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A role for the hypoblast (AVE) in the initiation of neural induction, independent of its ability to position the primitive streak

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Abstract

The mouse anterior visceral endoderm (AVE) has been implicated in embryonic polarity: it helps to position the primitive streak and some have suggested that it might act as a "head organizer", inducing forebrain directly. Here we explore the role of the hypoblast (the chick equivalent of the AVE) in the early steps of neural induction and patterning. We report that the hypoblast can induce a set of very early markers that are later expressed in the nervous system and in the forebrain, but only transiently. Different combinations of signals are responsible for different aspects of this early transient induction: FGF initiates expression of *Sox3* and *ERNI*, retinoic acid can induce *Cyp26A1* and only a combination of low levels of FGF8 together with Wnt- and BMP-antagonists can induce *Otx2*. BMP- and Wnt-antagonists and retinoic acid, in different combinations, can maintain the otherwise transient induction of these markers. However, neither the hypoblast nor any of these factors or combinations thereof can induce the definitive neural marker *Sox2* or the formation of a mature neural plate or a forebrain, suggesting that the hypoblast is not a head organizer and that other signals remain to be identified. Interestingly, FGF and retinoids, generally considered as caudalizing factors, are shown here to play a role in the induction of a transient "pre-neural/pre-forebrain" state.

Keywords: Anterior visceral endoderm; Gastrulation; Head organizer; Forebrain induction; Anterior–posterior patterning; Chick embryo

Introduction

During early vertebrate development, part of the ectoderm is induced to acquire neural character (neural plate, which gives rise to the entire central and most of the peripheral nervous systems). Around the same time, the neural plate starts to become regionalized, becoming subdivided into gross regions (forebrain, midbrain, hindbrain and spinal cord) which later become refined. Induction and regionalization appear to begin at the same developmental stage, which has aroused interest concerning the precise relationships between them. One model ("multiple organizers"), first proposed in the 1930s, postulates that distinct signals from different embryonic tissues are responsible for inducing specific regions of the nervous system corresponding to head, trunk and tail (Holtfreter, 1933; Mangold, 1933; see also Niehrs, 2001). An alternative model (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954) suggests instead that initial inductive signals from a single organizer are responsible for inducing a territory with forebrain character, parts of which are subsequently posteriorized (caudalized) by signals from other tissues.

Some recent findings have been taken to support either one or the other model. For example, FGFs (Cox and Hemmati-Brivanlou, 1995; Pownall et al., 1996; Muhr et al., 1997, 1999; Hardcastle et al., 2000; Koshida et al., 2002; Rentzsch et al., 2004), retinoids (Blumberg, 1997; Chen et al., 2001; Dupe and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005) and Wnts (McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Houart et al., 2002; Nordstrom et al., 2002; Shimizu et al., 2005) have all been shown to act as caudalizing agents, supporting Nieuwkoop's model. On the other hand, different combinations of BMP antagonists, Wnt antagonists and FGF, emanating from different regions of the early fish embryo, have been proposed to be responsible for independent induction of different parts of the

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nervous system (Agathon et al., 2003; Kudoh et al., 2004). There is still no agreement as to which model best explains how the nervous system is induced and subdivided.

Both models and most of the work performed to test them are based on observations in amphibians and more recently in teleost embryos. In amniotes, the idea of separate organizers responsible for inducing different regions was recently re-introduced as a result of the findings that an extraembryonic tissue, the anterior visceral endoderm (AVE), is required for head formation (Thomas and Beddington, 1996; Varlet et al., 1997; Acampora et al., 1998; Rhinn et al., 1998; Shawlot et al., 1999; Dufort et al., 1998; Ding et al., 1998; Liguori et al., 2003) and that grafts of the organizer, Hensen's node, generally produce an ectopic nervous system lacking the forebrain (Beddington, 1994). However, grafts of the AVE to an ectopic site do not induce either nervous system or forebrain unless combined with other tissues, including ectoderm from the region fated to become forebrain (Tam and Steiner, 1999). To complicate matters further, the AVE is also important in orienting the axis of the embryo by influencing the site at which gastrulation begins: it emits Nodal antagonists which block primitive streak formation and thus indirectly defines the caudal side of the embryo (Waddington, 1932, 1933; Foley et al., 2000; Bertocchini and Stern, 2002; Perea-Gomez et al., 2002). It has therefore been difficult to determine whether the AVE is directly involved in nervous system induction and/or its regional subdivision, independently from its ability to position the primitive streak.

The chick embryo is a powerful system in which to address these questions because it contains a large extraembryonic region (area opaca) which does not contribute to the nervous system yet can be readily induced to form a complete CNS by grafts of Hensen's node (Waddington, 1932, 1933, 1937; Gallera and Nicolet, 1969; Dias and Schoenwolf, 1990; Storey et al., 1992) and because in vivo electroporation or grafts of cells secreting particular factors allow analysis of the time-course of responses of the ectoderm at any stage and in any region. This approach has started to reveal that neural induction and early patterning comprise a sequence of steps, each characterized by unique molecular markers (Stern, 2005). Here we explore the role of the hypoblast (the chick equivalent of the mouse AVE) in the early steps of neural induction and patterning. We report that the hypoblast induces a set of very early markers (some of which are later expressed in the nervous system and in the forebrain), but only transiently. Different combinations of signals are responsible for different aspects of this transient early induction: FGF can initiate expression of *Sox3* and *ERNI*, retinoic acid can induce *Cyp26A1* and only a combination of low levels of FGF8 with Wnt- and BMP-antagonists can induce *Otx2*. BMP antagonists can maintain the otherwise transient induction of some of these markers. However, none of these factors or any combination thereof can induce the definitive neural marker *Sox2* or a mature neural plate, suggesting that further signals remain to be identified. The hypoblast itself, either alone or in combination with these factors, is also unable to induce these states, arguing that it is not a true "head organizer". Surprisingly, however, FGF and retinoids, generally considered as caudalizing factors, appear to underlie the earliest induction of a "pre-neural/pre-forebrain" state reminiscent of that proposed by Nieuwkoop.

Materials and methods

Embryos and grafting experiments

Fertilized hens' eggs (Brown Bovan Gold) were incubated at 38°C to the desired stages. Host embryos were incubated to stages 3–3$^+$ (Arabic numerals for primitive streak and later stages: Hamburger and Hamilton, 1951). They were placed in modified New Culture (Stern and Ireland, 1981) and a small region of the germ wall (area opaca endoderm) removed from the inner third of the lateral area opaca. Since hypoblast grafts into the area opaca do not contribute cells to the ectoderm (Foley et al., 2000), chick embryos were used as both donors and hosts. Donor embryos were incubated to stages XII–XIII (Roman numerals for pre-primitive streak stages: Eyal-Giladi and Kochav, 1976); the central region of the hypoblast was peeled from the epiblast and grafted into the cleared region of the host. Hosts were incubated for 1–20 h.

Cell pellets (see below) or beads were grafted into the same region of stage 3–3$^+$ hosts. FGF8b (R&D or Sigma) was diluted at 25 μg/ml, 50 μg/ml or 100 μg/ml. 25 μg/ml does not induce *brachyury*. 100 μg/ml always induces *brachyury*, whereas only some batches do so at 50 μg/ml — only those that did not were used. These were incubated with heparin beads as described (Streit et al., 2000).

All *trans*-retinoic acid (RA) (Sigma) and Citral (Sigma) were dissolved in DMSO and diluted to the appropriate concentration (Citral: 10^{-4} M; RA at various concentrations as described in the Results section) before incubating with AG1X2 beads (Biorad) for 1 h. Beads were rinsed with DMSO and then PBS prior to grafting. PBS-AG1X2 or -heparin beads were used as controls.

Cell culture and transfection

COS-1 cells were grown in DMEM containing 10% calf serum and transfected with *Chordin* (Streit et al., 1998), *Dkk-1* (gift of E. Laufer), *Crescent* (gift of P. Pfeffer) or *XCerS* (gift of E. De Robertis; see Bertocchini and Stern, 2002) using lipofectamine (Invitrogen). 24 h later, pellets containing 1500 cells were generated from hanging drop cultures so that the proportion of cells expressing each protein constituted one third of the total cell number (500) to keep levels consistent between experiments. Pellets were transplanted 36–48 h after transfection.

