An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo

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Background: In *Xenopus* embryos, fibroblast growth factors (FGFs) and secreted inhibitors of bone morphogenetic protein (BMP)-mediated signalling have been implicated in neural induction. The precise roles, if any, that these factors play in neural induction in amniotes remains to be established.

Results: To monitor the initial steps of neural induction in the chick embryo, we developed an *in vitro* assay of neural differentiation in epiblast cells. Using this assay, we found evidence that neural cell fate is specified *in utero,* before the generation of the primitive streak or Hensen's node. Early epiblast cells expressed both *Bmp4* and *Bmp7,* but the expression of both genes was downregulated as cells acquired neural fate. During prestreak and gastrula stages, exposure of epiblast cells to BMP4 activity *in vitro* was sufficient to block the acquisition of neural fate and to promote the generation of epidermal cells. *Fgf3* was also found to be expressed in the early epiblast, and ongoing FGF signalling in epiblast cells was required for acquisition of neural fate and for the suppression of *Bmp4* and *Bmp7* expression.

Conclusions: The onset of neural differentiation in the chick embryo occurs *in utero,* before the generation of Hensen's node. *Fgf3, Bmp4* and *Bmp7* are each expressed in prospective neural cells, and FGF signalling appears to be required for the repression of *Bmp* expression and for the acquisition of neural fate. Subsequent exposure of epiblast cells to BMPs, however, can prevent the generation of neural tissue and induce cells of epidermal character.

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Received: **8 December 1999**
Revised: **19 January 2000**
Accepted: **15 February 2000**

Published: **30 March 2000**

Current Biology 2000, 10:421–429

0960-9822/00/$ – see front matter

Background

The induction of neural tissue represents an early and fundamental step in the generation of the vertebrate nervous system. During this process, pluripotential ectodermal cells are directed to a neural fate. Much of our understanding of neural induction in vertebrates derives from studies performed in *Xenopus* embryos. Here, the embryonic ectoderm initially expresses members of the bone morphogenetic protein (BMP) class of transforming growth factor-β (TGF-β) proteins, and signals mediated by these factors can promote the differentiation of epiblast cells into epidermal ectoderm [1,2]. Several BMP inhibitors that are secreted by organiser cells have been shown to induce neural differentiation, apparently by blocking BMP signalling activity within the ectoderm or by repressing the expression of genes encoding BMPs [3–8]. Collectively, these studies have led to the prevailing view that BMP inhibitors derived from Spemann's organiser at gastrula stages have a critical role in neural induction in *Xenopus*.

In amniotes, however, the role of BMP inhibitors derived from the organiser/node as neural inducers remains ambiguous [9,10]. In mice, neural differentiation proceeds in the absence of activities of the genes encoding the BMP inhibitors noggin, chordin or follistatin, and neural plate cells are also generated in mice lacking both noggin and chordin genes [11–13]. In addition, mouse embryos mutant for the *HNF3β* gene lack a recognisable node, and both noggin and chordin expression are absent, but still these embryos form neural tissue [14]. In both chick and mouse, cells in the region of the organiser/Hensen's node can induce ectopic neural cells when transplanted to competent regions of the ectoderm [15,16]. But in avian embryos, chordin alone is unable to mimic the activity of Hensen's node to induce neural differentiation in prospective extraembryonic tissue [9]. Moreover, in chick, the temporal patterns of expression of noggin, chordin and follistatin do not coincide with the ability of the organiser tissue to induce ectopic neural cells [9,15,17]. These studies leave unresolved the extent to which views of neural induction derived from studies in *Xenopus* are applicable in amniotes.

As a first step to examine the identity of signals that influence the differentiation of neural tissue in the chick embryo, and to determine the time at which these signals operate, we have developed an *in vitro* assay of neural cell

differentiation. Explants derived from definitive embryonic tissue, the area pellucida, differentiated into neural tissue *in utero*, before the initiation of gastrulation. Prospective neural cells expressed genes for fibroblast growth factors (FGFs), and we found that FGF signalling was required for epiblast cells to acquire an overt neural character. Moreover, FGFs acted to repress *Bmp* expression in epiblast cells, providing a potential link between the signalling activities of FGFs and the extinction of BMP signalling during the induction of neural tissue.

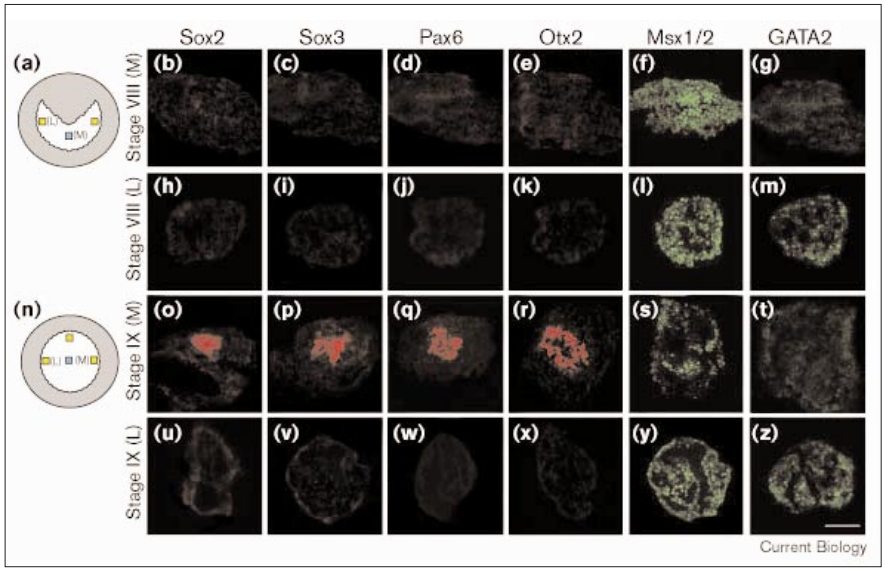
Results

In previous studies, we found that, at Hamburger and Hamilton (HH) stage 3, the definitive primitive streak stage, prospective neural cells isolated from the chick epiblast and grown *in vitro* acquire forebrain character, as assessed by a series of molecular markers [18]. To extend these studies, we examined whether cells in younger prestreak-stage chick embryos also acquired neural character when grown *in vitro*. We monitored the expression of several transcription factors to assess the early neural or epidermal character of epiblast cells. Sox3 is initially expressed widely by epiblast cells but its expression gradually becomes restricted to neural plate cells [19], whereas Sox2 expression is initially restricted to neural plate cells [19]. The expression of Otx2 defines neural cells normally located at levels rostral to the hindbrain [20], whereas the expression of Pax6 defines cells at forebrain, hindbrain and spinal cord levels of the neural tube [21]. Although not all of these markers are initially restricted exclusively to neural cells, the coincidence in their expression serves to define neural cells in an unambiguous manner. The expression of Msx1/2 by early chick embryos is found in cells located at the border between the epidermis and the neural plate [22], and Msx1 activity can induce epidermal cells and block the generation of neural tissue, at least in *Xenopus* [23]. GATA2 is expressed in the lateral regions of prestreak embryos in an anterior^{high} to posterior^{low} pattern and, from late gastrula stages, its expression defines the entire non-neural ectoderm and is excluded from the prospective neural plate [24]. Thus, the coincidence in expression of Msxl/2 and GATA2 defines non-neural cells of epidermal character.

