP4 in establishing the epidermal side of the boundary, where BMP2 could act from the lower germ layers and BMP4 within the ectoderm itself. In addition, we demonstrate a *DLX5*-inducing signal triggered from the midline, but active only at the neural plate-epidermis boundary ('X' in Fig. 8). Our explantation experiments indicate that the *DLX5*-suppressing and neuroectoderm-permitting signal stems from the lower germ layers ('Y'). It would not be surprising if the molecular nature of this regulatory mechanism in the neural plate area were BMP antagonism.

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