Electroporation

Cyp26A1 (gift of M. Maden) was cloned into the pCIG expression vector upstream of IRES-GFP. It was electroporated into the inner third of the lateral area opaca (Sheng et al., 2003) at 1.5 μg/μl and embryos cultured for 2 h prior to grafting a hypoblast and/or cell pellets. Hosts were re-incubated for 20 h. Expression of the transgene was assayed by fluorescence, in situ hybridization for *Cyp26A1* and/or immunostaining for GFP.

mRNA in situ hybridization and immunostaining

Whole mount in situ hybridization using DIG-labeled RNA-probes was performed as described (Streit and Stern, 2001). Embryos were sectioned after staining to confirm expression in the host ectoderm since pellets of transfected cells often display non-specific staining and because the hypoblast itself expresses some of these markers.

Results

The hypoblast induces transient expression of early, but not definitive, anterior neural markers

The mouse anterior visceral endoderm (AVE) has been shown to be required for forebrain development (Thomas and

Beddington, 1996; Varlet et al., 1997; Acampora et al., 1998; Rhinn et al., 1998; Shawlot et al., 1999; Dufort et al., 1998; Ding et al., 1998; Liguori et al., 2003). However, grafts of the AVE do not induce a forebrain when grafted to ectopic sites unless combined with the Early Gastrula Organizer (EGO) as well as with prospective forebrain epiblast (Tam and Steiner, 1999), leaving open the question of whether the AVE plays any direct role in forebrain induction. In the chick embryo, the equivalent of the AVE is the hypoblast (see Foley et al., 2000; Bertocchini and Stern, 2002). Markers for anterior and neural tissue have been ordered in a hierarchy according to when they are first expressed both in normal development and after grafting an organizer (Hensen's node) (Stern, 2005), which offers an opportunity to investigate whether the hypoblast can induce any of them.

The time-course of induction of early markers of neural tissue (*Sox3*, *ERNI*, *Sox2*) and forebrain (*Otx2*, *Cyp26A1*, *Ganf*) was analyzed, following a hypoblast graft from a stage XII–XIII donor embryo into the area opaca of a stage 3 host. *Sox3* (5/8) and *ERNI* (6/8) are induced first, within 1–2 h of incubation (Figs. 1A, B). *Otx2* (8/9) and *Cyp26A1* (6/8) are induced after 3–4 h (Figs. 1G, H, K, L). All markers are expressed strongly after 4- to 6-h incubation (6/6 each for *Sox3*, *ERNI*, *Otx2*, *Cyp26A1*) and sections confirm expression in the ectoderm (Figs. 1I–L, I′–L′). However, neither the definitive neural marker *Sox2* (0/8) nor *Ganf* (0/8), the earliest known marker of anterior neural tissue, was induced. Weaker expression of all markers is observed after 8- to 10-h incubation (*Sox3*: 3/5; *ERNI*: 4/7; *Otx2*: 2/6; *Cyp26A1*: 1/7; Figs. 1M–P). By 10–12 h only *ERNI* remains, while Sox3, Otx2 and Cyp26A1 are lost (Figs. 1Q–T) and no expression at all remains after 13 (*Sox3*: 0/6; *ERNI*: 0/6, *Otx2*: 0/6, *Cyp26A1*: 0/6) (Figs. 1U–X), 15 or 17 h (not shown).

It is possible that the hypoblast loses its inducing ability with time as stage 4 hypoblasts are unable to induce *Sox3* or *Otx2* (Foley et al., 2000). To test this, a hypoblast graft was performed and the host incubated for 8 h; a second hypoblast was then grafted to the same site and the host incubated for a further 8 h. None of the markers was expressed (*Sox3* 0/6; *ERNI* 0/8; *Otx2* 0/8; *Cyp26A1* 0/8), suggesting that signals other than those produced by the hypoblast are required to maintain expression of genes induced by this tissue. These results support and extend previous observations (Foley et al., 2000; Streit et al., 2000) and suggest that the hypoblast can induce, but only transiently, expression of early, "pre-neural" markers in the epiblast.

Induction is independent of brachyury, which is inhibited by the hypoblast

It is possible that the transient induction described above is due to induction of mesendoderm by the hypoblast, which in turn induces the other markers. To test this, we examined whether *brachyury* (*bra*; an early mesendodermal marker expressed in the primitive streak and node) is induced by the hypoblast. Not only does the hypoblast fail to induce *bra* (0/4; Fig. 2A), but it can also block its expression: heparin beads soaked in high concentrations of FGF (100 μg/ml FGF8) applied to the area opaca for 4–6 h induce *bra* (4/4; Fig. 2B, left), but this is abolished when a hypoblast is co-transplanted with the FGF beads (4/4; Fig. 2B, right). Furthermore, grafts of hypoblast into a region of endogenous *bra* expression (the primitive streak and emerging mesoderm of a stage 3^+ host) downregulate *bra* within 4 h (4/4; Fig. 2C). These results are consistent with previous observations that the chick hypoblast inhibits primitive streak formation (Bertocchini and Stern, 2002) and hence mesendoderm induction.

FGF mimics the ability of the hypoblast to induce Sox3 and ERNI within 1–2 h

At pre-primitive streak stages, FGF8 is expressed in the hypoblast and could therefore be involved in its induction of *Sox3* and *ERNI* (Streit et al., 2000). To test this, FGF8 beads were grafted into the area opaca. Within 2 h, 100 μg/ml FGF8 induces *Sox3* at low levels (5/8; Fig. 3C), 50 μg/ml FGF8 induces *ERNI* at low levels (4/4; Fig. 3B) and 100 μg/ml at higher levels (4/4; Fig. 3D) in the absence of *bra* (0/4 at each concentration). This is a similar time frame to the induction of *Sox3* and *ERNI* by the hypoblast. *Sox3* (6/6) and *ERNI* (6/6) expression was still observed after 6-h incubation with 50 μg/ml FGF8 (Figs. 3E, F) in the absence of *bra* (0/4), consistent with Streit et al. (2000).

To determine whether FGF signaling is required for the hypoblast to induce these genes, beads soaked in the FGF antagonist SU5402 were grafted together with a hypoblast and the embryos incubated for 4–6 h. A reduction of induced *Sox3* expression was observed near the bead (8/14) (Figs. 4A, B). SU5402 can block induction of *ERNI* by a node (Streit et al., 2000) but it did not block *ERNI* induction by the hypoblast (0/8; Fig. 4C). Together, these results suggest that FGF signaling can mimic the effects of a hypoblast graft in inducing *Sox3* and *ERNI* and that FGF activity is required for induction of at least the former.

FGF does not induce Cyp26A1 or Otx2 except indirectly through mesendoderm

Unlike *Sox3* and *ERNI*, which are induced after 1–2 h, *Otx2* and *Cyp26A1* induction by a hypoblast requires 3–4 h. FGF8 was tested for its ability to induce these markers. Neither 2-h incubation with 50–100 μg/ml nor 6 h with 50 μg/ml FGF8 beads induces *Otx2* or *Cyp26A1* (0/6 for each concentration). In contrast, *Otx2* is induced in 4–6 h by 100 μg/ml FGF8 beads (3/7; Fig. 5A). However, under these conditions, *bra* is also induced (4/4).

We noticed that *Otx2* is always induced at a distance from the bead, in a crescent shape pointing towards the embryo proper, suggesting that a miniature primitive streak may have been induced by FGF (see Bertocchini et al., 2004). To test whether mesoderm is required for the induction of *Otx2* by FGF, FGF8 beads were co-transplanted with cells secreting the Nodal antagonist XCerS (Piccolo et al., 1999); *Otx2* was no