Specification of neural cells in chick is initiated *in utero*

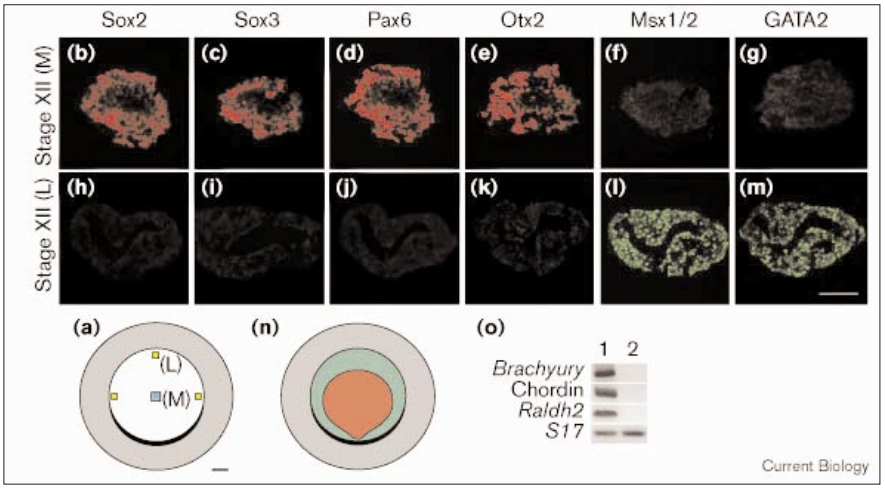
We first attempted to define the time at which epiblast cells acquire neural character when isolated and grown *in vitro*. Epiblast tissue was isolated from medial and lateral regions of the area pellucida of Elyal-Giladi and Kochav stage VIII–IX chick embryos [25] (Figure 1a,n). Explants were grown *in vitro* for 40 hours and then assayed for the generation of epidermal and neural marker expression. Stage VIII medial explants generated Msx1/2$^+$ cells, whereas no expression of Sox2, Sox3, Otx2, Pax6 or GATA2 was detected. Stage VIII lateral explants generated Msx1/2$^+$ cells and GATA2$^+$ cells (Figure 1b–m). Stage IX medial explants generated Sox2$^+$ cells, Sox3$^+$ cells, Otx2$^+$ cells, Pax6$^+$ cells and also generated Msx1/2$^+$ cells in non-overlapping regions of individual explants, but no GATA2$^+$ cells were detected (Figure 1o–t). Stage IX

Figure 1



Specification of cells in stage VIII and IX chick embryos. **(a)** Schematic representation of an Elyal-Giladi and Kochav stage VIII embryo. The area pellucida (white) is indicated. The boxed areas indicate where the medial (M, blue) and lateral (L, yellow) area pellucida explants were isolated for *in vitro* studies. **(b–g)** Stage VIII medial explants cultured for 40 h ($n = 6$) did not generate (b) Sox2$^+$ cells, (c) Sox3$^+$ cells, (d) Pax6$^+$ cells, (e) Otx2$^+$ cells or (g) GATA2$^+$ cells, but did generate (f) Msx1/2$^+$ cells (95 ± 5% cells per section). **(h–m)** Stage VIII lateral explants cultured for 40 h ($n = 6$) did not generate (h) Sox2$^+$ cells, (i) Sox3$^+$ cells, (j) Pax6$^+$ cells or (k) Otx2$^+$ cells, but did generate (l) Msx1/2$^+$ cells (95 ± 5% cells per section) and (m) GATA2$^+$ cells. **(n)** Schematic representation of a stage IX embryo. The area pellucida and the regions where medial and lateral explants were derived are indicated. **(o–t)** Stage IX medial explants cultured for 40 h ($n = 6$) generated (o) Sox2$^+$ cells, (p) Sox3$^+$ cells, (q) Pax6$^+$ cells, (r) Otx2$^+$ cells (25 ± 10% cells per section) and (s) Msx1/2$^+$ cells (35 ± 10% cells per section), but no (t) GATA2$^+$ cells. **(u–z)** Stage IX lateral explants cultured for 40 h ($n = 6$) did not generate (u) Sox2$^+$ cells, (v) Sox3$^+$ cells, (w) Pax6$^+$ cells or (x) Otx2$^+$ cells, but did generate (y) Msx1/2$^+$ cells (95 ± 5% cells per section) and (z) GATA2$^+$ cells. The scale bar in (z) represents 100 μM and applies to (b–m, o–z).

Figure 2



Specification of cells of the stage XII chick embryo. **(a)** Schematic representation of an Elyal-Giladi and Kochav stage XII embryo. The area pellucida (white) and area opaca (grey) are indicated. Boxed areas indicate where medial (M, blue) and lateral (L, yellow) explants were taken for *in vitro* studies. **(b–g)** Stage XII medial explants cultured for 40 h ($n = 6$) generated (b) Sox2$^+$ cells, (c) Sox3$^+$ cells, (d) Pax6$^+$ cells and (e) Otx2$^+$ cells (95 ± 5% cells per section), but did not generate (f) Msx1/2$^+$ cells or (g) GATA2$^+$ cells. **(h–m)** Stage XII lateral explants cultured for 40 h ($n = 6$) did not generate (h) Sox2$^+$ cells, (i) Sox3$^+$ cells, (j) Pax6$^+$ cells or (k) Otx2$^+$ cells but did generate (l) Msx1/2$^+$ cells (95 ± 5% cells per section) and (m) GATA2$^+$ cells (70 ± 10% cells per section). **(n)** Specification map of the stage XII embryo. The explants that generated Sox2$^+$ cells, Sox3$^+$ cells, Otx2$^+$ cells and Pax6$^+$ cells are indicated in red. Cells expressing Msx1/2 are located lateral to prospective neural cells at all positions of the embryo (green). **(o)** RT–PCR analysis of RNA isolated from an HH stage 4 embryo and from stage XII medial explants. *Brachyury*, chordin and *Raldh2* were expressed in stage 4 embryos (lane 1) but not in stage XII medial explants cultured for 40 h (lane 2). The scale bar shown in (a) represents 300 μM; the one in (m) represents 100 μM and applies to (b–m).

lateral explants, in contrast, generated only Msx1/2$^+$ cells and GATA2$^+$ cells (Figure 1u–z). These results provide evidence that medial epiblast cells acquire the capacity to express neural markers *in utero*, soon after stage VIII.

