Anterior identity is established in chick epiblast by hypoblast and anterior definitive endoderm

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Summary

Previous studies of head induction in the chick have failed to demonstrate a clear role for the hypoblast and anterior definitive endoderm (ADE) in patterning the overlying ectoderm, whereas data from both mouse and rabbit suggest patterning roles for anterior visceral endoderm (AVE) and ADE. Based on similarity of gene expression patterns, fate and a dual role in 'protecting' the prospective forebrain from caudalising influences of the organiser, the chick hypoblast has been suggested to be the homologue of the mouse anterior visceral endoderm. In support of this, when transplanted to chick embryos, the rabbit AVE induces anterior markers in the chick epiblast. To re-evaluate the role of the hypoblast/ADE (lower layer) in patterning the chick ectoderm, we used rostral blastoderm isolates (RBIs) as an assay, that is, rostral regions of blastoderms transected at levels rostral to the node. RBIs are, therefore, free from the influences of Hensen's node and ingressing axial mesoderm – tissues that are able to induce *Ganf*, the earliest specific marker of anterior neural plate. We demonstrate, using such RBIs (or RBIs dissected to remove the lower layer with or without tissue replacement), that the hypoblast/ADE (lower layer) is required and sufficient for patterning anterior positional identity in the overlying ectoderm, leading to expression of *Ganf* in neuroectoderm. Our results suggest that patterning of anterior positional identity and specification of neural identity are separable events operating to pattern the rostral end of the early chick embryo. Based on this new evidence we propose a revised model for establishing anteroposterior polarity, neural specification and head patterning in the early chick that is consonant with that occurring in other vertebrates.

Key words: Blastula, Expression, Forebrain, Gastrula, Head, Induction, Organiser, Markers, Patterning, Specification, Trunk/tail, Visceral endoderm

Introduction

Early patterning of the vertebrate central nervous system involves a complex and interwoven set of spatiotemporal inductions, tissue movements and patterning mechanisms. Two major tasks are achieved early on: the definition of anterior positional identity and the segregation of neural tissue identity. In mouse (Beddington and Robertson, 1998; Beddington and Robertson, 1999), zebrafish (Houart et al., 1998; Koshida et al., 1998) and *Xenopus* (Jones et al., 1999), signalling centres have been identified that are distinct from the classical organiser and capable of establishing anterior positional identity separately from neural specification. In chick, therefore, positional and tissue identity may also be separable. Three mechanisms could exist for establishing initial anterior positional and tissue identity in the epiblast/prospective neural plate: first, neural specification leads to neuralised tissue, which by default is anterior in character (Mangold, 1933; Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954; Spemann, 1931; Spemann, 1938); second, positional identity is conferred by a separate mechanism upon neuralised tissue, which is initially positionally neutral (Waddington and Needham, 1936); and third, initial anterior positional identity is established in the epiblast independent of and before neural specification occurs. The latter two possibilities require that anterior positional identity be established separately from neural specification.

In chick, the process of neural induction begins before the onset of gastrulation, with competence being conferred by FGF signals emanating from the posterior of the embryo (Muhr et al., 1999; Streit et al., 2000; Wilson et al., 2001; Wilson and Edlund, 2001; Wilson et al., 2000). The cellular interactions leading to the specification of competent tissue as neural, and the timing over which they occur, remain unclear. Although transplants of posterior epiblast can induce transient expression of pre-neural markers such as *Sox3* in epiblast, stable expression of *Sox2* in specified neuroectoderm requires both central and posterior epiblast cells to come together at mid-streak stages (3+ or 3c/d), forming a functional organiser (Streit et al., 2000). Further support for the timing of neural specification at mid-streak stages comes from explant studies in which competent tissue at stage 3d, but not 3c, cultured in isolation, was able to self differentiate, developing the columnar neuroepithelial morphology of specified neuroectoderm, as well as having stable expression of *Sox2* (Darnell et al., 1999).

The relative spatiotemporal positions of early embryonic tissues (Fig. 1) suggest that several tissues could function

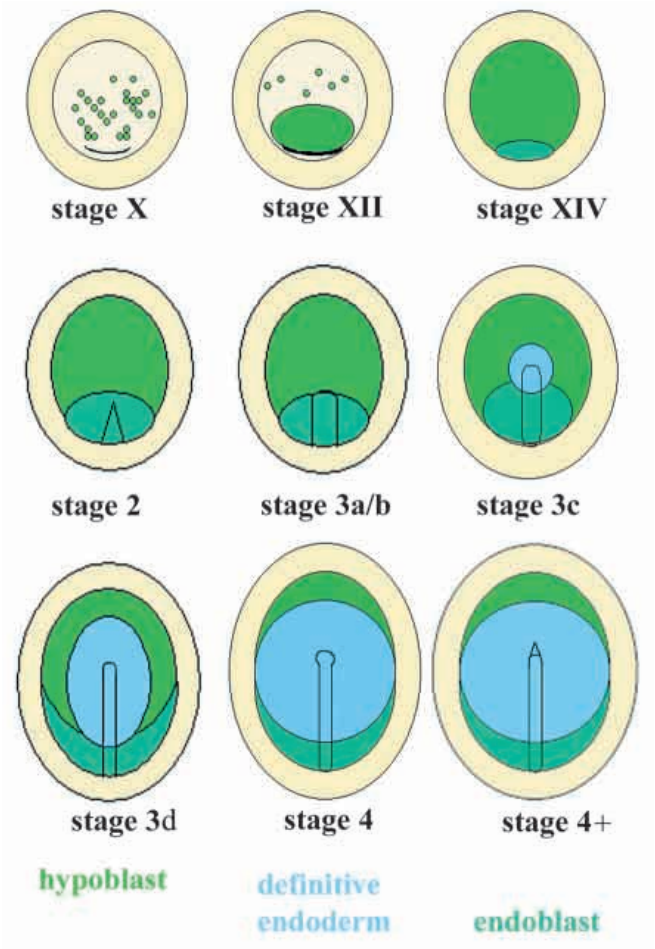

Fig. 1. Early chick embryonic stages and formation of the lower layer. At stage X, detached cells originating from epiblast lie in the subgerminal cavity (green circles). Koller's sickle (black crescent) and the posterior marginal zone produce a sheet of lower layer cells extending rostrally, forming the primary hypoblast (green sheet) by stage XIV. Endoblast (dark green) formation from the caudal part of the embryo then begins. Stage 2, a broad, short triangular streak forms in the caudal part of the embryo. The streak begins to elongate at stage 3a/b, but only reaches the centre of the area pellucida at stage 3c (equivalent to HH stage 3+) with definitive endoderm ingressing through the rostral tip of the streak (blue). Stage 3a and 3b are difficult to separate in practice (stage 3a, a short and broad linear streak with no groove yet visible; stage 3b, a longer narrower linear streak with visible primitive groove). The defining feature of stage 3a/b is that the streak has not yet reached the centre of the area pellucida, the widest zone across the left/right axis of the embryo. Stage 3c, the elongated and grooved streak extends to the centre, with stage 3d characterised by the rostral extension of the streak beyond the centre point. Maximum streak extension is at stage 4, with a noticeable change in the morphological character of the ectoderm, with the onset of neural specification at stage 3d. By stage 4, definitive endoderm has almost completely ingressed and both primary hypoblast and endoblast have been displaced towards the rostral and caudal poles of the embryo, respectively. Definitive endoderm spreads by a polonaise movement; after ingressing through the tip of the streak, the cells spread rostrally and then laterally. A noticeable swelling at the rostralmost part of the streak indicates the formation of Hensen's node. Stage 4+ heralds the beginning of ingression of axial mesoderm, recognised by a triangular-shaped ingression rostral to Hensen's node.