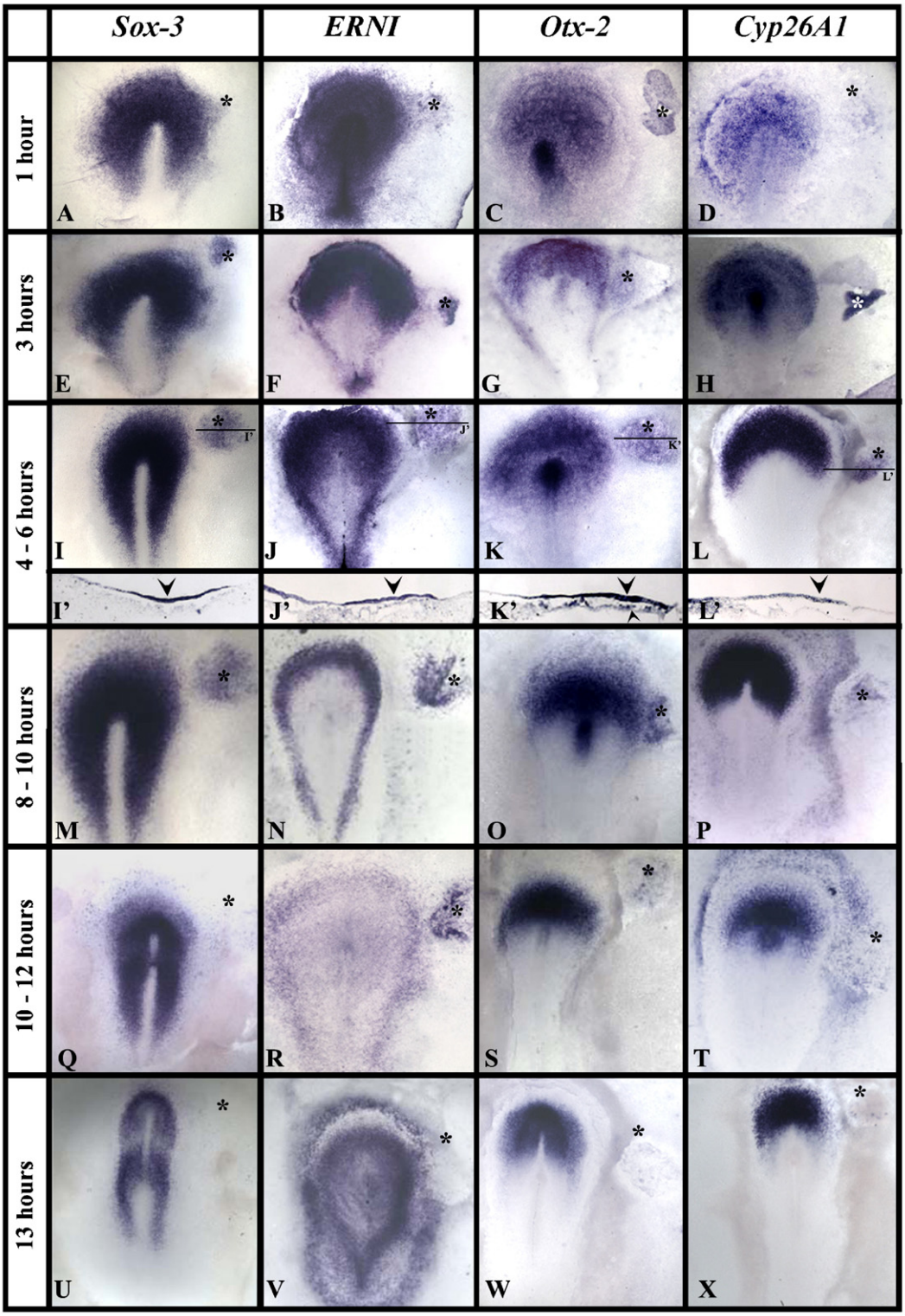

Fig. 1. The hypoblast transiently induces *Sox3* and *ERNI*, followed by *Otx2* and *Cyp26A1*. (A–D) 1 h incubation after a hypoblast graft induces *Sox3* (A) and *ERNI* (B) but *Otx2* is only observed in the underlying hypoblast and not the ectoderm (C) and *Cyp26A1* is also absent (D). (E–H) 3 h after a hypoblast graft, *Sox3* (E) and *ERNI* (F) are still expressed and *Otx2* (G) and *Cyp26A1* (H) start to be induced. (I–L) At 4- to 6-h incubation, there is strong induction of all markers: *Sox3* (I), *ERNI* (J), *Otx2* (K) and *Cyp26A1* (L). (I′–L′) sections through embryos incubated for 4–6 h reveal induced expression (arrowheads) of *Sox3* (I′), *ERNI* (J′), *Otx2* (K′) and *Cyp26A1* (L′) in the ectoderm of the area opaca. (Note: *Otx2* is also expressed in the underlying hypoblast graft; small arrowhead: K′). (M–P) by 8- to 10-h incubation, *Sox3* (M), *ERNI* (N), *Otx2* (O) and *Cyp26A1* (P) are still expressed. However, after 10–12 h (Q–T), *Sox3* (Q) is lost, while *ERNI* (R) is still present, *Otx2* (S) and *Cyp26A1* (T) are no longer detectable. (U–X) All markers are lost by 13 h: *Sox3* (U), *ERNI* (V), *Otx2* (W) and *Cyp26A1* (X). There is no reappearance of expression either after 15 h or 17 h. * indicates the position of the hypoblast graft.

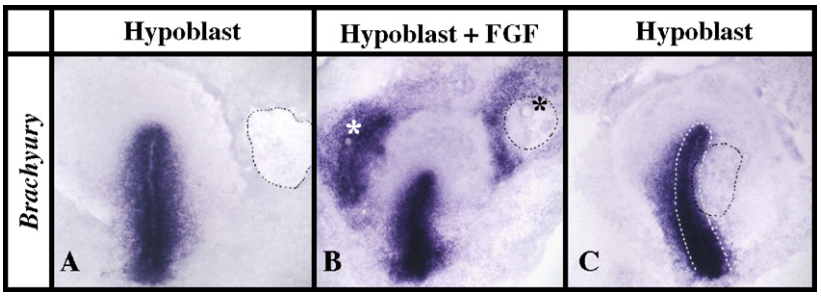

Fig. 2. *brachyury* is repressed by the hypoblast (grafts of which are outlined in black). (A) Hypoblast grafts do not induce *brachyury*. (B) A 100 μg/ml FGF8 bead grafted on both sides of the area opaca (*) can induce *brachyury* (left) but is prevented from doing so when a hypoblast is also grafted (right). (C) A hypoblast graft also represses endogenous *brachyury* expression in the primitive streak (outlined in white) and emerging lateral mesoderm.

longer induced in most of these embryos (1/5; Fig. 5B), suggesting that *Otx2* induction by FGF is indirect.

Although blocking FGF signaling reduces induction of *Sox3* by the hypoblast, the opposite is found for *Otx2*. SU5402 beads together with a hypoblast graft increase the intensity of *Otx2* induction near the bead (10/16) (Figs. 4D, E). *Cyp26A1* is unaffected (0/10; Fig. 4F). Conversely, a hypoblast graft plus 50 μg/ml FGF8 decreases induction of *Otx2*, compared to hypoblast alone (7/10; Figs. 4G, H). Other markers are unaffected (*Sox3*: 0/4; *ERNI*; 0/4; *Cyp26A1*: 0/6). These results suggest that FGF is not responsible for the induction of *Otx2* by the hypoblast; rather, it reduces *Otx2* induction, although we cannot rule out a requirement for an initial or very low level of FGF signaling or the possibility that FGF is required in combination with other factors.

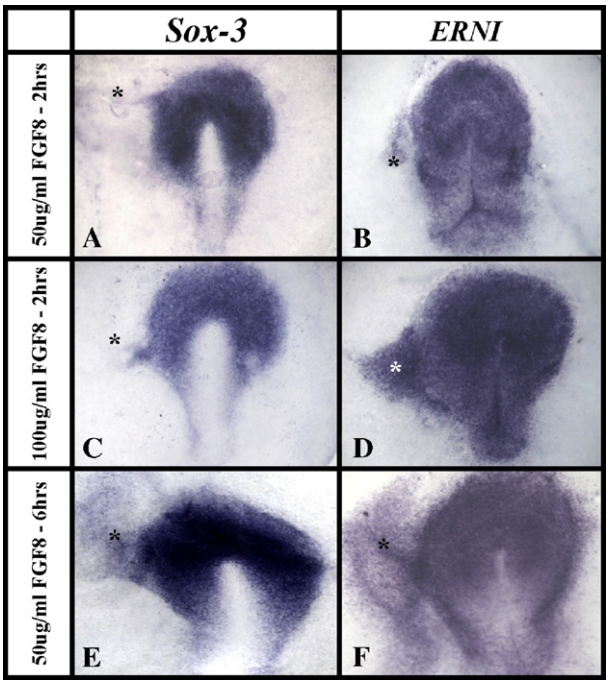

Fig. 3. Direct induction of *Sox3* and *ERNI* by FGF8. (A, B) 2-h incubation with 50 μg/ml FGF8 does not induce *Sox3* (A) but induces *ERNI* weakly (B). (C, D) 2-h incubation with 100 μg/ml FGF8 induces *Sox3* weakly (C) and *ERNI* strongly (D). (E, F) 50 μg/ml FGF8 induces both *Sox3* (E) and *ERNI* (F) strongly after 6 h in the absence of *bra*. * indicates bead graft.