We next isolated medial and lateral explants from stage XII embryos (Figure 2a). At this stage, medial explants generated Sox2$^+$ cells, Sox3$^+$ cells, Otx2$^+$ cells and Pax6$^+$ cells, but no expression of Msxl/2 or GATA2 was detected (Figure 2b–g). Lateral explants generated Msxl/2$^+$ cells and GATA2$^+$ cells (Figure 2h–m).

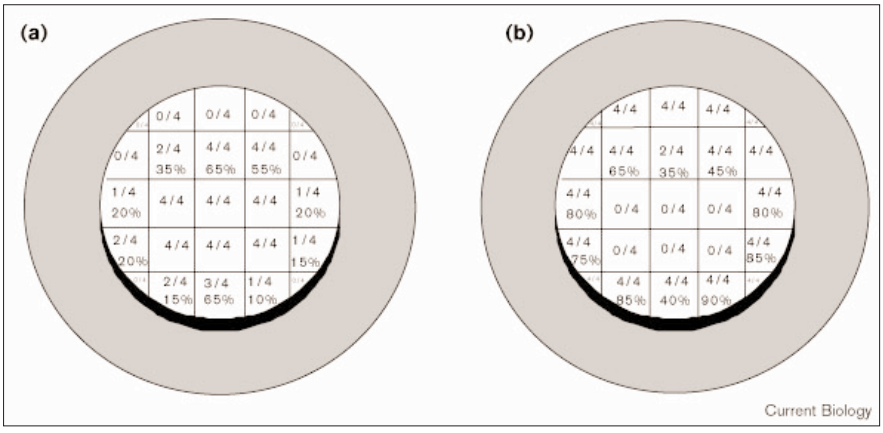
To define in more detail the location of prospective neural cells in stage XII embryos, we dissected the epiblast into 100 explants and monitored the generation of neural and epidermal cells after 40 hours of culture. Explants isolated from the central region of the epiblast generated Sox2$^+$ cells, Sox3$^+$ cells, Otx2$^+$ cells and Pax6$^+$ cells and no Msx1/2$^+$ cells (Figure 3a). Explants isolated from the peripheral region of the epiblast generated Msxl/2$^+$ cells, but no expression of Sox2, Sox3, Otx2 or Pax6 was detected (Figure 3b). Most or all individual explants isolated from the region between the central and peripheral epiblast generated both Msxl/2$^+$ cells and Sox2$^+$ cells, Sox3$^+$ cells, Otx2$^+$ cells and Pax6$^+$ cells in non-overlapping domains of the explants (Figure 3a,b). Explants that generated Sox2$^+$ cells, Sox3$^+$ cells, Otx2$^+$ cells and Pax6$^+$ cells covered approximately two-thirds of the area pellucida. This central region was oval in shape and extended posteriorly towards the centre of the Koller's sickle (Figure 2n). Cells that acquired Msx1/2 expression were located peripheral to prospective neural cells at all anteroposterior positions of the area pellucida (Figure 2n). Cells that acquired GATA2 expression were located in the most peripheral regions of the area pellucida except, in the most posterior region of the embryo, where epiblast cells failed to express GATA2. Thus, at stage XII, prospective neural cells are located in the central region of the embryo, and prospective epidermal cells are found in the peripheral region of the embryo.

Fate maps and lineage analyses have provided evidence that the epiblast of stage XII embryos consists of a mosaic of presumptive ectodermal, mesodermal and neuroectodermal cells [26]. To examine whether mesoderm, a potential source of neural-inducing signals, is generated during culture of medial explants from stage XII, we monitored the expression of three mesodermal markers *Brachyury*, chordin and *Raldh2*, by reverse transcription (RT)–PCR. *Brachyury* is expressed in mesoderm precursor cells located in the primitive streak, in Hensen's node and in axial mesoderm [27]. Chordin is expressed in Hensen's node and subsequently in axial mesoderm [9], and *Raldh2* is selectively expressed in paraxial mesoderm [28]. No expression of *Brachyury*, chordin or *Raldh2* was detected in stage XII medial explants after 40 hours of culture (Figure 2o, lane 2). Thus, in stage XII medial explants, neural markers are expressed in the absence of the generation of mesoderm.

Downregulation of *Bmp4* and *Bmp7* expression in prospective neural cells

In *Xenopus*, the exposure of ectodermal cells to BMPs has been shown to block the generation of neural tissue and

Figure 3



Specification map of the stage XII embryo. The area pellucida was dissected into 25 pieces, which were each further divided into four explants. The incidence of explants that generated **(a)** Sox2$^+$, Sox3$^+$, Pax6$^+$ and Otx2$^+$ cells and **(b)** Msx1/2$^+$ cells are depicted. When explants generated cells of both neural and epidermal character, the percentage of cells is indicated. Neural and epidermal cells were always located in non-overlapping regions. Percentages are not indicated where values are < 5% or > 90%. Similar results were obtained from three independent stage XII embryos.

to induce epidermal character [1]. The expression of *Bmp4* is downregulated in the prospective neural plate cells during neural induction [1,2], and there is evidence implicating *Msx1* in the BMP-mediated induction of epidermal tissue [23]. Similarly, in the embryonic region of prestreak chick embryos, *Bmp4* and *Bmp7* are expressed at low levels and are downregulated before the formation of the primitive streak [9].

To determine whether the extinction of *Bmp* expression correlates with the generation of neural cells in chick epiblast explants, we examined whether *Bmp4* and *Bmp7* expression is downregulated in stage XII medial and lateral explants grown in culture. *Bmp4* and *Bmp7* were initially expressed in both stage XII medial and lateral epiblast tissue prior to growth *in vitro* (Figure 4a, lanes 1,5). The expression of both *Bmps* was downregulated in medial explants (Figure 4a, lane 2), but was maintained in lateral explants (Figure 4a, lane 6) after 40 hours *in vitro*. Thus, the extinction of *Bmp4* and *Bmp7* expression by prospective neural cells correlates with the acquisition of neural character.