potentially in anteroposterior patterning. Candidate tissues able to produce 'organising' signals include a population of central epiblast (CE) cells (Darnell et al., 1999; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Healy et al., 2001; Lawson and Schoenwolf, 2001a; Lawson and Schoenwolf, 2001b; Schoenwolf et al., 1989b; Streit et al., 2000), and the underlying lower layer, the hypoblast and ingressing anterior definitive endoderm (ADE). The CE population is a group of epiblast cells rostral to the tip of the primitive streak between stages 2 and 4. They are in a position equivalent to that of the mouse early gastrula organiser (EGO), which has been shown to have a role in head patterning when combined with epiblast and anterior visceral endoderm (AVE) (Tam and Steiner, 1999). As the primitive streak extends forward, the CE population becomes incorporated into the streak (Garcia-Martinez et al., 1993; Garcia-Martinez and Schoenwolf, 1993; Joubin and Stern, 1999; Lawson and Schoenwolf, 2001a; Lawson and Schoenwolf, 2001b; Schoenwolf and Alvarez, 1989; Schoenwolf et al., 1989a; Schoenwolf et al., 1989b; Schoenwolf et al., 1992; Smith and Schoenwolf, 1991). Early fate-mapping studies used quail/chick chimaeras and fluorescent dye injections to determine the fate of cells in the rostral streak (Garcia-Martinez et al., 1993; Garcia-Martinez and Schoenwolf, 1993; Schoenwolf et al., 1992; Selleck and Stern, 1991). Homotopic and isochronic cell grafts from stage 3a/b rostral streak contributed extensively to head mesenchyme and foregut endoderm, whereas a small proportion was also detected in notochord and the median hinge-point cells (i.e. the future floor plate of the neural tube). Stages 3c-4 rostral streak cells contributed mainly to notochord and median hinge-point cells, although a small number were traced to the head mesenchyme and foregut endoderm.

Other tissues in chick with putative 'organising' ability are those comprising the lower layer – the hypoblast and ADE. Based on similar gene expression patterns, fate and a dual role in 'protecting' the prospective forebrain from caudalising influences of the organiser, the hypoblast at stage XII/XIII has been proposed to be the homologue of the mouse anterior visceral endoderm (Foley et al., 2000). The hypoblast forms the primitive endoderm underlying the epiblast from stage X/XI until the ADE begins to ingress at stage 3a, displacing the hypoblast rostrally. Transplant experiments of the hypoblast only, produced transient induction of *Sox3* and *Otx2* (Foley et al., 2000). These authors argue that the hypoblast, therefore, protects the epiblast against caudalising influences rather than influencing cell fate. Neither hypoblasts from stage XIV, nor the ADE present under the epiblast from stage 3a until the beginning of axial mesoderm ingression from stage 4+, have previously been implicated in anterior patterning. Potentially both planar and vertical signalling mechanisms operate in patterning positional identity through central epiblast, and lower layer hypoblast and ADE, respectively.

Owing to contradictory results and gaps in our understanding regarding the role of CE, the hypoblast and ADE, we have tested these tissues for a role in establishing anterior positional identity. Previous chick transplant studies have failed overall to show a role for lower layer tissues in determining cell fate (Foley et al., 2000). Removing the lower layer has also met with little previous success, mostly because of the embryos' ability to recover and replace ablated tissues, such as the hypoblast at early stages (Vanroelen et al., 1982),

or to tissue being removed later than the time in question (Withington et al., 2001). We used the transection assay (Fig. 2) (Darnell et al., 1999; Healy et al., 2001; Schoenwolf et al., 1989b; Yuan et al., 1995a) to address what the role for these lower layer tissues is, and determined that anterior positional identity seems to be separable from neural specification. Isolating rostral epiblast (prospective anterior neural plate) from the influences of Hensen's node and ingressing axial mesoderm was crucial, because ingressing axial mesoderm from stage 4+ has a role in the induction of *Ganf*, the earliest specific maker of anterior neural plate (Knoetgen et al., 1999). The expression patterns of *Sox2*, the most definitive early neural specification marker identified to date (Rex et al., 1997; Streit et al., 2000; Streit et al., 1997; Uchikawa et al., 2003), and *Ganf* were re-examined and used to define anterior and neural identity. We show that anterior positional identity is established and maintained in the epiblast by the hypoblast at stage 3a/b and ADE at stages 3d and 4, apparently separately from neural specification, and we propose a revised model for establishing anteroposterior polarity, neural specification and head patterning, based on this new evidence.

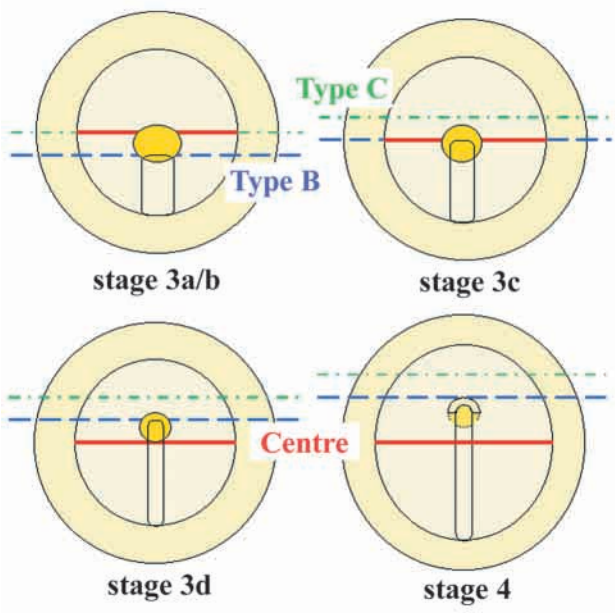

Fig. 2. Transections. At all stages shown, transections were either Type B (broken blue line) or Type C (broken green line). Type B transections were made at the rostral extent of the ingressing primitive streak, excluding the extending streak and later ingressing axial mesoderm from the rostral blastoderm isolates (RBIs). Type C transections were made by measuring 125 μm rostral to the tip of the primitive streak (approximately one node diameter) and then transecting through all layers. The centre of the area pellucida is marked by a red line and assists in staging before transecting the embryo. The yellow circle represents the central epiblast (CE) population of cells. This group of cells is known to have organising properties and is rostral to the extending streak at stage 3a/b. At stages 3c, the CE is becoming incorporated into the extending streak. By stage 3d and 4, CE is incorporated into the rostral streak and forms part of Hensen's node. At each of the stages note the relative positions of the CE, the rostral extent of the primitive streak, the centre of the area pellucida, and the level of type B and type C transections.

Materials and methods

Incubation and staging

Hens' eggs (White Leghorn) were incubated at 38°C for desired stages. Prestreak embryos were staged according to Eyal-Giladi and Kochav (Eyal-Giladi and Kochav, 1976) (EGK; Roman numerals), and Hamburger and Hamilton (Hamburger and Hamilton, 1951) for primitive streak and later stages (HH; Arabic numbers), with HH stage 3 (gastrula) embryos refined according to Schoenwolf and co-workers (Fig. 1) (Chapman et al., 2002).