Otx2 is induced by low concentrations of FGF8 and Wnt and BMP antagonists

Although the hypoblast expresses the Wnt antagonists *Dkk1* and *crescent* and the Wnt, BMP and Nodal antagonist *Cerberus*, neither cells secreting Dkk1 or Crescent, nor cells secreting Chordin, nor a combination of Wnt and BMP antagonists, can induce *Sox3*, *ERNI*, *Otx2* or *Cyp26A1* in 4–6 h (0/6 for each combination). Likewise, beads soaked in low concentrations of FGF8 (25 μg/ml) grafted together with cells secreting either Wnt or BMP antagonists do not induce *Otx2* (0/6) or *Cyp26A1* (0/6). However, low FGF8 together with both Wnt- and BMP-antagonists does induce *Otx2* (7/11; but not *Cyp26A1*; 0/6) (Fig. 5C) in the absence of *bra* (0/5) (Fig. 5D). Despite this, *Sox2* was not induced after overnight incubation (0/8), suggesting that the epiblast induced to express *Sox3*, *ERNI*, and *Otx2* does not have definitive neural plate or forebrain character, consistent with the findings of Linker and Stern (2004).

RA is sufficient and necessary to induce Cyp26A1

The above results indicate that, while the hypoblast induces *Cyp26A1* in the area opaca within 3–4 h, neither FGF8, Wnt- or BMP-antagonists, nor any combination of these can. Since retinoic acid (RA) can induce *Cyp26* in chick limb buds (Martinez-Ceballos and Burdsal, 2001) and *RALDH2* is expressed in the hypoblast at stage 4 (Halilagic et al., 2003), RA is a good candidate inducer of *Cyp26A1* from the hypoblast. To test this, RA beads were grafted into the area opaca for different lengths of time (Figs. 6A–D) and at different concentrations (Figs. 6E–H). In all cases, *Cyp26A1* was induced (4/4 for each experiment). In addition, the endogenous domain of expression of *Cyp26A1* in the epiblast was shifted dramatically towards the RA source. RA failed to induce any of the other markers (*Sox3*: 0/8; *ERNI*: 0/8; *Otx2*: 0/10; Figs. 7I–L). Interestingly, even the lowest RA concentration induces *Cyp26A1* within 1–2 h (4/4), 1–3 h faster than hypoblast grafts.

To investigate whether RA is required for the hypoblast to induce *Cyp26A1*, beads soaked in the RA inhibitor Citral were grafted together with a hypoblast and the hosts incubated for 4–6 h. This abolished *Cyp26A1* induction in most cases (2/10 expressing) as compared to grafts of a hypoblast and a control

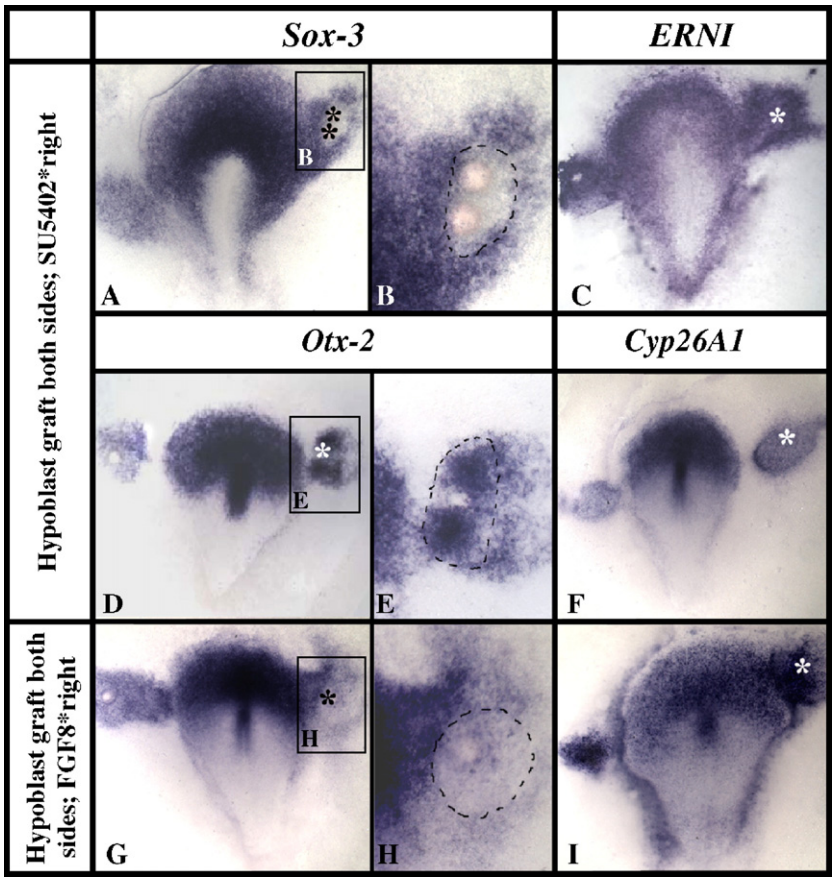

Fig. 4. FGF signaling can modulate expression of genes induced by the hypoblast. (A–C) SU5402 blocks induction of *Sox3* by the hypoblast (A) (shown at higher magnification in panel B), while *ERNI* (C) is not altered. (D–E) SU5402 potentiates hypoblast induction of Otx2. (F) In contrast, *Cyp26A1* is unaffected. (G–I) Beads with 50 μg/ml FGF8 reduce induction of *Otx2* by the hypoblast (G, H), but do not alter *Cyp26A1* expression (I). * indicates position of SU5402 or FGF8 beads. Hypoblasts are grafted on both left and right sides of the area opaca.

bead (10/10; Fig. 6J). These results strongly implicate RA in the induction of *Cyp26A1* by the hypoblast.

Antagonism between RA and FGF

The above results suggest that FGF8 can induce *Sox3* and *ERNI*, while RA induces *Cyp26A1*. RA and FGF8 beads were grafted together into the area opaca, and the hosts incubated for 4–6 h to test whether all markers are induced. Expression of *Sox3* (8/10) and *ERNI* (6/7) was much reduced in the vicinity of the beads compared to FGF8 alone (compare Fig. 7E with Fig. 3E and Fig. 7F with Fig. 3F) and little or no *Otx2* expression was observed ($n=10$; Fig. 8C). *Cyp26A1* induction by RA was also repressed by addition of FGF. In this instance, it is still induced in a broad domain around the RA bead but expression comes to an abrupt halt close to the FGF bead (6/10; Fig. 7H). These results suggest that RA and FGF, or the states induced by them, antagonize each other.

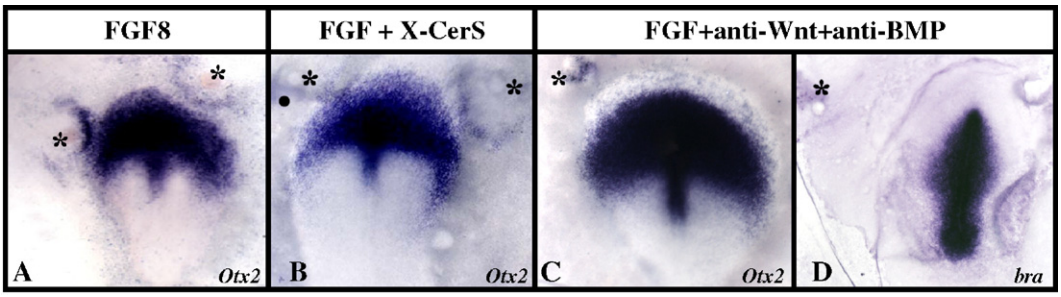

Fig. 5. Induction of *Otx2* by factors. (A) 100 μg/ml FGF8 beads induce a crescent of *Otx2* expression. (B) Co-grafting of XCer-S-expressing cells (black circle) blocks 100 μg/ml-FGF8 (*) induction of *Otx2*. (C–D) *Otx2* (C), but not *bra* (D), can be induced by 25 μg/ml FGF8 plus Chordin-, Dkk1- and Crescent-expressing cell pellets (*).