BMP4 induces epidermal character in epiblast cells

In stage XII embryos, *Bmp7* is expressed at high levels in the area opaca [9], a region located adjacent to the lateral area pellucida cells which acquire epidermal character. Moreover, Msx1/2 can be induced by BMP signalling in chick embryos [29,30]. We therefore determined whether the exposure of prospective neural epiblast cells to BMPs induces epidermal character and blocks the acquisition of neural character. Stage XII medial epiblast explants grown in the presence of BMP4 (1.3 nM) for 40 hours generated Msxl/2$^+$ cells, GATA2$^+$ cells and a few Otx2$^+$ cells, but no Sox2$^+$, Sox3$^+$ or Pax6$^+$ cells were detected (Figure 5a–f). BMP4 was also able to block the generation of Sox2$^+$, Sox3$^+$, Otx2$^+$ and Pax6$^+$ cells in HH stage 2 prospective neural plate explants (data not shown). However, explants of the prospective rostral neural plate, isolated from HH stage 4 embryos cultured in either the absence or the presence of BMP4 (1.3 nM) generated Sox2$^+$ cells, Sox3$^+$ cells and Otx2$^+$ cells, but no Pax6$^+$, Msx1/2$^+$ or GATA2$^+$ cells were detected (Figure 5g–r). Thus, BMP4 induces the generation of epidermal cells in prestreak and early gastrula stage epiblast explants. By late gastrula stages, however, prospective neural cells appear to be refractory to the actions of BMP4.

FGF signalling is required for the specification of neural cells

We next examined the pattern of expression of genes encoding BMP inhibitors in the early epiblast. No expression of the genes encoding the secreted BMP inhibitors noggin, chordin, follistatin or *Caronte* was detected in stage XII medial or lateral epiblast explants at the time of isolation or after 40 hours in culture (Figure 2o and data not shown). Thus, the generation of neural cells in stage XII medial explants is not likely to require the activity of these BMP inhibitors.

FGFs have been implicated in neural induction in the chick embryo [31,32], and *Fgf3* is expressed in early gastrula stage embryos [33]. We therefore tested whether *Fgf3* was expressed by epiblast cells in the area pellucida of prestreak stage XII embryos. *Fgf3* expression was detected in stage XII medial and lateral explants both at the time of isolation and after 40 hours of culture (Figure 4a, lanes 1,2 and 5,6).

FGF3 signalling is mediated mainly through activation of the FGF receptors (FGFRs) 1b and 2b [34,35]. The *FgfR2b* gene was expressed in stage XII medial explants at the time of isolation and after 40 hours of culture (Figure 4b, lanes 1,2). To test whether ongoing FGF signalling was required for the acquisition of neural character by epiblast cells, we cultured stage XII medial

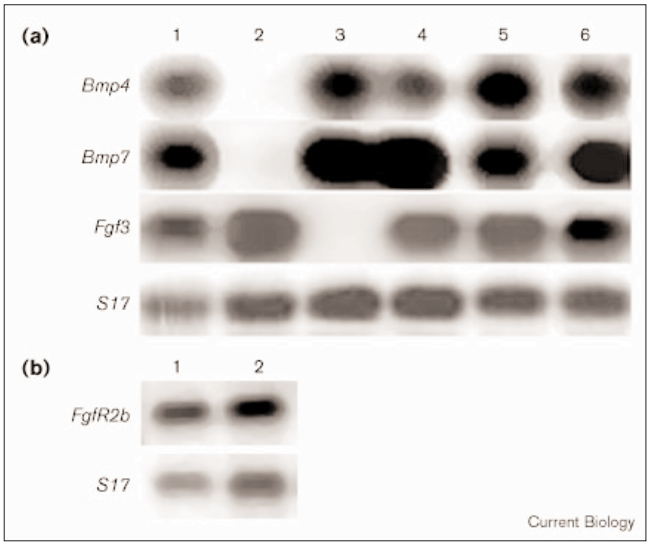
explants in the presence of different concentrations of the FGF receptor tyrosine kinase inhibitor SU5402 [18,36]. SU5402 has been shown to attenuate FGF signalling in chick neural plate explants and in chick embryos [37] without affecting the signalling pathways mediated by BMP and Hedgehog factors [18]. In the presence of SU5402 (2 μM), stage XII medial explants grown for 40 hours *in vitro* generated Msxl/2$^+$ cells and GATA2$^+$ cells. In contrast, no Sox2$^+$, Sox3$^+$, Otx2$^+$ or Pax6$^+$ cells were generated (Figure 6a–f). In addition, *Fgf3* expression was not detected (Figure 4a, lane 3). Addition of FGF1 (33 nM) in the presence of SU5402 (2 μM) to stage XII medial explants was able to overcome the block in neural differentiation. Sox2$^+$, Sox3$^+$, Otx2$^+$ and Pax6$^+$ cells, but few Msxl/2$^+$ cells and no GATA2$^+$ cells were detected (data not shown). These findings support the idea that the action of SU5402 is mediated by blockade of FGF signalling rather than by inhibition of other signalling pathways. They also suggest that ongoing FGF signalling is required to maintain the potential for neural differentiation in cells in stage XII medial epiblast explants, and also to maintain *Fgf3* expression.

FGF signalling downregulates *Bmp* expression in prospective neural cells

The results described above indicate that attenuation of FGF signalling and exposure to BMP4 can each suppress the acquisition of neural character in stage XII medial explants. In view of these findings, we considered whether FGF signalling might have a role in the repression of *Bmp* expression in epiblast cells of prospective neural character. To test this, stage XII medial explants were exposed to SU5402 (2 μM) and the expression of *Bmp4* and *Bmp7* analysed. In marked contrast to the normal loss of *Bmp4* and *Bmp7* expression, the expression of both genes was maintained in the presence of SU5402 (Figure 4a, lane 3).