In situ hybridisation

In situ hybridisation was performed as described previously (Chapman et al., 2002). Embryos were then cleared in 80% glycerol/PBS, embedded in 20% gelatin, fixed with 4% PFA and sectioned using a Leica vibratome at 40-50 μm. Embryos were imaged with a SPOT, Coolsnap or Zeiss Axiocam digital camera. The following markers were used: *Sox2*, specified neuroectoderm (R. Lovell-Badge): *Wnt8c*, ingressing mesodermal cells (J. Dodd): *Ganf*, earliest marker of anterior neuroectoderm (A. Zaraisky); *Fgf8*, primitive streak (G. Martin); *Chordin*, primitive streak, Hensen's node and ingressing axial mesoderm (A. Graham); *Crescent*, hypoblast and ADE (P. Pfeffer).

Embryo culture and transection

Transection of embryos was performed as described by Darnell et al. (Darnell et al., 1999). In experiment 1, embryos were transected to determine the effect of separating rostral tissues from the primitive streak, prospective node and ingressing mesoderm. Embryos were transected at the rostralmost level of the streak (Type B), or 125 μm rostral to the streak (Type C), at stages 3a-4+ and cultured on an agar/albumen substrate with no added culture media for 24 hours (Fig. 2). The blastoderm isolates were then processed for *Ganf* and *Sox2* transcripts (Table 1). In experiment 2, the lower layer of rostral isolates was removed to determine whether this layer has a role in patterning the rostral epiblast. Isolates were cultured in collagen: 3.3 mg/ml rat tail collagen (Roche) was prepared in 0.2% acetic acid. 480 μl collagen, 36 μl DEPC-H_2O, 60 μl 10× DMEM and 20 μl 0.75% bicarbonate solution were added together on ice. Rostral and caudal isolates of each transected embryo were embedded, and after 30 minutes at 37°C in a 5% CO_2 incubator, carbonated Neurobasal medium supplemented with Glutamax was added. Embryos were transected (Type B) in saline (123 mM), followed by removal of the lower layer using tungsten needles (0.125 mm tungsten wire, WPI). No enzymatic treatments were used. RBIs with an intact lower layer served as controls (Table 2). To test for mesoderm in the RBIs, in experiment 3, transected embryos were fixed immediately and then processed for *Wnt8c* expression. Experiments 4 and 5 were designed to test sufficiency of the lower layer to induce *Ganf*: either rostral (experiment 4) or caudal (experiment 5) lower layer was recombined with rostral epiblast in collagen culture for 24 hours and then processed for *Ganf* transcripts.

Results

Terminology – formation of tissues in the early embryo

Before discussing our results, it is important for the purposes of clarity to define the terminology that we will be using. This is especially true because early development of the avian embryo is complex, and different investigators often use different terms, or even the same terms but in different ways.

The lower (ventral) layer is formed by polyingression of cells from the overlying epiblast at stage X/XI, forming islands of cells in the subgerminal cavity (Fig. 1) (Harrison et al., 1991; Lawson and Schoenwolf, 2001a). Together with cells moving

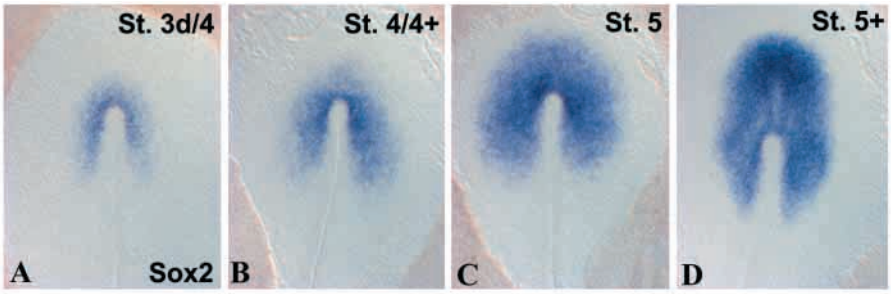

Fig. 3. *Sox2* expression enlarges progressively with neural specification. A panel of whole-mount embryos probed for *Sox2* transcripts by in situ hybridisation. Anterior is towards the top. (A) At stage 3d, neural specification of the prospective neural ectoderm occurs. The first cells with detectable expression of *Sox2* lie in close proximity rostral and lateral to the definitive streak. (B) As neuralisation proceeds and Hensen's node forms, expression of *Sox2* expands outward from the streak (stages 4/4+). The caudal boundary of expression remains constant at this stage. (C) By stage 5, the neural plate has pan-neural expression of *Sox2* over the whole of the still flat neuroectoderm. (D) At stage 5+, the node regresses, drawing the posterior boundary of expression caudally. The ventral neural plate looks lighter in colour as the neural tube begins to form, becoming narrower as the neural folds move medially. Some variation in transcript levels occurs with stronger expression of *Sox2* in the anteriormost neural plate.

rostrally from Koller's sickle and the posterior marginal zone (PMZ), a complete lower layer is formed, called the primary hypoblast (Callebaut et al., 1999; Stern and Canning, 1990; Vakaet, 1970). The endoblast (secondary hypoblast) forms at stage XIII/XIV, with cells moving rostrally from Koller's sickle and the PMZ. Primary and secondary hypoblast form a continuous sheet of cells under the epiblast by stage XIV/2 – the primitive endoderm. The primitive streak forms at stage 2, as a triangularly shaped structure that elongates rostrally. Definitive endoderm begins ingression through the rostral end of the primitive streak from stage 3a to stage 4/4+, by which time the lower layer has fully displaced the hypoblast sheet rostral to the embryo, forming the germ cell crescent (Lawson and Schoenwolf, 2003). At stages 3a/b the rostral streak gives rise to the ADE, including the midline prechordal plate endoderm (PCPE) that lies beneath the forebrain. During subsequent development, the prechordal plate endoderm buds off proliferative mesoderm and together with ingressing mesoderm, forms a middle layer, the prechordal plate mesoderm, which contributes to the head mesenchyme (Seifert et al., 1993). Axial mesoderm ingresses through the rostral streak from stage 4+ (the head process), and consists of a mixed cell population of prechordal plate mesoderm and rostral notochord, which intercalates between the neuroectoderm and ADE (Foley et al., 1997; Vesque et al., 2000). The molecular basis for the spatial separation of these two populations is unclear, although SEM studies of the morphological movements have been described (England, 1984; England and Wakely, 1977; England et al., 1978; Wakely and England, 1979). The laying down of more caudal notochord occurs as Hensen's node and the definitive streak regress caudally. Intercalation of the fan-shaped prechordal plate mesoderm results in the prechordal plate mesoderm coming to partially overlie the PCPE.

Sox2 is a pan-neural marker expressed from the onset of neural specification

Sox2 is the earliest pan-neural marker stably expressed in the specified neuroectoderm (Rex et al., 1997; Streit et al., 2000; Streit et al., 1997). Embryos were tested for expression of *Sox2* from stage XI/XII (not shown), with expression first detected at stage 3d, as expected (Fig. 3A). Expression began just rostral and lateral to Hensen's node and later expanded rostrally and laterally toward the outer boundary of the neural plate, away from the streak (stages 4/4+) (Fig. 3B). This pattern suggests that neural specification occurs in a spatiotemporal manner across the prospective neuroectoderm. The caudal boundary of expression remained constant, suggesting that the first cells to express *Sox2* are not the anteriormost neuroectoderm, but rather are the more caudal neural plate. By stage 5, *Sox2* was expressed throughout the neural plate, which is still flat prior to formation of the head fold and neural tube (Fig. 3C). Concomitant with node regression, the caudal boundary of *Sox2* expression extended caudally through convergent extension (Fig. 3D). The ventral neural plate was lighter in colour as the neural tube formed, while the neural plate narrowed as the neural folds rose up and moved medially, with varying levels of expression within the rostrocaudal length of the neural plate, with stronger expression of *Sox2* in the rostralmost neural plate. In summary, *Sox2* is detected from stage 3d onwards and is the earliest available stable marker of specified neuroectoderm.