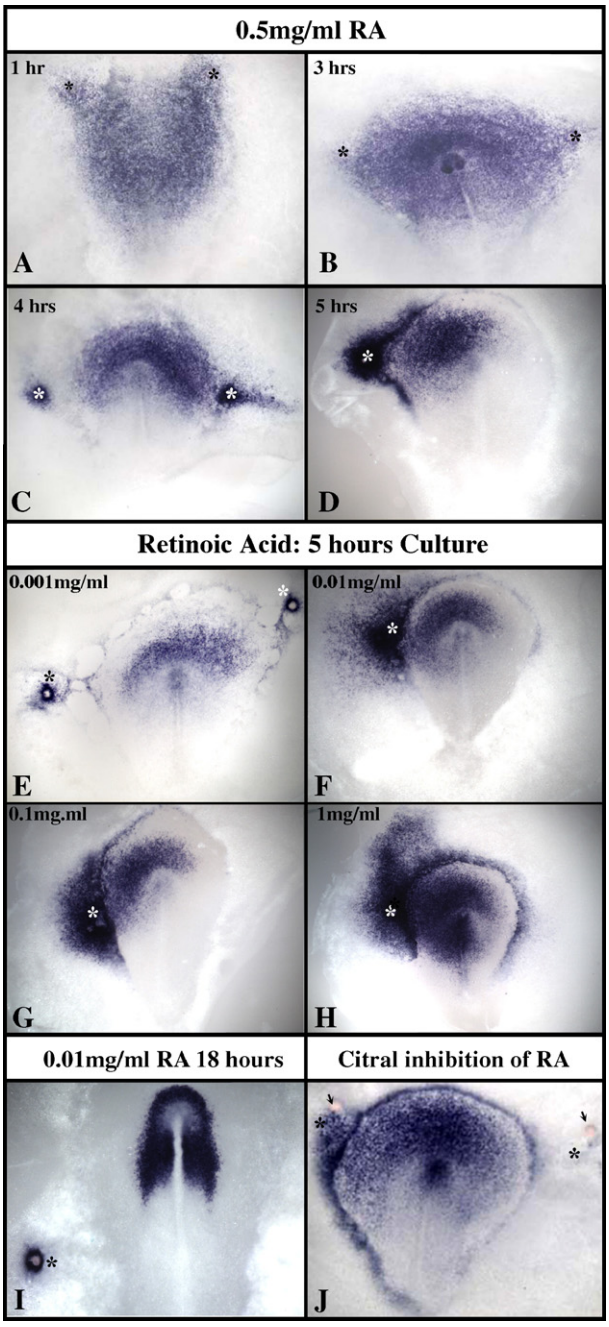

Fig. 6. Induction of *Cyp26A1* by RA. (A–D) 0.5 mg/ml RA induces *Cyp26A1* within 1 h (A) and the intensity of induced expression increases for 5 h (B–D). (E–H) After 5-h incubation, *Cyp26A1* is induced weakly by 1 μg/ml (E) and the intensity and area of staining increase with the dose of RA (F–H). (I) *Cyp26A1* can be maintained by 10 μg/ml RA following overnight culture. * indicates RA bead in above. (J) *Cyp26A1* induction by the hypoblast (grafted on both sides of the embryo: *) is prevented by a bead coated with the RA antagonist Citral (arrow on left) but not by a control bead (arrow on right). Note that unilateral RA bead grafts affect endogenous expression in the host (D, F, G, H).

Both Chordin and Wnt antagonists can maintain the induction of Sox3 and ERNI by the hypoblast

It was previously shown that Hensen's node grafts induce transient expression of *Sox3* and that this induction is maintained when the node is replaced by the BMP antagonist Chordin 5 h after the node graft (Streit et al., 1998). It has also been proposed that Wnt antagonism in combination with FGF can induce neural fates through inhibition of BMP signaling (Wilson et al., 2001; Wilson and Edlund, 2001). Since hypoblast grafts can only induce transient expression of *Sox3*, we tested whether antagonists of BMP and/or Wnt can maintain the transient induction of early markers by a hypoblast. Hypoblasts were either grafted at the same time as cell pellets secreting various factors and incubated for 20 h, or a hypoblast graft was followed 6 h later by cells secreting the factors and further incubation for 14 h. The results obtained with both methods were indistinguishable. When cell pellets secreting any factor or various combinations thereof were grafted alone, no induction of any marker was observed (Chordin, Dkk1, Crescent, Dkk1+Crescent, Chordin+Dkk1+Crescent; 0/8 for each marker: *Sox3*, *ERNI*, *Otx2*, *Cyp26A1*, *Sox2*; Table 1). When a hypoblast was grafted with Chordin-secreting cells, intense expression of both *Sox3* (20/20; Fig. 8A) and *ERNI* (6/6; Fig. 8B) was maintained after 20-h incubation. Furthermore, sections revealed that the area opaca had thickened and cells were more columnar, resembling an early neural plate ($n=10$; Figs. 8M–P) and that the maintenance of *Sox3* and *ERNI* was not confined to the region immediately adjacent to the cell pellet but extended much further (Figs. 8M, O). Interestingly, expression of *Sox3* in the epiblast directly above the center of the pellet was often reduced or eliminated (Figs. 8N, P). A similar lack of *Sox3* expression is observed in the midline of the neural plate of normal embryos, which lies directly above the notochord (Rex et al., 1997). Maintenance of *Sox3* (5/5; Fig. 8E) and, to a lesser extent, of *ERNI* (5/6; Fig. 8F) is observed when cells secreting the Wnt antagonists Dkk1 and/or Crescent are grafted together with a hypoblast and hosts incubated for 18–20 h. Neither *Otx2* (Chordin: 0/6; Dkk1+Cres: 0/8) nor *Cyp26A1* (Chordin: 0/6; Dkk1+Cres: 0/6) expression is maintained in these experiments (Figs. 8C–D, G–H), suggesting that different signals are required to maintain these markers.

Otx2 is maintained only by combination of a hypoblast graft with both BMP- and Wnt-antagonists

Since neither BMP- nor Wnt-antagonists alone can maintain *Otx2* induction by a hypoblast, a combination of all three (Chordin+Dkk1+Cres together with a hypoblast) was tested. After 20 h, *Sox3* (5/5; Fig. 8I) and *ERNI* (5/5; Fig. 8J) were again maintained; however, this time *Otx2* was also maintained (5/8; Fig. 8L). *Otx2* is briefly expressed in the organizer at stage 3^{+}–4 (Bally-Cuif et al., 1995); to ensure that an organizer is not being induced in the area opaca, *bra* was tested following a hypoblast+BMP antagonists+Wnt antagonists graft. No expression was seen (0/8). Although these results are consistent with the idea (Glinka et al., 1997) that antagonism of both BMP and Wnt pathways promotes anterior specification, this is not sufficient; other signals from the hypoblast are also required since overexpression of Wnt-

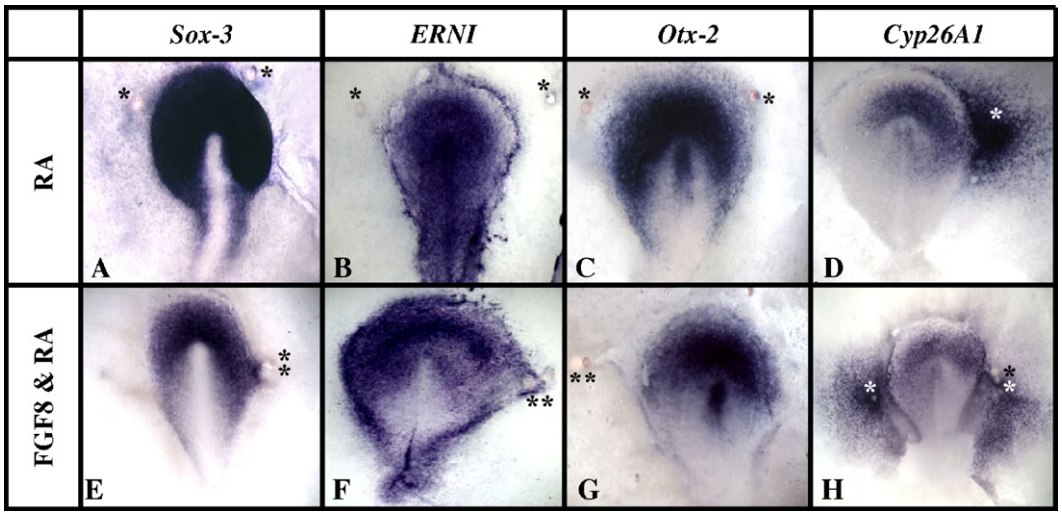

Fig. 7. RA does not induce other markers and antagonizes induction by FGF. (A–D) RA does not induce *Sox3* (A), *ERNI* (B) or *Otx2* (C) but does induce *Cyp26A1* (D). (E–G) Co-grafting FGF8 and RA beads suppresses expression of *Sox3* (panel E; compare with FGF8 induction in Fig. 3E), reduces that of *ERNI* (panel F; compare with Fig. 3F), and does not induce *Otx2* (panel G; compare with Fig. 5A) as compared to FGF8 beads alone. (H) RA (left: white*) induces *Cyp26A1*, while FGF8 (right: black*) rostral to a RA bead (right: white*) blocks induction of *Cyp26A1* in this vicinity. * indicates bead positions.

and BMP-antagonists alone (Chordin+Dkk1+Cres) cannot induce *Otx2*.