To examine whether blockade of BMP signalling could result in the generation of neural cells under these conditions, we cultured stage XII medial explants in the presence of both SU5402 (2 μM) and *Xenopus* noggin (64 nM). Under these conditions, stage XII medial explants generated Sox2$^+$ cells, Sox3$^+$ cells, Otx2$^+$ cells and Pax6$^+$ cells (Figure 6g–j). In addition, *Fgf3* expression was maintained, and no Msxl/2$^+$ cells or GATA2$^+$ cells were detected after 40 hours in culture (Figure 4a, lane 4; Figure 6k,l). Under these conditions, *Bmp4* and *Bmp7* were still expressed (Figure 4a, lane 4). Similar results were obtained when stage XII medial explants were cultured with either medium containing chick chordin (data not shown) or soluble isoforms of the dominant negative BMP receptors IA/Fc and IB/Fc (0.5 μM each) (Figure 6m–r). Thus, the attenuation of FGF signalling appears to prevent the downregulation of *Bmp* expression in epiblast explants. Nevertheless, the blockade of BMP signalling activity under these conditions is sufficient to promote the acquisition of neural character and to maintain *Fgf3* expression. Together, these results suggest that ongoing FGF signalling during early phase of neural cell differentiation functions to attenuate BMP signalling, at least in part by repressing *Bmp* expression in prospective neural epiblast cells.

Figure 4

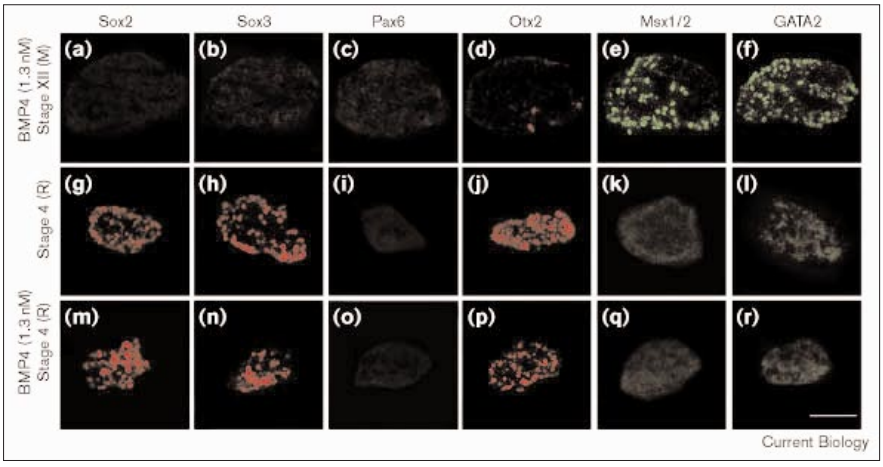


Acquisition of neural character is accompanied by attenuation of *Bmp4* and *Bmp7* expression. **(a)** RT–PCR and Southern blot analysis of PCR products derived from RNA isolated from Elyal-Giladi and Kochav stage XII explants. Lane 1, stage XII medial explants before culture ($n = 10$); lane 2, stage XII medial explants after 40 h of culture ($n = 6$); lane 3, stage XII medial explants cultured in the presence of 2 μM SU5402 ($n = 6$); lane 4, stage XII medial explants cultured in the presence of 2 μM SU5402 and 64 nM noggin ($n = 6$); lane 5, stage XII lateral explants before culture ($n = 10$); lane 6, stage XII lateral explants after 40 h of culture ($n = 6$). *Bmp4* and *Bmp7* were expressed before culture in both stage XII medial and lateral explants (lanes 1,5) and were downregulated in medial but not lateral explants after 40 h of culture (lanes 2,6). *Bmp4* and *Bmp7* were expressed when stage XII medial explants were cultured in the presence of 2 μM SU5402 (lane 3), and 2 μM SU5402 and 64 nM noggin (lane 4). *Fgf3* was expressed in both stage XII medial and lateral epiblast explants both before (lanes 1,5) and after 40 h of culture (lanes 2,6). *Fgf3* expression was extinguished when stage XII medial explants were cultured in the presence of 2 μM SU5402 (lane 3). *Fgf3* expression was maintained when stage XII medial explants were cultured in the presence of both 2 μM SU5402 and 64 nM noggin (lane 4). **(b)** *FgfR2b* was expressed in stage XII medial explants before (lane 1) and after (lane 2) 40 h of culture. Expression of the ribosomal gene *S17* was used as a control for loading.

Discussion

In this study, we have provided evidence that the initial steps in the specification of neural cell fate in the chick embryo are initiated *in utero*, and that early epiblast cells require ongoing FGF signalling to promote neural character and to repress *Bmp* expression. We discuss these

Figure 5



BMP4 induces epidermal character in prospective neural cells at Elyal-Giladi and Kochav stage XII, and neural cells become refractory to the action of BMP4 by stage 4 (HH 4). **(a–f)** Stage XII medial (M) explants ($n = 6$), when cultured in the presence of BMP4 (1.3 nM), generated (e) Msx1/2$^+$ cells (90 ± 10% cells per section) and (f) GATA2$^+$ cells (85 ± 10% cells per section), and generated few (d) Otx2$^+$ cells, but did not generate (a) Sox2$^+$ cells, (b) Sox3$^+$ cells or (c) Pax6$^+$ cells. **(g–l)** Stage 4 (HH 4) rostral (R) explants cultured alone generated (g) Sox2$^+$ cells, (h) Sox3$^+$ cells and (j) Otx2$^+$ cells (95 ± 5% cells per section), but no (i) Pax6$^+$ cells, (k) Msx1/2$^+$ cells or (l) GATA2$^+$ cells. **(m–r)** Stage 4 (HH 4) explants cultured in the presence of 1.3 nM BMP4 generated (m) Sox2$^+$ cells, (n) Sox3$^+$ cells and (p) Otx2$^+$ cells (95 ± 5% cells per section), but no (o) Pax6$^+$ cells, (q) Msx1/2$^+$ cells or (r) GATA2$^+$ cells. The scale bar in (r) represents 100 μM and applies to (a–r).

findings in the context of current views of neural induction in vertebrate embryos.

Specification of neural cells begins *in utero*

In the chick embryo, signals from Hensen's node are sufficient to induce the formation of neural tissue in extra-embryonic regions [15,38,39]. Our results provide evidence, first, that the specification of neural cells in chick begins much earlier than the time of Hensen's node formation, shortly after the formation of the area pellucida *in utero*; second, that the neural fate of epiblast cells has been consolidated by the late gastrula stage; and, third, that in early prestreak embryos cells in the medial region of the area pellucida are specified to a neural fate and that cells in the lateral region are specified to an epidermal fate.