Ganf is expressed in the neuroectoderm from stage 4

The time course of *Ganf* expression was examined by in situ hybridisation from stage X/XI to stage 17. *Ganf* transcripts were detected from stage 4 (Fig. 4A), earlier than previously reported (Knoetgen et al., 1999). This is important because Knoetgen and colleagues reported that *Ganf* is expressed only once ingressing axial mesoderm underlies the anterior neural plate. Our wholemounts and sagittal sections (Fig. 4A,B) reveal a gap between *Ganf* expression (blue) and *Chordin* (red), which is a marker of Hensen's node and ingressing axial mesoderm. In addition, previous studies using scanning electron microscopy of dissected blastoderms show that ingressed mesoderm is absent rostral to the node at this stage (Lawson and Schoenwolf, 2001a). Furthermore, unlike in mouse, where transcripts of the *Anf* homologue *Hesx1* are found in both neuroectoderm and the underlying anterior visceral endoderm (Hermesz et al., 1996), expression in chick was detected only in neuroectoderm fated to become the forebrain rostral to the ZLI (Kazanskaya et al., 1997). At later stages, *Ganf*-positive mesodermal cells may be present, as in mice and frogs, but this is not the case at stage 4 (Fig. 4A,B) (Kazanskaya et al., 1997). At stage 4/4+ the definitive streak has extended maximally (Fig. 4C) and ingression of axial mesoderm begins. Although these axial cells migrate rostrally, Hensen's node regresses caudally with the streak, laying down

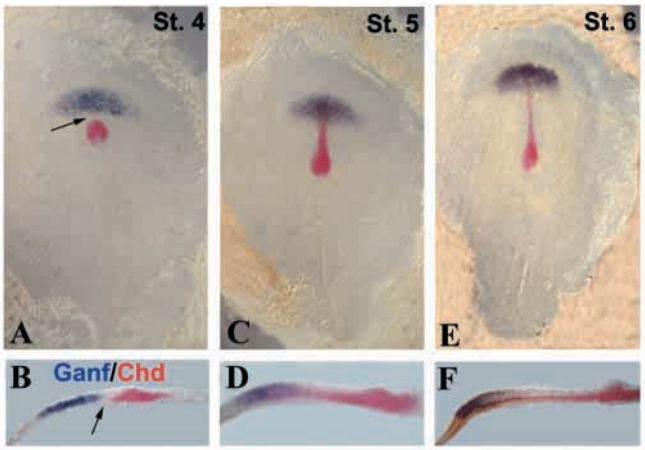

Fig. 4. Whole-mount in situ hybridisation of *Ganf*. Embryos stained for *Ganf* (blue) and *Chordin* (red). (A,C,E) Whole-mount, dorsal view with anterior towards the top of the page. (B,D,F) Sagittal sections (40-50 μm) with anterior towards the left. (A,B) At stage 4 (18 hours) *Ganf* is expressed for the first time. Arrows indicate the gap between neuroectodermal *Ganf* expression and *Chordin* in Hensen's node and ingressing axial mesoderm. (C,D) At stage 5, axial mesoderm underlies *Ganf* expressing tissue and there is some suggestion that mesodermal cells may also express *Ganf* at this point. (E,F) The headfold begins to form at stage 6 (24 hours) and the *Ganf*-positive zone narrows as convergent extension takes place in advance of neural tube formation.

the notochord. The domain of expression of the axial mesodermal marker *Chordin* lengthens during this process (Fig. 4C-F). *Ganf* expression narrows mediolaterally from stage 5 as the neural plate extends rostrally and begins to fold (Fig. 4E), leading to formation of the neural tube. *Ganf* expression becomes progressively restricted, until at stage 17 when expression is detected only in the floor of Rathke's pouch (not shown), a region fated to form part of the anterior pituitary. Double in situ hybridisation confirms that *Ganf* expression is co-localised with the rostralmost *Sox2* domain, beginning in stage 4 embryos (Fig. 5). These data indicate that *Ganf* is expressed before ingression of axial mesoderm at stage 4/4+ and may, therefore, be induced by tissues other than Hensen's node and the axial mesoderm.

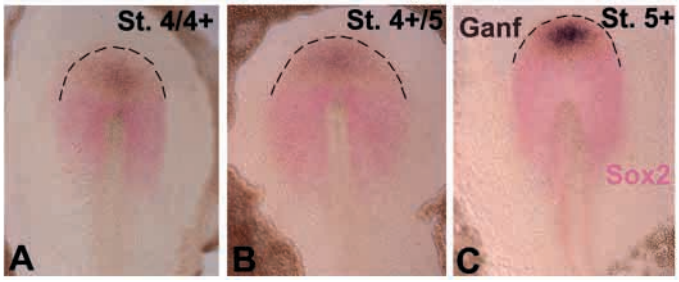

Fig. 5. *Ganf* is colocalised within the *Sox2* expression domain. Anterior is towards the top. *Sox2* alone results in a pink colour, and where *Ganf* and *Sox2* overlap a brown colour results. In all the embryos tested, *Ganf* is always colocalised within the *Sox2* expression domain. (A) Stage 4/4+: a broken line marks the rostral extent of *Sox2* expression. *Ganf* is strongest directly rostral to Hensen's node. (B) *Sox2* expression extends rostrally as neural specification occurs throughout the neural plate at stages 4+/5. The *Ganf* expression domain has also enlarged, but remains within the *Sox2* expression region. (C) By stage 5+, the streak has begun to regress (note gap between *Ganf* expression and Hensen's node) and *Sox2* expression extends more caudally (pink).

Ganf expression in RBIs requires neural specification

Transected rostral blastoderm isolates (RBIs) were processed for *Sox2* (Fig. 6) and *Ganf* (Fig. 7) transcripts to establish the percentage of type B and C RBIs with expression at each stage (experiment 1). Results are summarised in Table 1, with stage 3a/b, type B, RBIs (directly rostral to the node) expressing *Sox2* in 6/11 cases and *Ganf* in 7/11 cases. This number drops at stage 3c when the central epiblast (CE) cells, which have the ability to act as an organiser or inducer of an organiser (Darnell et al., 1999), become incorporated into the extending streak and are thus excluded from the RBIs, with only 3/11 isolates expressing *Sox2* and 2/13 expressing *Ganf*. At stage 3d, with neural specification, 7/11 *Sox2* and 6/9 *Ganf*-expressing RBIs are detected. Likewise at stage 4, 8/8 and 6/10 RBIs expressed *Sox2* and *Ganf* transcripts, respectively, with later stages all expressing *Ganf*. Control embryos (stages 3a-4) were processed immediately after transection to ensure the accuracy of the transection. *Fgf8* (primitive streak) and *Chordin* (primitive streak, Hensen's node and ingressing axial mesoderm) transcripts were detected only in the caudal isolate and not the RBI (6/6, each marker, not shown). *Sox2* and *Ganf* were not expressed in the RBIs from type C transections (125 μm rostral to streak) until stage 3d when 1/8 RBIs was positive for *Ganf* transcripts, followed by stage 4 when 3/8 expressed *Ganf* (Table 1). This result is consistent with the position of CE cells and the expression pattern of *Sox2*, the leading edge of which does not extend more than 125 μm rostral to the node until stage 4 (Fig. 5). As *Sox2* is expressed in a spatiotemporal manner spreading from Hensen's node toward the outer edge of the neural plate, RBIs resulting from Type C transections would have had to receive the signals resulting in neural specification before being transected, and CE cells would be excluded from all the isolates. RBIs that had not received signals to undergo neural specification would be unable to express *Ganf*, as is the case here. This and other studies show that *Ganf* expression only occurs in neural specified cells after receiving signals from the organizer, CE and/or ingressing