Cyp26A1 is induced and maintained by RA

Despite the maintenance of *Sox3*, *ERNI* and *Otx2*, *Cyp26A1* was still not maintained in the above experiments (0/8; Figs. 8C, G, K). Since *Cyp26A1* is induced by RA and since the hypoblast is only a weak source of RA, a further source may be required to maintain *Cyp26A1* expression. Indeed, *Cyp26A1* is both induced and maintained by RA alone, if the concentration is sufficiently high: at the lowest concentration (1 μg/ml), induction is transient (1/5 maintain expression overnight), which may be due partly to degradation of the RA by the induced *Cyp26A1*. Higher RA concentrations (10 μg/ml) do sustain overnight expression of *Cyp26A1* (4/4; Fig. 7I).

Maintenance of early markers is not sufficient to define a definitive anterior neural state

Despite the maintenance of expression of some markers by some combinations of factors described above, the neural plate marker *Sox2* is not induced by any combination (hypoblast+Chordin: 0/20; +Wnt antagonists: 0/8; +Chordin+Wnt antagonists: 0/8). We also tested a combination of hypoblast+Wnt antagonists+Chordin+electroporation of a *Cyp26A1* expression construct; *Sox2* is still not induced (0/10) (Table 1). Likewise we tested whether *Ganf* could be induced by a combination of hypoblast+Wnt antagonists+Chordin with or without *Cyp26A1*; it was not (0/8 for each combination). Therefore, even when all of the early induced markers are maintained in the epiblast, this is not sufficient for induction of the definitive neural marker *Sox2* or for the formation of a rostral CNS expressing a full complement of regional and neural markers.

Discussion

What state does the hypoblast induce in the epiblast?

Many genes expressed broadly at pre-primitive streak stages are markers for specific tissues or regions at later stages of development. Two of the genes used here, *Otx2* and *Cyp26A1*, start to be expressed in a broad domain of the pre-streak epiblast (including both prospective neural and non-neural regions) and eventually become restricted to the forebrain. For both genes, there is a brief intermediate stage (3^{+}–4) at which *Otx2* (Bally-Cuif et al., 1995) and *Cyp26A1* (Blentic et al., 2003) are also expressed in Hensen's node. The other two markers used (*ERNI* and *Sox3*) are also initially expressed in a broad domain of the epiblast but later become restricted either to the entire neural plate (*Sox3*; Uwanogho et al., 1995; Rex et al., 1997) or to its border (*ERNI*; Streit et al., 2000; Streit, 2004). All of these genes can be induced transiently by the hypoblast in the area opaca, unlike the later-appearing, definitive neural plate and forebrain markers *Sox2* and *Ganf*. What does the early phase of co-expression of these markers represent?

Another gene whose initial expression is identical to those of the four genes studied here and which is also transiently induced by the hypoblast (Knezevic and Mackem, 2001 and our unpublished observations) is *Not1*/*CNot*. This also becomes restricted to the node during primitive streak stages, but then segregates to the notochord (Knezevic et al., 1995; Knezevic and Mackem, 2001; Stein and Kessel, 1995). As shown here for *Cyp26A1*, *Not1* is induced by retinoic acid (Knezevic and Mackem, 2001). Regardless of whether the grafted hypoblast remains in place or whether it is replaced with a new hypoblast some hours later, the expression of all these markers disappears and the epiblast in contact with the graft never develops expression of later markers or acquires neural plate or forebrain character. These considerations strongly suggest that neither the

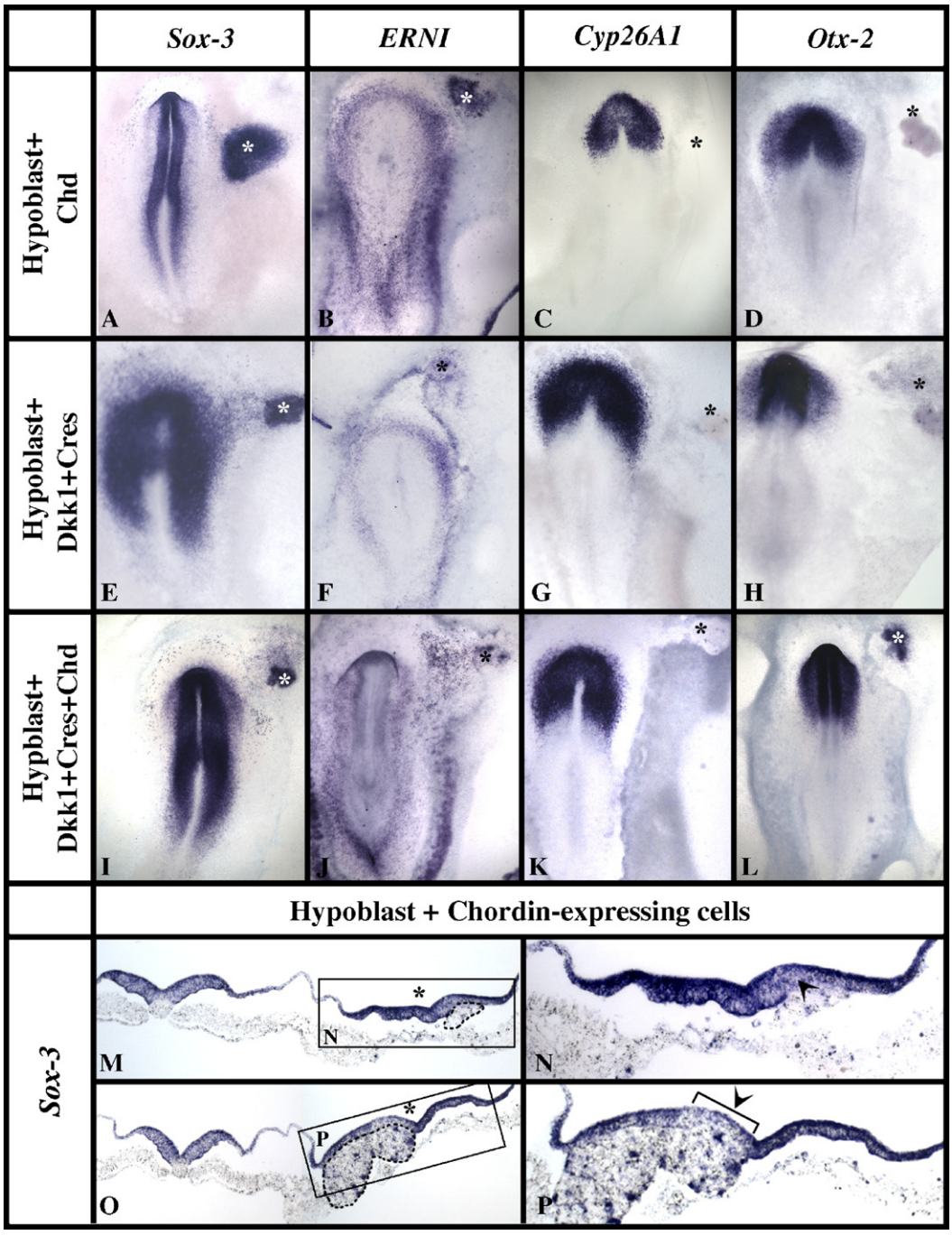

Fig. 8. Maintenance of genes induced by the hypoblast by pellets of factor-secreting cells. (A–D) Chordin-expressing cells maintain *Sox3* (A) and *ERNI* (B) but not *Cyp26A1* (C) or *Otx2* (D). (E–H) Dkk1+Crescent also maintain *Sox3* (E) and *ERNI* (F) but not *Cyp26A1* (G) or *Otx2* (H). (I–L) A combination of Chordin+Dkk1+Crescent maintains expression of *Sox3* (I) and *ERNI* (J) but not of *Cyp26A1* (K), and *Otx2* is now maintained (L). * indicates position of graft. (M–P) Sections through *Sox3*-stained embryos grafted with a hypoblast and Chordin-secreting cells. * indicates the induced 'early neural plate' in the area opaca and the black, dashed line demarcates the cell pellet. Panels N and P are higher magnification views of panels M and O, respectively. Arrow heads point to a reduced expression of *Sox3*.

early induction of these markers nor their normal expression domains before gastrulation can be considered to represent a true neural or forebrain state. For this reason, we refer to the state induced by the hypoblast as "pre-neural/pre-forebrain", but it should be emphasized that the cells within this synexpression domain are not committed to any particular fate and can (as many of them normally do) still give rise to components in any germ layer, including mesoderm and endoderm.