The differential patterns of expression of *Dlx5* [40] and *GATA2* [24] by cells in the medial and lateral regions of prestreak embryos provide independent evidence for a distinction in the fates of cells in these regions. In addition, the pattern of expression of *GATA2* at early stages indicates that the prospective epidermis is patterned along its anteroposterior axis [24]. Collectively, these results support the idea that the specification of neural and epidermal cells in chick is initiated *in utero*, before the generation of the primitive streak and Hensen's node.

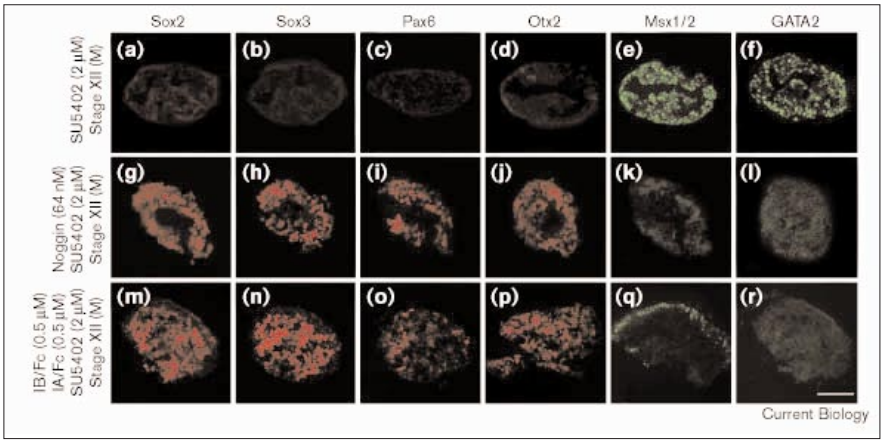
In *Xenopus*, Wnt signalling at blastula stages has recently been implicated in the downregulation of *Bmp4* expression in prospective neural plate cells and in the induction of neural cells [41]. In the chick, however, a nuclear-localised form of β-catenin, a marker of active Wnt signalling, is detected in cells in the lateral but not in the medial region of stage VIII–X embryos [42]. The involvement of Wnt signalling in the generation of neural and epidermal cells may therefore differ in *Xenopus* and avian embryos.

BMP signalling promotes the generation of epidermal cells at the expense of neural tissue

In *Xenopus* embryos, BMP inhibitors derived from the Spemann's organiser region are believed to contribute to neural induction by sequestering BMP protein [2]. In chick, noggin and chordin appear unable to induce neural cells in extra-embryonic tissue [9,10]. Moreover, neither noggin, chordin or follistatin exhibit temporal patterns of expression that correlate with the ability of Hensen's node to induce ectopic neural tissue [9,15,17]. Therefore, there remains little evidence that the generation of neural ectoderm in amniotes results from the blockade of BMP signalling by secreted inhibitors.

Nevertheless, in embryonic regions of prestreak stage chick embryos, *Bmp4* and *Bmp7* are expressed at low levels and the expression of both genes is extinguished in prospective neural cells before the formation of the primitive streak. Thus, the extinction of BMP expression from the prospective neural plate may represent a common link in neural induction in different vertebrate embryos. In different vertebrate organisms, *Bmp7* is expressed at high levels in cells of the extra-embryonic region of prestreak embryos, that is, adjacent to the lateral region of area pellucida that generates epidermal cells when cultured *in vitro*. Consistent with these observations, application of BMP4 to the area pellucida at prestreak stages promotes the generation of cells of epidermal character and prevents the generation of neural tissue. Thus, it is possible that, in

Figure 6



The FGFR tyrosine kinase inhibitor SU5402 inhibits the acquisition of neural character. **(a–f)** Stage XII medial (M) explants ($n = 6$), when cultured in the presence of 2 μM SU5402, did not generate (a) Sox2$^+$ cells, (b) Sox3$^+$ cells, (c) Pax6$^+$ cells or (d) Otx2$^+$ cells, but did generate (e) Msx1/2$^+$ cells (95 ± 5% cells per section) and (f) GATA2$^+$ cells (85 ± 10% cells per section). **(g–l)** Stage XII medial explants cultured in the presence of both 2 μM SU5402 and 64 nM noggin generated (g) Sox2$^+$ cells, (h) Sox3$^+$ cells, (i) Pax6$^+$ cells and (j) Otx2$^+$ cells (95% ± 10% cells per section), but no (k) Msx1/2$^+$ cells or (l) GATA2$^+$ cells. **(m–r)** Stage XII explants cultured in the presence of both 2 μM SU5402 and the soluble dominant-negative BMP receptors IA/Fc and IB/Fc (0.5 μM each) generated (m) Sox2$^+$ cells, (n) Sox3$^+$ cells, (o) Pax6$^+$ cells and (p) Otx2$^+$ cells (80 ± 10% cells per section), but no or very few (q) Msx1/2$^+$ cells (10 ± 5% cells per section) and no (r) GATA2$^+$ cells were generated. The scale bar in (r) represents 100 μM and applies to (a–r).

the chick embryo, the repression of *Bmp4* and *Bmp7* expression may be causally linked with the acquisition of neural character and that cells that acquire epidermal character maintain *Bmp4* and *Bmp7* expression.

FGF signalling in epiblast cells is required for the acquisition of neural character

In both *Xenopus* and chick embryos, FGF has been suggested to play a role in neural induction [31,32,43,44]. Our results provide evidence that *Fgf3* is expressed in early epiblast cells before the formation of the primitive streak and that ongoing FGF signalling is required for effective repression of *Bmp4* and *Bmp7* expression in epiblast cells and for the acquisition of neural character. Signalling by FGF3 is mediated primarily through the activation of FGFRs 1b and 2b [34,35]. Genetic evidence in mouse shows that the FGFR2 is required for pregastrulation development [45] and, thus, it remains possible that in mouse, as in chick, an early phase of FGF signalling is required to promote neural character.

Our results show also that exposure of epiblast cells to noggin, chordin or soluble dominant-negative BMP receptors is sufficient to promote the generation of neural cells under conditions when FGF signalling is attenuated and *Bmp* expression is maintained in area pellucida cells. These results raise the possibility that an important role of FGF signalling during early neural differentiation is to attenuate BMP signalling by repressing the expression of *Bmps* in epiblast cells of prospective neural character.