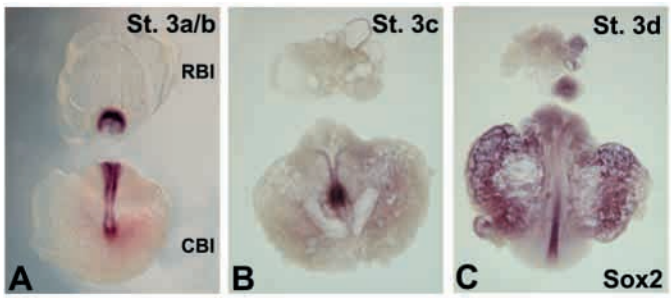

Fig. 6. *Sox2* expression in transected embryos. (A-C) RBIs to the top and CBI below, processed after 24 hours in agar/albumen culture. (A,C) Stage 3a/b and 3d RBIs have expression of Sox2 in most cases (see Table 1), whereas (B) stage 3c RBIs do not. All caudal isolates have expression of the pan-neural marker.

Table 1. *Sox2* and *Ganf* expression in rostral blastoderm isolates (RBIs) of normal transections

	Stage								
	3a/b		3c		3d		4		4+
Type	B	C	B	C	B	C	B	C	B
Sox2	6/11	0/2	3/11	0/3	7/11	0/4	8/8	-	-
%	54.5	0	27.2	0	63.6	0	100	-	-
Ganf	7/11	0/10	2/13	0/9	6/9	1/8	6/10	3/8	3/3
%	63.6	0	15.4	0	66.7	12.5	60	37.5	100

RBIs probed for *Sox2* and *Ganf* expression. Type B transected at the rostralmost level of the streak, and Type C transected 125 μm rostral to the streak (approximately one node diameter). Isolates cultured on agar/albumen substrate. Central epiblast (CE) present in RBIs at stage 3a/b results in neuralised tissue. At stage 3c, CE is incorporated into extending streak and is excluded from RBIs. Loss of neural specification results in loss of *Ganf* expression. From stage 3d onwards, neural specification exclusion of CE from RBIs has no effect. Type C RBIs all exclude CE have no *Ganf* expression at stages 3a/b and 3c, while from stage 3d onwards neural specification has begun and type C RBIs are expected to express *Ganf* and *Sox2* in increasing numbers, as specified tissue is included in the RBIs.

axial mesoderm (Knoetgen et al., 1999). As transections exclude the organiser and ingressing axial mesoderm, this raises the question of whether neural specification alone is sufficient for expression of *Ganf* or whether an additional signal from the CE or lower layer might be required. To test the hypothesis that CE or lower layer is required, we removed the lower layer from RBIs at each stage and determined whether *Ganf* was expressed. As type B transections were regarded as the most informative, no further type C transections were performed.

Ganf expression in RBIs with the lower layer removed

To determine a role for the lower layer in induction of *Ganf*, embryos were probed for transcripts after removal of the lower layer from RBIs (experiment 2) (Fig. 7 and Table 2). To determine the presence or absence of the lower layer, control RBIs, with the lower layer intact or removed, were processed immediately after transection for *Crescent*. Intact RBIs had strong expression in the lower layer (8/8), as expected, while in lower-layer deficient RBIs *Crescent* was absent (4/4). All caudal isolates expressed *Crescent* (12/12, data not shown). For longer-term culture, RBIs were embedded in collagen, with either the lower layer removed, or in control isolates, left intact to ensure that the collagen itself or culture medium did not affect *Ganf* expression. First, in the control RBIs transected at stage 3a/b, 11/19 RBIs (57.9%) expressed *Ganf*. By stage 3c, the percentage reduces as expected when CE cells are excluded, with only 5/19 (26.3%) of the RBIs with detectable expression. Once neural specification occurs at stage 3d the percentage rises again to 58.3% in 7/12 RBIs. This result is the same as for stage 4 isolates, 7/12 RBIs expressing *Ganf*. By contrast, removal of the lower layer results in a decreased number of RBIs expressing *Ganf*. This indicates that in addition to neural specification, the lower layer [i.e. hypoblast and anterior definitive endoderm (ADE)], are required for *Ganf* expression (see Table 2). Each stage tested suggests a progressive role for the lower layer. At stage 3a/b the lower layer, rostral to the primitive streak, consists almost exclusively of hypoblast, with ADE only beginning to ingress through the primitive streak. When this layer is removed, only 2/17 isolates (11.8%) continue to express *Ganf*, compared with 11/19 in controls. *Sox2* expression was ascertained for RBIs from stage 3a/b embryos in which the lower layer was removed. 6/9 (66.7%) isolates were positive for *Sox2* transcripts. This suggests that removal of the lower layer has no effect on the number of RBIs that are neuralised. This result further suggests that CE is not the inducer of *Ganf* in the epiblast, although it is crucial for neural specification and the induction of *Sox2*.

At stage 3c, the number of RBIs with *Ganf* transcripts is similar to that of intact embryos, 5/19, compared with 4/15

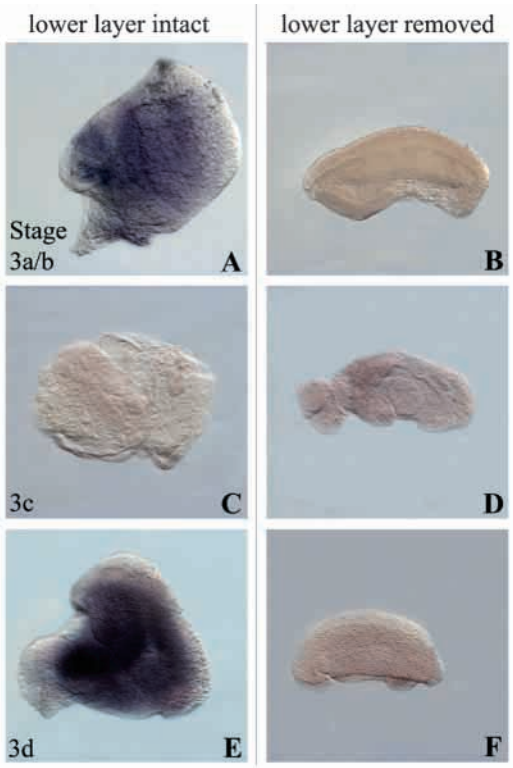

Fig. 7. *Ganf* expression in intact and lower layer deficient transected embryos. RBIs of type B transections. Isolates are cultured in collagen gel to maintain tissue integrity following lower layer excision. All CBIs were positive for *Ganf* expression (not shown). (A,B) Stage 3a/b. Intact RBI has *Ganf* expression (A), whereas RBI with lower layer removed (B) is negative for transcripts. CE is present in RBIs and is required for neural specification to occur, whereas the hypoblast is required for *Ganf* expression. (C,D) Stage 3c. Both control (C) and experimental (D) RBIs are negative for transcripts. (E,F) Stage 3d. As for stage 3a/b, except that the RBI does not include CE cells. *Ganf* expression occurs because the rostral tissue is already neuralised. Loss of the lower layer indicates the requirement for hypoblast and ADE.