Roles of the hypoblast in early axial development and the signals involved

Before and during early gastrulation, several important patterning events occur. These include positioning of the primitive streak (Bertocchini and Stern, 2002; Perea-Gomez et al., 2002), a set of complex morphogenetic movements ("Polonaise": convergence of epiblast to the posterior midline

Table 1
Ability of various factors to maintain expression of markers induced by the hypoblast

Hypoblast +	*Sox3*	*ERNI*	*Cyp26A1*	*Otx2*	*Sox2*
Chordin	++++	+++	−	−	−
Dkk1 + Cres	++++	++	−	−	−
Chordin + Dkk1 + Cres	++++	++	−	++	−
Chordin + Dkk1 + Cres + CYP26A1					−

and anterior movement along this midline) (Hatada and Stern, 1994; Thomas and Beddington, 1996; Foley et al., 2000; Kimura et al., 2001) and the initiation of neural induction (Streit et al., 2000; Wilson et al., 2000). The results of the present study along with previous reports suggest that the hypoblast (or its equivalent in mouse, the anterior visceral endoderm, AVE) is important for all of these.

Formation of the primitive streak is initiated by cooperation between Vg1 and Wnt signals, which induce Nodal. Together with FGF, Nodal induces the mesendoderm of the primitive streak (Skromne and Stern, 2001; Bertocchini and Stern, 2002; Bertocchini et al., 2004). However, primitive streak formation only occurs some time after induction of Nodal because the underlying hypoblast emits the Nodal antagonist Cerberus whose function is to block premature streak formation. Nodal signaling is released from inhibition when the hypoblast is displaced away from the posterior edge of the embryo by the endoblast (another extraembryonic tissue, similar to mouse visceral endoderm, VE), which triggers primitive streak formation (Bertocchini and Stern, 2002). Similar events take place in the mouse, where Nodal is inhibited by both Cerberus and Lefty-1 (Perea-Gomez et al., 2002).

Fate maps reveal that, before gastrulation, the prospective forebrain territory is located close to the posterior midline. Just prior to primitive streak formation, as a result of the "Polonaise" movements of the epiblast, this territory moves anteriorly to occupy its final position just in front of where the future organizer will be located (Hatada and Stern, 1994). This anterior movement mirrors the spreading of the underlying hypoblast sheet, which occurs at the same rate. When the hypoblast is rotated by 90°, new movements are initiated in the host epiblast which cause the primitive streak to bend and a reorientation of the embryonic axis (Waddington, 1932; Foley et al., 2000). The signals responsible are unknown, but Wnt signaling may be important (Kimura-Yoshida et al., 2005). A correlation between movement of the prospective forebrain from a distal to an anterior position and similar movements of the adjacent AVE have also been observed in the mouse (Thomas and Beddington, 1996), and mutants with impaired movements have a shortened axis and forebrain defects (Ding et al., 1998: Acampora et al., 1998; Liu et al., 1999; Huelsken et al., 2000; Perea-Gomez et al., 2001; Kimura et al., 2001; Liguori et al., 2003).

Neural induction requires a very early FGF signal (Streit et al., 2000; Wilson et al., 2000). FGF8 induces *Sox3* and *ERNI* as quickly as does a graft of a node, and inhibition of FGF signaling blocks not only the induction of these genes by a node but also all subsequent steps of neural induction, including *Sox2* expression (Streit et al., 2000). The hypoblast, along with an early population of middle layer cells, both of which express *FGF8*, had been implicated in this initial step, but the inducing ability of the hypoblast had not been tested. Here we show that the hypoblast can indeed induce transient expression not only of *Sox3* and *ERNI* but also of *Otx2* and *Cyp26A1*. However, our results reveal that FGF is not the only signal involved in induction of these genes by the hypoblast because this factor cannot induce either of the latter two and because inhibition of FGF signaling by SU5402 does not completely abolish all of the effects of hypoblast grafts. We show that retinoic acid induces *Cyp26A1* and that it is required for this induction. The RA-synthesizing enzyme *RALDH2* is expressed in the hypoblast at least at stage 4 (as well as in prechordal mesendoderm, PME; Blentic et al., 2003; Halilagic et al., 2003).

In conclusion, the chick hypoblast is involved in all of these important early events of axis formation and FGF and RA are two of the critical signals. However, other signals remain to be identified. In frog and fish embryos, FGF, RA and Wnt pathways have all been shown to be strong caudalizing factors, posteriorizing the neurectoderm directly as well as indirectly through mesoderm patterning (RA: Blumberg, 1997; Kaiser et al., 2003; Molotkova et al., 2005, FGF: Cox and Hemmati-Brivanlou, 1995; Pownall et al., 1996; Hardcastle et al., 2000; Rentzsch et al., 2004; Wnt: McGrew et al., 1997; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Houart et al., 2002; Nordstrom et al., 2002; Shimizu et al., 2005). Here we show that FGF and RA are involved in the very earliest stages of neural induction and polarity, highlighting the importance of cellular context in determining the effects of signaling pathways. Unexpectedly, our findings raise the possibility that the initial "activation" proposed in the Nieuwkoop model (Nieuwkoop and Nigtevecht, 1954; corresponding here to the initial induction of *Sox3*+*Otx2*+*Cyp26A1*) might rely on the same signals as the "transformation" responsible for caudalization. The relative levels, duration and timing of the signals are likely to be important in determining which of these activities they will manifest.

Transient nature of hypoblast-induced markers and differential maintenance by BMP and Wnt antagonism

All of the genes induced by hypoblast grafts are only expressed transiently and disappear after 10- to 12-h incubation even if the hypoblast is replaced with a fresh one. In the embryo, the hypoblast becomes restricted anteriorly while the primitive streak elongates, Hensen's node develops and the head process and PME emerge from it. Genes initially induced in the epiblast by the hypoblast might require signals emanating from these tissues to sustain and refine expression. The PME and head process are involved in anterior neural development (Foley et al., 1997; Rowan et al., 1999) and express BMP- and Wnt-antagonists (*chordin* and *noggin*: Connolly et al., 1997; Dale et al., 1997; McMahon et al., 1998; Hongo et al., 1999; Streit and Stern, 1999; Bachiller et al., 2000; Anderson et al., 2002; *Dkk1* and *crescent:* Pfeffer et al., 1997; Kazanskaya et al., 2000; Pera and De Robertis, 2000; Mukhopadhyay et al., 2001; del Barco Barrantes et al., 2003). These tissues are therefore the most

likely sources of the signals responsible for maintenance of hypoblast-induced markers.

Different combinations of factors are required to maintain expression of each of the four genes analyzed. *Sox3* and *ERNI*, expressed throughout the prospective neural plate, can be maintained by either BMP or Wnt antagonism. However, only the combined antagonism of both BMP and Wnt pathways can maintain expression of the prospective fore/midbrain marker *Otx2* and only RA can maintain expression of *Cyp26A1*. Our experiments do not allow us to determine whether these factors act on the epiblast itself or indirectly, through the hypoblast. For example, they might prolong or enhance the viability of the hypoblast itself, which appears to be limited both in the embryo and after grafting (see Knoetgen et al., 1999; Foley et al., 2000). Nevertheless, the finding that different signals are required to maintain different markers suggests that this is not the main mechanism by which they act.

Initiation of "pre-neural", "pre-fore/midbrain" markers and definitive neural induction

Even when expression of all four markers is sustained for 18–20 h by additional factors, no expression of *Sox2* or *Ganf* is ever observed, and a mature neural plate does not develop. Therefore, the state generated in the epiblast even after maintaining expression of markers initially induced by the hypoblast might be equivalent to the prospective neural plate at stage 4 and not beyond. Other signals required to progress to a mature, regionalized neural plate are likely to emanate from the node and/or emerging axial mesendoderm (see above). Consistent with this, a thickened epithelium resembling the prospective neural plate from stage 4–5 embryos develops in some cases, but this does not elevate or fold. Strikingly, both the thickening and the expression of the markers extend some distance away from the region directly adjacent to the source of Wnt- or BMP-antagonists, as if the hypoblast could somehow aid in the spread of the signals emanating from the cell pellets. Another interesting observation is that the region of ectoderm directly above Chordin-expressing cell pellets shows reduced levels of *Sox3* expression. In the embryo, the floor plate, which does not express *Sox3* (Rex et al., 1997), lies directly above the notochord (which produces Chordin), suggesting that a floor plate-like state may be triggered by BMP antagonists at this site.

Comparison with other model organisms

This and previous studies in the chick embryo (Foley et al., 1997, 2000; Knoetgen et al., 1999, 2000; Bertocchini and Stern, 2002) suggest that the hypoblast influences embryonic polarity and neural development in several, experimentally separable ways. It contributes to positioning the site where the primitive streak will arise, to direct the movements of the adjacent epiblast, which move the prospective forebrain region from an initial posterior site to its final anterior location and, as shown here, it is also able to induce expression of marker genes which are later expressed either throughout the nervous system (*Sox3*) or in the fore- and midbrain (*Otx2*, *Cyp26A1*). However, other signals are required both to maintain this initial expression and to initiate expression of definitive markers of the neural plate and of anterior neural identity (*Sox2*, *Ganf*).