Cells in the lateral region of stage XII epiblast cells express *Fgf3*, but these cells do not downregulate *Bmp* expression *in vitro*. Moreover, in these lateral epiblast regions, noggin, alone or in combination with FGF1, an activator of all FGFRs, was not sufficient to induce cells of neural character (data not shown). Thus, FGF signalling is not sufficient to repress BMP expression in prospective epidermal cells, nor does the blockade of BMP signalling at this stage appear to be sufficient to impose neural character. This distinction could be explained if medial but not lateral epiblast cells are exposed to additional signals that are normally required for the induction of neural character. Alternatively, lateral cells may become refractory to the actions of FGF at early stages. Despite these uncertainties, the present explant assay system may provide a means of characterising in more detail the mechanism through which epiblast cells acquire neural fates at early stages of chick embryogenesis.

Materials and methods

Preparation of inducing factors

COS-7 cells were transfected with chick chordin (in pMT23) [46] using Lipofectamine (Gibco BRL). The chordin-conditioned medium was prepared by incubating transfected COS cells for 48 h in OPTI-MEM medium, and was used undiluted. Human recombinant BMP4 and FGF1 (R & D systems) were used at concentrations of 1.3 nM and 33 nM, respectively. Recombinant mouse dominant-negative BMP receptors IA/Fc and IB/Fc (R & D systems) were used at a final concentration of 0.5 μM. Purified recombinant *Xenopus* noggin was used at a concentration of 64 nM. The FGF inhibitor SU5402 (Calbiochem) was used at a concentration of 2 μM.

Isolation and growth of explants

Explants were isolated from stage VIII–XII [25] chick embryos, as described in the text (Figures 1 and 2). Epiblast ectoderm located rostral to the tip of the primitive streak was isolated from stage 2 and stage 4 [47] chick embryos. Explants were cultured for 40 h at 37°C on a collagen bed in OPTI-MEM medium supplemented with N-2 (Gibco BRL) in the presence or absence of factors as described in the text.

Immunohistochemistry

Immunohistochemical localisation of proteins was performed as previously described [48]. Neural tissue was defined with rabbit anti-Sox2 antibody provided by R. Lovell-Badge [49], rabbit anti-Sox3 antibody, mouse

anti-Pax6 and rabbit anti-Otx2 antibodies [20], provided by E. Boncinelli. Msx1/2 were detected with monoclonal antibody 4G1. GATA2 was detected with mouse anti-GATA2 IgG (Santa Cruz Biotechnology).

RT–PCR analysis

RT–PCR was performed on pooled stage XII medial and lateral explants. Total RNA was prepared from the following: either 10 pooled uncultured explants or six pooled explants cultured for 40 h with or without factors as described in the text. Control RNA was prepared from the embryonic region of a HH stage 4 embryo. Total RNA was reverse transcribed under standard conditions. The ribosomal gene *S17* was used as a semi-quantitative control to ensure that equivalent concentrations of RNA were transcribed. Oligonucleotide primer sets included: *S17* [50]; *Bmp-4* 5′-CTACTATGCCAAGTCCTGCT-3′; 5′-TCGCTGAAATCCA-CATAGA-3′; *Bmp*-7 5′-TTCAGGTTTGATCTCTC-3′, 5′-AATAGAG-CACTGAGATG-3′; and *Fgf-3* 5′-AAACAGCGTCTTCAGTATCC-3′, 5′-AGGCTCTCTCGGTATCTCAC-3′; *Brachyury* [18]; chordin 5′-TAAGGGCAGGAATGTGGAAC-3′, 5′-TTGTCCCCATCAAAGTAGC-3′; *Raldh2* [51] and *FgfR2b* 5′-GCTGAAGTGCTGACACTGTA-3′, 5′-TAGCACACCAAATGACCATA-3′. PCRs were performed at an annealing temperatures of 50°C (*S17*, *Bmp4* and *Bmp7*), or 55°C. (*Fgf3*, *Brachyury, FgfR2* and *Raldh2*) or 60°C (chordin). *Fgf3, S17*, *Bmp4* and *Bmp7* were amplified for 25 cycles and subjected to Southern blot analysis. *FgfR2b* was amplified for 35 cycles. *Brachyury*, chordin and *Raldh2* were subjected to 35 cycles of amplification from RNA prepared from stage XII medial explants after 40 h of culture, while RNA prepared from the control HH stage 4 embryo was amplified for 30 cycles under parallel conditions.

Acknowledgements

We are grateful to members of the Edlund lab for helpful discussions and to C. Stern for discussion of unpublished results. T.E. is supported by the Swedish Medical Research Council and by the Foundation for Strategic Research. R.H. is supported by the NIH. T.M.J. is supported by the NIH and is an Investigator of the Howard Hughes Medical Institute.