Table 2. Transected rostral blastoderm isolates (RBIs) tested for *Ganf* expression with the rostral lower layer intact or removed

	Stage of transection							
	3a/b		3c		3d		4	
Lower layer tissues present	H intact	H removed	H and ADE intact	H and ADE removed	H and ADE intact	H and ADE removed	H and ADE intact	H and ADE removed
Numbers of isolates	11/19	2/17	5/19	4/15	7/12	3/15	7/12	0/8
%	57.9	11.8	26.3	26.7	58.3	20	58.3	0

H, hypoblast; ADE, anterior definitive endoderm.

Type B RBIs cultured in collagen and probed for *Ganf* expression. At each stage, control transections were performed with the lower layer remaining intact in RBIs. RBIs with the lower layer removed are shown on the right. Central epiblast (CE) is present in stage 3a/b RBIs; lower layer is hypoblast only. Removing hypoblast dramatically reduces RBIs with *Ganf* expression. At stage 3c, RBIs exclude CE and ADE is not fully specified. Removal of the lower layer has no affect on *Ganf* expression in the few neuralised RBIs. At stage 3d/4, exclusion of CE has no effect as neural specification has occurred. Removal of the hypoblast and specified ADE results in a reduction of *Ganf* expression in RBIs.

isolates lacking the lower layer. The lack of neural specification in these RBIs is due to the exclusion of CE cells and results in low numbers of RBIs expressing *Ganf*. Lower layer removal, now composed of hypoblast and ADE, has no effect on the numbers of RBIs expressing *Ganf*, indicating that the inducing activity of the lower layer may be no longer required. Interestingly, the ADE expresses only *Crescent*, *Cerberus*, *Hex* and *Otx2* at stage 3c, whereas at stage 3d, *Lim1* and *Hnf3β* are also induced (Chapman et al., 2002). This may indicate that at stage 3c the ADE cannot perform a maintenance role, but by stage 3d has developed sufficiently to do so. At stage 3d, removal of hypoblast and ADE again causes a reduction in the numbers of embryos expressing *Ganf*, 3/15 (20%) compared with 7/12 intact RBIs. This is indicative of a maintenance function being lost. At stage 4 this effect is even more pronounced with 0/8 isolates (7/12 intact RBIs) positive for *Ganf* transcripts, suggesting that although the lower layer is responsible for initial induction and maintenance of anterior identity in epiblast, factors from other tissues may be required to maintain and even stabilise the expression. Tissue candidates for this role include Hensen's node and ingressing axial mesoderm, which have been identified previously as important in *Ganf* expression (Knoetgen et al., 1999). In summary, removal of the lower layer in RBIs demonstrates that vertical signalling by the lower layer is required for the expression of the anterior neuroectoderm marker *Ganf*, apparently separately from and before neural specification, and that following neural specification the lower layer has a maintenance role, without the involvement of mesoderm.

Mesodermal cells are not detected in RBIs

Patterning of the rostral ectoderm in intact isolates could be due to the presence of mesodermal cells that are inadvertently included in the RBIs when transecting (experiment 3). When the lower layer is removed in these isolates, any mesodermal cells present could conceivably also be stripped away, resulting in loss of *Ganf* expression. To test this we, transected embryos from stages 3a/b through to stage 4 and tested for *Wnt8c* expression, which marks ingressing mesoderm (Fig. 8). Isolates were fixed immediately after transection, and in no case were *Wnt8c* transcripts detected by in situ hybridisation in the RBIs (n=29; stage 3a/b, n=12; stage 3c, n=7; stage 3d, n=7; stage 4, n=3). The caudal blastoderm isolates from these transections acted as controls for the presence of *Wnt8c* and were positive for mesodermal cells in all cases.

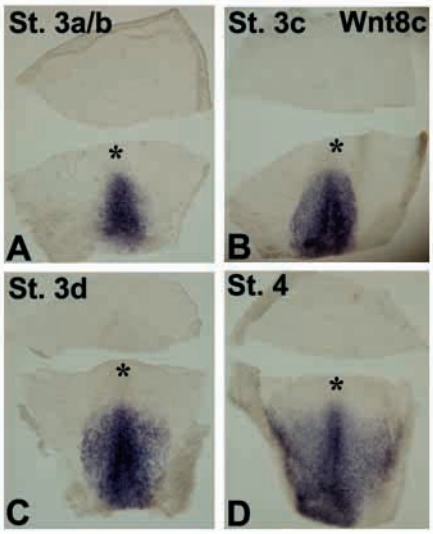

Fig. 8. Mesoderm is excluded from RBIs. (A-D) Transected isolates at the level of the node at stage 3a/b-4 tested for the presence of mesoderm in RBIs (top) and CBIs (below) immediately after transecting (asterisks indicate the nodes). In all cases, the CBIs were positive for *Wnt8c* transcripts, as expected, whereas the RBIs were negative.

Rostral endoderm is sufficient to induce *Ganf* in RBIs, whereas caudal endoderm is not

Having established a requirement for the lower layer in patterning regional identity in the overlying ectoderm we wanted to know whether the hypoblast tissue at stage 3a/b was sufficient (Fig. 9). Loss of *Ganf* expression could be due simply to damage to the epiblast while removing the lower layer. To test this, RBIs at stage 3a/b were stripped of the lower layer and then rostral ectoderm and hypoblast were recombined in collagen culture for 24 hours, followed by processing for *Ganf* expression (experiment 4). *Ganf* was detected in 7/11 cases (63.6%), the same as in intact RBIs, indicating that the lower layer is sufficient to induce *Ganf* in the rostral ectoderm (Fig. 9A). Caudal endoderm (lateral to the primitive streak) at stage 3a/b was used to test whether another regional population of endoderm cells could substitute for the rostral lower layer (experiment 5). Embryos were transected as normal, the rostral endoderm removed and then rostral ectoderm was recombined in collagen culture with the caudal endoderm. In none of the

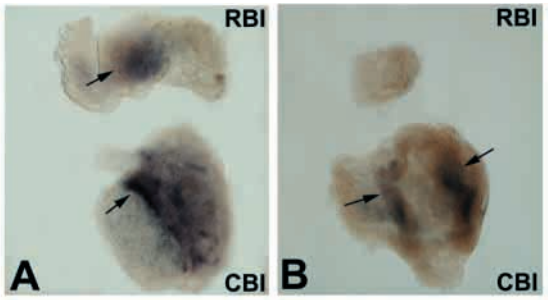

Fig. 9. The lower layer is sufficient for *Ganf* induction in rostral epiblast. RBIs (top) and CBIs (below) cultured in collagen gel. (A) After transection, rostral ectoderm recombined with rostral hypoblast is sufficient to induce *Ganf* expression (top arrow). The CBI is also positive for *Ganf* expression (bottom arrow). (B) Caudal endoderm taken from a position lateral to the streak is unable to induce *Ganf* expression (0/6) when recombined with rostral ectoderm. CBI is positive for *Ganf* expression (arrows).

cases was *Ganf* induced (0/6) (Fig. 9B). This was interesting because even if the caudal endoderm is not sufficient to induce *Ganf*, a small number of cases might be expected to express *Ganf*, raising the possibility that the caudal endoderm was not only insufficient to induce *Ganf* but actually inhibited *Ganf* expression, although the later possibility remains to be tested more vigorously.