In the mouse, numerous experiments have established that the equivalent tissue, the AVE, is required for head development (Thomas and Beddington, 1996; Varlet et al., 1997; Acampora et al., 1998; Rhinn et al., 1998; Dufort et al., 1998; Ding et al., 1998; Shawlot et al., 1999; Liguori et al., 2003). Some of the activities listed above for the chick hypoblast have also been demonstrated for the mouse AVE, including inhibition of Nodal signaling (through Cerberus and Lefty-1) and thus primitive streak formation (Perea-Gomez et al., 2002). The AVE also contributes to the correct positioning of the A–P axis—for example in embryos mutant for either *Otx2*, *Cripto*, *Wnt3* or β-catenin, the AVE fails to move anteriorly and the adjacent epiblast either lacks anterior markers or expresses them at the distal tip and they do not resolve to the rostral pole (Ding et al., 1998: Acampora et al., 1998; Liu et al., 1999; Huelsken et al., 2000; Kimura et al., 2001; Perea-Gomez et al., 2001; Liguori et al., 2003). However, no study to date has succeeded in demonstrating that the AVE has the ability to induce either a forebrain or neural tissue, or any marker gene for these, in tissues not normally fated to express it. Indeed, a particularly important study revealed that a forebrain can only be induced when the AVE is combined with both the Early Gastrula Organizer (EGO) and with epiblast from the prospective forebrain region (Tam and Steiner, 1999). This finding is consistent with those in the present paper. Together, these results in mouse and chick suggest that the hypoblast/AVE contributes to polarizing the embryo along its future anterior–posterior axis by positioning the primitive streak, by orienting cell movements and by initiating the expression of genes required for neural and forebrain development, but is not sufficient to induce a forebrain without signals emanating from other tissues.

Are these properties unique to amniotes, or do functionally equivalent tissues exist in other vertebrates? Teleost fish have an extraembryonic tissue: the yolk syncytial layer (YSL) (Kimmel et al., 1995). Based on the fact that the movements of the YSL appear to direct cell movements of the overlying ectoderm (Long, 1983; D'Amico and Cooper, 2001), that it expresses some of the same genes found in the AVE/hypoblast (Ho et al., 1999; Chang and Calame, 2002) and that it appears to promote anterior identity through BMP and Wnt inhibition (Fekany et al., 1999; Fekany-Lee et al., 2000), the YSL could be homologous to the AVE/hypoblast. It remains to be established whether the YSL can induce gene expression in naive ectoderm and, if so, whether there is a transient quality to this induction.

In *Xenopus*, homologues of the AVE/hypoblast are not as readily identifiable because there is no obvious "extraembryonic" tissue. One possible candidate is the yolky vegetal endoderm which contributes more to gut contents than to gut lining. Although the yolky vegetal tissue does influence embryonic polarity (specifying "dorsal", equivalent to "posterior" in amniotes in being the site where gastrulation begins; see Stern et al., 2006), the other functions of the hypoblast/AVE have not been demonstrated for this tissue. The anterior

embryonic endoderm shares expression of some genes with the AVE/hypoblast (*Hex*: Newman et al., 1997; Thomas et al., 1998; *Xblimp1*: de Souza et al., 1999; *Cerberus*: Bouwmeester et al., 1996; Belo et al., 1997; Biben et al., 1998 and *Dkk1*: Glinka et al., 1998; Osada and Wright, 1999; Schneider and Mercola, 1999). However, its ablation does not prevent forebrain development (Schneider and Mercola, 1999). On the other hand, this operation can only be done rather later in development (well into the gastrula stage) than the equivalent experiments in the mouse. Rather than the murine AVE being an evolutionary adaptation to mammalian head development (Knoetgen et al., 1999; de Souza and Niehrs, 2000), it seems more likely in most vertebrates that embryonic polarity as a whole (specifying the site where gastrulation begins, where dorsal mesoderm including the organizer form, and the future location of the tail end of the embryo) is initially regulated partly through the action of an extraembryonic tissue (AVE, hypoblast, YSL and perhaps yolky vegetal cells). However, there may be some differences between species in the precise sources of signals that cooperate to induce and/or reinforce development of the most rostral central nervous system.

In amniotes, the prospective fore- and midbrain regions at the primitive streak stage occupy a large proportion of the epiblast (Tam, 1989; Lawson and Pedersen, 1992; Hatada and Stern, 1994) and may be specified early (Muhr et al., 1999), while more caudal prospective hindbrain and spinal cord regions of the neuraxis arise progressively from a relatively small 'stem zone' located adjacent to the node as the axis elongates (Delfino-Machin et al., 2005). In contrast, in anurans, the entire CNS axis appears to be laid down by the late gastrula stage and elongates mainly by convergent extension (Keller, 1976; Keller and Danilchik, 1988; Keller et al., 1992), while teleosts appear to use a combination of both mechanisms (Woo and Fraser, 1995). Thus, in most vertebrates, with the possible exception of anuran amphibians, the AVE/hypoblast/YSL plays an important role in distancing the future forebrain/midbrain region from putative caudalizing influences from the organizer (Foley et al., 2000; Kimura et al., 2001; Kimura-Yoshida et al., 2005).

Significance to models of A–P patterning of the CNS

Of many models proposed to explain how the nervous system is induced and initially subdivided into broad regions along the head–tail axis, two have been particularly influential. One, proposed by Holtfreter (1933) and Mangold (1933), suggested that distinct organizers exist in the axial mesendoderm (archenteron roof) which are responsible individually for inducing "head", "trunk" and "tail" regions of the overlying neural plate. It was originally thought that these inductive events take place at the neurula stage. More recent results have pushed back the time of neural induction to earlier stages of development, and some now believe that the distinct signals arise from tissues other than definitive mesendoderm long before the end of the gastrula stage (e.g. Glinka et al., 1997; Agathon et al., 2003; Kudoh et al., 2004). The initial findings that the AVE is required for normal head development in the mouse have even been taken by some as evidence that the AVE itself is a "head organizer". Our present results and those of Foley et al. (2000) and Tam and Steiner (1999) suggest that this is not the case: although the AVE/hypoblast is required for head development, it cannot induce a forebrain.

An alternative was proposed by Nieuwkoop and Nigtevecht (1954), the "activation–transformation" model. It proposes that all of the initially induced neural tissue is anterior (rostral) in character, and that parts of this are subsequently transformed (caudalized) by signals from the organizer. Although considerable evidence has since been obtained for transforming signals (FGFs, Wnts and retinoids) and that BMP inhibition alone was proposed to be responsible for generating rostral CNS, a finding apparently inconsistent with this model is that older organizers only generate a caudal nervous system (Mangold, 1933; Dias and Schoenwolf, 1990; Storey et al., 1992). To accommodate this and other findings, a modification of this model was suggested, including an extra step. It was postulated that neural induction is initiated by induction of an unstable, "pre-neural/pre-prosencephalic" state; a second step (stabilization) then maintains this and converts the initial territory into a definitive fore/midbrain, finally followed by a caudalization step as proposed by Nieuwkoop (Stern, 2001). Our present data partly support this model: we show that the hypoblast induces expression of *Sox3*, *Otx2* and *Cyp26A1* (which later in development are markers for neural and forebrain tissue, respectively) in the epiblast, but this induction is only transient and can be stabilized by signals from other tissues. However, our findings also suggest that activation and stabilization are still not sufficient either for definitive neural induction or for establishing fore/midbrain identity. Other, as yet unidentified, signals are required, likely to emanate from the organizer or from cells derived from it, such as the axial mesendoderm. Although our results tend to be more consistent with the Nieuwkoop model (or its recent modification) than with Holtfreter's and Mangold's proposal (and its updated versions), they cannot provide definitive proof for either.

Conclusions

The data presented here suggest that, in addition to its role in positioning the primitive streak (Bertocchini and Stern, 2002; Perea-Gomez et al., 2002) and directing epiblast cell movements (Foley et al., 2000; Kimura-Yoshida et al., 2005), the hypoblast plays a role in establishing an initial "pre-neural/pre-prosencephalic" territory in the epiblast of the avian embryo. The signals include a combination of FGF and RA signaling, perhaps together with Wnt- and BMP-antagonists. This induction is transient, but can be stabilized by RA and continued Wnt- and BMP-inhibition. However, a definitive neural state requires additional factors remaining to be identified. A particularly tantalizing observation is that FGF and RA, two "transforming" (caudalizing) signals in the Nieuwkoop model, also appear to mediate the initial "activation" step.

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