References

1. Wilson PA, Hemmati-Brivanlou A: **Induction of epidermis and inhibition of neural fate by Bmp-4.** *Nature* 1995, **376:**331-333.
2. Fainsod A, Steinbeisser H, De Robertis EM: **On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo.** *EMBO J* 1994, **13:**5015-5025.
3. Sasai Y, Lu B, Steinbeisser H, De Robertis EM: **Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*.** *Nature* 1995, **376:**333-336.
4. Lamb TM, Knecht AK, Smith WC, Stachel SE, Economides AN, Stahl N, Yancopolous GD, Harland RM: **Neural induction by the secreted polypeptide noggin.** *Science* 1993, **262:**713-718.
5. Hemmati-Brivanlou A, Kelly OG, Melton DA: **Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity.** *Cell* 1994, **77:**283-295.
6. Hansen CS, Marion CD, Steele K, George S, Smith WC: **Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3.** *Development* 1997, **124:**483-492.
7. Kroll KL, Salic AN, Evans LM, Kirschner MW: **Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation.** *Development* 1998, **125:**3247-3258.
8. Bouwmeester T, Kim S, Sasai Y, Lu B, De Robertis EM: **Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer**. *Nature* 1996, **382:**595-601.
9. Streit A, Lee KJ, Woo I, Roberts C, Jessell TM, Stern CD: **Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo.** *Development* 1998, **125:**507-519.
10. Streit A, Stern CD: **Neural induction. A bird's eye view.** *Trends Genet* 1999, **15:**20-24.
11. McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP: **noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite.** *Genes Dev* 1998, **12:**1438-1452.
12. Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A: **Multiple defects and perinatal death in mice deficient in follistatin.** *Nature* 1995, **374:**360-363.
13. Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, *et al.*: **The organizer factors Chordin and noggin are required for mouse forebrain development.** *Nature* 2000, **403:**658-661.
14. Ang SL, Rossant J: **HNF-3 beta is essential for node and notochord formation in mouse development.** *Cell* 1994, **78:**561-574.
15. Storey KG, Crossley JM, De Robertis EM, Norris WE, Stern CD: **Neural induction and regionalisation in the chick embryo.** *Development* 1992, **114:**729-741.
16. Beddington RS: **Induction of a second neural axis by the mouse node.** *Development* 1994, **120:**613-620.
17. Levin H: **The roles of activin and follistatin signaling in chick gastrulation.** *Int J Dev Biol* 1998, **42:**553-559.
18. Muhr J, Graziano E, Wilson S, Jessell TM, Edlund T: **Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos.** *Neuron* 1999, **23:**689-702.
19. Rex M, Orme A, Uwanogho D, Tointon K, Wigmore PM, Sharpe PT, Scotting PJ: **Dynamic expression of chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue.** *Dev Dyn* 1997, **209:**323-332.
20. Mallamaci A, Di Blas E, Briata P, Boncinelli E, Corte G: **OTX2 homeoprotein in the developing central nervous system and migratory cells of the olfactory area.** *Mech Dev* 1996, **58:**165-178.
21. Li HS, Yang JM, Jacobson RD, Pasko D, Sundin O: **Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens.** *Dev Biol* 1994, **162:**181-194.
22. Streit A, Stern CD: **Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity.** *Mech Dev* 1999, **82:**51-66.
23. Susuki A, Ueno N, Hemmati-Brivanlou A: ***Xenopus* msx1 mediates epidermal induction and neural inhibition by BMP4.** *Development* 1997, **124:**3037-3044.
24. Sheng G, Stern CD: **Gata2 and gata3: novel markers for early embryonic polarity and for non-neural ectoderm in the chick embryo.** *Mech Dev* 1999, **87:**213-216.
25. Eyal-Giladi H, Kochav S: **From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of development of the chick.** *Dev Biol* 1976, **49:**321-227.
26. Hatada Y, Stern CD: **A fate map of the epiblast of the early chick embryo.** *Development* 1994, **120:**2879-2889.
27. Kispert A, Ortner H, Cooke J, Herrmann BG: **The chick Brachyury gene: developmental expression pattern and response to axial induction by localized activin.** *Dev Biol* 1995, **168:**406-415.
28. Swindell EC, Thaller C, Sockanathan S, Petkovich M, Jessell TM, Eichele G: **Complementary domains of retinoic acid production and degradation in the early chick embryo.** *Dev Biol* 1999, **216:**282-296.
29. Barlow AJ, Francis-West PH: **Ectopic application of recombinant BMP-2 and BMP-4 can change patterning of developing chick facial primordia.** *Development* 1997, **124:**391-398.
30. Watanabe Y, Le Douarin NM: **A role for BMP-4 in the development of subcutaneous cartilage.** *Mech Dev* 1996, **57:**69-78.
31. Alvarez IS, Araujo M, Nieto MA: **Neural induction in whole chick embryo cultures by FGF.** *Dev Biol* 1998, **199:**42-54.
32. Storey KG, Goriely A, Sargent CM, Brown JM, Burns HD, Abud HM, Heath JK: **Early posterior neural tissue is induced by FGF in the chick embryo.** *Development* 1998, **125:**473-484.
33. Mahmood R, Kiefer P, Guthrie S, Dickson C, Mason I: **Multiple roles for FGF-3 during cranial neural development in the chicken.** *Development* 1995, **121:**1399-1410.
34. Mathieu M, Chatelain E, Ornitz D, Bresnick J, Mason I, Kiefer P, Dickson C: **Receptor binding and mitogenic properties of mouse fibroblast growth factor 3. Modulation of response by heparin.** *J Biol Chem* 1995, **270:**24197-24203.
35. Mathieu M, Kiefer P, Mason I, Dickson C: **Fibroblast growth factor (FGF) 3 from *Xenopus laevis* (XFGF3) binds with high affinity to FGF receptor 2.** *J Biol Chem* 1995, **270:**6779-6787.
36. Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR, Schlessinger J: **Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors.** *Science* 1997, **276:**955-960.
37. Rodriguez Esteban C, Capdevila J, Economides AN, Pascual J, Ortiz A, Izpisua Belmonte JC: **The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry.** *Nature* 1999, **401:**243-251.

38. Dias MS, Schoenwolf GC: **Formation of ectopic neurepithelium in chick blastoderms: age-related capacities for induction and self-differentiation following transplantation of quail Hensen's nodes.** *Anat Rec* 1990, **228:**437-448.
39. Storey KG, Selleck MA, Stern CD: **Neural induction and regionalisation by different subpopulations of cells in Hensen's node.** *Development* 1995, **121:**417-428.
40. Pera E, Stein S, Kessel M: **Ectodermal patterning in the avian embryo: epidermis versus neural plate.** *Development* 1999, **126:**63-73.
41. Baker JC, Beddington RSP, Harland RM: **Wnt signaling in *Xenopus* embryos inhibits BMP4 expression and activates neural development.** *Genes Dev* 1999, **13:**3149-3159.
42. Roeser T, Stein S, Kessel M: **Nuclear beta-catenin and the development of bilateral symmetry in normal and LiCl-exposed chick embryos.** *Development* 1999, **126:**2955-2965.
43. Launay C, Fromentoux V, Shi DL, Boucaut JC: **A truncated FGF receptor blocks neural induction by endogenous *Xenopus* inducers.** *Development* 1996, **122:**869-880.
44. Lamb TM, Harland RM: **Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern.** *Development* 1995, **121:**3627-3636.
45. Arman E, Haffner-Krausz R, Chen Y, Heath JK, Lonai P: **Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development.** *Proc Natl Acad Sci USA* 1998, **95:**5082-5087.
46. Roelink H, Augsburger A, Heemskerk J, Korzh V, Norlin S, Ruiz i Altaba A, *et al.*: **Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord.** *Cell* 1994, **76:**761-775.
47. Hamburger V, Hamilton HL: **A series of normal stages in the development of the chick embryo.** *J Morph* 1951, **88:**49-92.
48. Yamada T, Placzek M, Tanaka H, Dodd J, Jessell TM: **Control of cell pattern in the developing nervous system polarizing activity of the floor plate and notochord.** *Cell* 1991, **64:**635-647.
49. Kamachi Y, Uchikawa M, Collignon J, Lovell-Badge R, Kondoh H: **Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction.** *Development* 1998, **125:**2521-2532.
50. Tanabe Y, Roelink H, Jessell TM: **Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation.** *Curr Biol* 1995, **5:**651-658.
51. Sockanathan S, Jessell TM: **Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons.** *Cell* 1998, **94:**503-514.

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