Discussion

Anterior identity and neural specification appear to be separable patterning events

The transection assay provides evidence that suggests that the establishment of anterior positional identity in the epiblast is a separate event from neural specification. This evidence was obtained by using the earliest available specific anterior neural identity marker *Ganf*, as only tissue that is both neural and anterior in character expresses *Ganf* (Knoetgen et al., 1999). Transection separates prospective anterior neural plate from the influence of the node (the classical organiser), preventing ingression of axial mesoderm, whereas removing the lower layer from these RBIs provides insight into the inductive ability of component tissues at various stages. Our results demonstrate a novel role for the lower layer hypoblast and ADE in patterning overlying epiblast.

After transection at stage 3a/b, the epiblast still neuralises, as indicated by the expression of the definitive pan-neural marker *Sox2* (Rex et al., 1997). Neural specification in RBIs depends on the presence of a population of central epiblast (CE) cells with 'organising' ability, acting either as an organiser or inducer of an organiser (Darnell et al., 1999). After removal of Hensen's node in whole embryos and tissue isolates, the organiser reconstitutes (Joubin and Stern, 2001; Psychoyos and Stern, 1996; Yuan et al., 1995a; Yuan et al., 1995b; Yuan and Schoenwolf, 1998; Yuan and Schoenwolf, 1999). We have not determined whether the same mechanism operates in the rostral blastoderm isolates, although markers of notochord (*Not1*), node (*Shh*) and primitive streak (*Brachyury/T*) were detected in RBIs, suggesting that the organiser is reconstituted (Darnell et al., 1999). Therefore, tissue identified as able to specify neural identity in RBIs was present at stage 3a/b and reduced at stage 3c as the primitive streak extended rostrally, incorporating the CE cells (Darnell et al., 1999; Lawson and Schoenwolf, 2001a; Lawson and Schoenwolf, 2001b), although long-range neural specification signalling, prior to transection, cannot be ruled out entirely. When lower layer, composed only of hypoblast, was included in the RBI, *Ganf* was expressed at the same frequency as *Sox2*. By contrast, removing lower layer from these RBIs resulted in the loss of *Ganf* expression, but did not affect neural specification. *Ganf* was not transiently induced, suggesting that lower layer signals are required to establish positional identity in the overlying epiblast before neural specification at stage 3d.

Hypoblast seems to be required for only a brief period, because in stage 3c transections, removal of the hypoblast does not abolish *Ganf* expression. Only a small proportion of RBIs undergo neural specification at this stage, as CE cells are excluded from transected RBIs. By contrast, concomitant with neural specification at stage 3d, transection does not affect the neural character of RBIs, whereas removal of lower layer still leads to a reduction in the percentage of RBIs with *Ganf* expression, similar to that for stage 3a/b. Hypoblast has been displaced rostrally by the ADE (including midline prechordal plate endoderm) now underling the region where *Ganf* is induced. The ADE may perform a maintenance role from stage 3d when expression of *Lim1* and *Hnf3β* is induced, in addition to *Crescent*, *Cerberus*, *Hex* and *Otx2* (Chapman et al., 2002). Further work will be needed to determine whether the ADE is directly involved in the induction of *Ganf*, or whether ADE maintains anterior character specified earlier by the inductive interaction with the hypoblast.

Reassessing current models of early chick development

There is an ongoing debate as to which lower layer tissue in the chick is equivalent to the mammalian AVE, and whether the hypoblast and ADE have any patterning role. In mouse, adjoining the rostral boundary of the primitive streak the early gastrula organiser (EGO), together with epiblast and AVE, is required for head formation (Martinez-Barbera and Beddington, 2001). Chick CE cells are in a position equivalent to the EGO and have 'head organiser' properties (i.e. the ability to induce neural identity) (Darnell et al., 1999; Garcia-Martinez et al., 1993; Healy et al., 2001; Schoenwolf et al., 1989b). Determining whether CE cells can be considered a true head organiser still requires that roles in neuralising naïve epiblast and re-patterning more caudal areas of the neural plate be demonstrated. At stage 2 and 3a/b, the CE population is rostral to the extending streak, but by stage 3c it becomes incorporated into the rostrally extending streak forming Hensen's node (Schoenwolf et al., 1989b). The node acts like a head organiser, establishing and refining neural identity, and maintaining and embellishing patterning in overlying neuroectoderm. The properties of axial mesoderm as it ingresses through Hensen's node at stage 4+ are reported to be the result of its origin in the node and vertical signals from the definitive endoderm as it intercalates between the upper and lower germ layers (Vesque et al., 2000). An anteriorising signalling centre in the lower layer could act as the source of signals that operate to further pattern the extending axial mesoderm, indicating a relay mechanism operates, where the

anterior endoderm patterns, directly or indirectly, the prechordal plate mesoderm, which in turn patterns the overlying neuroectoderm (Dale et al., 1997; Foley et al., 1997; Pera and Kessel, 1997). Our results suggest that hypoblast and ADE also have an earlier role in directly patterning the overlying epiblast.

With ingression of axial mesoderm through Hensen's node, head organiser ability is lost, perhaps allowing remaining cells to perform the role of trunk/tail organiser, refining the patterning of more caudal parts of the neural plate. An important related issue is whether neural identity is a neutral fate, with lower layer providing positional identity. In our experiments, removal of the lower layer does not affect neural specification, because RBIs still express the definitive pan-neural marker *Sox2*. The significance of the *Sox2* expression pattern, which expands progressively from medial tissue adjacent to Hensen's node and then laterally across the neuroectoderm, suggests that neural specification does not occur first in the most anteriorly positioned cells of the prospective neural plate. However, *Ganf* expression is lost when the lower layer is removed. Therefore, these data together support a model in which loss of anterior identity does not affect the neural character of tissue, suggesting that neural identity itself is neutral with respect to position.

The lower layer signals vertically to the overlying epiblast

Transplanted chick lower layer was unable to induce anterior neuroectodermal (ANE) markers in epiblast, whereas rabbit AVE and chick axial mesoderm induced *Ganf* (Knoetgen et al., 1999). A heterochronic shift in patterning of the ANE was proposed, with chick prechordal mesoderm taking over the role played by mouse AVE. Why did the transplanted chick tissue not induce expression of *Ganf*? The signals needed to induce positional identity were either no longer present (transplanted hypoblast was older than stage 3a/b), i.e. necessary signals could have been reduced by enzymatic treatments used to facilitate isolation of the lower layer, or the responding tissue was not neuralised and, therefore, not competent to express *Ganf*. Our data demonstrate that intact hypoblast at stage 3a/b is required for the neuroectoderm to express *Ganf*. Extirpation of hypoblast at stage 3 did not lead to loss of *Ganf* expression at later stages; however, stage 3 is highly dynamic and prospective ANE was not separated from the influence of the node or ingressing axial mesoderm, both of which are sufficient to induce the expression of *Ganf* (Knoetgen et al., 1999). Transections demonstrate that in RBIs the lack of a node does not affect induction of *Ganf*, whilst axial mesoderm begins ingressing only after *Ganf* expression has begun and, therefore, is unlikely to be the initial endogenous inducer. Thus, chick axial mesoderm is probably not the homologue of the mouse AVE and a heterochronic shift is unlikely. The later patterning role of axial mesoderm is important in refining regional neuroectoderm identity (Dale et al., 1997; Foley et al., 1997; Pera and Kessel, 1997), but initial anterior positional identity must be assigned to earlier endodermal tissues.

The modified Nieuwkoop model

An alternative hypothesis, a modified Nieuwkoop model, proposes that AVE and hypoblast at stage XII/XIII are equivalent tissues. However, the lower layer in this model is responsible for cell movements, rather than cell fate, directing cells away from the caudalising influence of Hensen's node (Foley et al., 2000). Transplanted hypoblast induced transient expression of *Sox3* and *Otx2* in epiblast, indicating signalling capability, but as expression was not maintained the authors suggested that anteriorising the epiblast is not the hypoblast's main role. Our data do not support the interpretation of the proposed early pre-forebrain state, defined by the expression of the pre-neural markers *Sox3* and chick ERNI (Streit et al., 2000). ERNI has been shown to be a retrotransposon only present in the Galliform genome, requiring clarification of its biological significance (Acloque et al., 2001), and *Sox3* is expressed in a mosaic of epiblast cells from prestreak stages across the entire area pellucida, only becoming restricted to the neuroectoderm after stage 3d (Rex et al., 1997). Rather, in our model successive inductive interactions anteriorises epiblast, with molecular signals establishing and maintaining anterior identity separately from neural specification. An explanation for the failure to maintain the initial *Sox3* and *Otx2* expression is that hypoblast is unable to either stabilise or maintain this expression, requiring later ADE to provide the necessary signals. More importantly, this early induction suggests that, as in the mouse, the ability to pattern the early embryo is not confined to Hensen's node and its derivatives.

A revised hypothesis for rostral patterning and head induction

We suggest a revised hypothesis, where successive inductive interactions between hypoblast/ADE and epiblast act to promote anterior character (Fig. 10). As hypoblast is displaced rostrally by the ADE, signals from ADE stabilise/maintain this rostral identity in the overlying epiblast/neuroectoderm. Rostrally located hypoblast (stages XII-XIV) is remarkably similar to the mouse AVE, expressing *Lim1*, *Hnf3b*, *Otx2*, *Gsc*, *Cerberus*, *Hex* and *Crescent* (Chapman et al., 2002). Genes expressed in ADE include *Crescent*, *Cerberus*, *Hex* and *Otx2*, whereas *Lim1* and *Hnf3b* are detected only after stage 3d (Chapman et al., 2002). *Ganf* is the earliest marker detected in the rostral epiblast in response to anteriorising signals from the lower layer and neural specification by the head organiser. Head organiser cells leave Hensen's node, as ingressing axial mesoderm, permitting the remaining population to perform the role of trunk/tail organiser. Changing gene expression reflecting this include *Otx2*, *Nodal* and *Dkk1*, which are lost from the streak at stages 5+/6, while *Bmp7* is now expressed in rostral streak from which it was previously excluded (Chapman et al., 2002). This novel hypothesis allows for separate signalling pathways to pattern anterior and neural identity, and for the hypoblast to direct cells with a rostral fate away from the caudalising influence of the trunk/tail organiser (Foley et al., 2000). It further takes into account results suggesting that definitive endoderm is required for patterning neural plate, as stage 4+ removal results in loss of the forebrain because of lack of vertical signals to ingressing head process, and also direct maintenance signals to the overlying neuroectoderm (Withington et al., 2001). Loss of these stabilising and maintenance signals results in the loss of forebrain identity. This hypothesis is further supported by the *Foxa2* conditional mouse mutant, where loss of *Foxa2* results in axial mesoderm losing its identity; anterior neuroectoderm

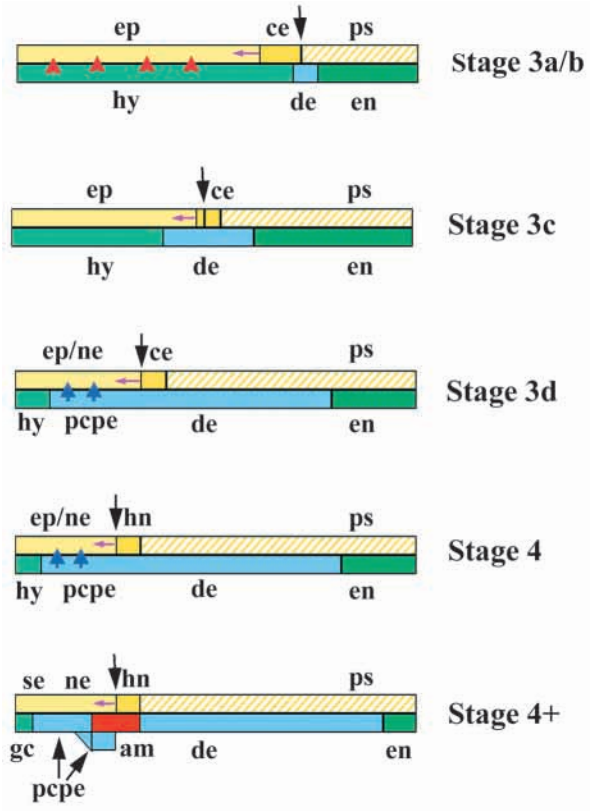

Fig. 10. Schematic representation of tissue positions in revised early patterning model. Anterior is towards the left, epiblast/neuroectoderm layer in yellow, with the rostralmost level of the primitive streak (hatched yellow) marked by vertical arrows. At stage 3a/b, note that CE is rostral to the streak, but becomes incorporated into the extending streak until it forms part of Hensen's node at stage 4. Definitive endoderm is represented by bar in blue. Only at stage 4+ does axial mesoderm begin ingressing (red). Hypoblast is responsible for establishing anterior identity in overlying epiblast at stage 3a/b (red arrowheads). Only at stage 3d and 4 is the prechordal plate endoderm fully specified and has a maintenance role in the overlying neuroectoderm layer (dark blue arrows). The CE population signals in the plane of the ectoderm and becomes incorporated into the rostral primitive streak, and together with Hensen's node forms the head organiser (horizontal arrows). After ingression of the axial mesoderm at stage 4+, a trunk/tail organiser function for Hensen's node is revealed. am, axial mesoderm; ce, central epiblast; de, definitive endoderm; ds, definitive streak; en, endoblast; ep, epiblast; gc, germ cell crescent; hy, hypoblast; hn, Hensen's node; ne, neuroectoderm; pcpe, prechordal plate endoderm; ps, primitive streak; se, stomodeal ectoderm.

in turn is not stabilised, resulting in forebrain truncation (Hallonet et al., 2002).

Hensen's node is, therefore, able to act as head and trunk/tail organiser, with spatiotemporally separated signals. The early head organiser producing neural identity in the plane of the ectoderm and the late organiser posteriorising more caudal neural plate. Anterior identity results from successive inductive interactions between the hypoblast, ADE and overlying epiblast, stabilised and refined later by ingressing axial mesoderm.

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