Neural induction by the node and placode induction by head mesoderm share an initial state resembling neural plate border and ES cells

Katherine Trevers[1], Ravindra Prajapati[2], Mark Hintze[2], Matthew Stower[1], Anna-Christina Strobl[1], Monica Tambalo[2], Ramya Ranganathan[2], Natalia Moncaut[1], Mohsin Khan[1], Claudio D Stern[1], Andrea Streit[2]

[1]University College London, [2]King's College London

Submitted to Proceedings of the National Academy of Sciences of the United States of America

Around the time of gastrulation in higher vertebrate embryos, inductive interactions direct cells to form central nervous system (neural plate) or sensory placodes. Grafts of different tissues into the periphery of a chick embryo elicit different responses: Hensen's node induces a neural plate, whereas head mesoderm induces placodes. How different are these processes? Transcriptome analysis in time-course reveals that both processes start by induction of a common set of genes, which later diverge. These genes are remarkably similar to those induced by an extraembryonic tissue, the hypoblast, and are normally expressed in the pre-gastrulation stage epiblast. Explants of this epiblast grown in the absence of further signals develop as neural plate border derivatives, and eventually express lens markers. We designate this state as "pre-border"; its transcriptome resembles embryonic stem cells. Finally, using sequential transplantation experiments, we show that the node, head mesoderm and hypoblast are interchangeable to begin any of these inductions, while the final outcome depends on the tissue emitting the later signals.

embryonic induction | organizer | gastrulation | neurulation | patterning

INTRODUCTION

During development, cell fate decisions are controlled by inductive interactions: instructive signals from one tissue cause a change of fate in adjacent responding cells. For example, signals from Spemann's organizer (Hensen's node in amniotes) can induce neural tissue from cells that otherwise develop into epidermis (1, 2) and the zone of polarizing activity (ZPA) induces and patterns digits in the limb bud (3). Thus, each inducer elicits a distinct and specific response. However classical transplantation experiments indicate that some inducers are interchangeable: Hensen's node and the ZPA alike result in digit duplication when grafted into anterior limb buds (4). These and similar observations have led to the suggestion that while inducing signals may be universal, it is the responding tissue that confers specificity to inductive events (5-7). Here we explore two well-characterized inductive events that occur at approximately the same time, but under the influence of signals from different tissues: the induction of neural plate by Hensen's node and the induction of placode progenitors by lateral head mesoderm (lHM).

The neural plate gives rise to the entire central nervous system, while sensory placodes contribute to the sense organs and cranial ganglia and arise from the ectoderm surrounding the anterior neural plate (the pre-placodal region, PPR) (8, 9). The neural plate is first defined by the appearance of *Sox2* and the PPR becomes molecularly distinct at head fold stages by the expression of *Six1*, *Six4* and *Eya1* or *-2*. At this stage, neural and placode precursors continue to be intermingled with each other as well as with future neural crest and epidermal cells in a territory denominated the "neural plate border" (9, 10), suggesting that they may initially share common properties. Both neural and PPR induction require FGF activity as well as antagonism to BMP and Wnt signaling (2, 11-14) although the relative contribution of each signal still needs to be unraveled.

We have previously identified response genes to mesoderm-derived signals (15); we now use transcriptional profiling of node-induced tissue, together with neural and pre-placodal tissue from normal embryos, to explore the similarities and differences between the two inductive events. We find that, despite the difference in inducing tissues and in the ultimate outcome, the initial response to the node is largely identical to the response to the lHM; this defines a distinct, common transcriptional state, which is very similar to the early pre-streak epiblast and to the neural plate border. When cells from the early epiblast are allowed to develop *in vitro* without further induction, they differentiate into neural plate border derivatives: lens, neural crest and neural plate. To demonstrate that this "pre-border" state is common to neural and pre-placodal induction, we show that induction of this state by short exposure to either the node or the lHM, as well as by the hypoblast (extraembryonic endoderm), can be subsequently directed to each set of fates by short exposure to the appropriate tissue. We propose that inductions by different inducing tissues start by a reprogramming step that converts cells to a common, "pre-border"-like state which is also similar to very early (pre-gastrulation) epiblast and to embryonic stem cells. Specific lineages diverge under the influence of later, specific signals.

Significance

It is generally believed that the outcome of many inductive interactions occurring during development is largely dependent on the responding tissue, the source of the signals playing a relatively minor part. Here we compare induction of the neural plate by the node, and of placodes by head mesoderm, and show that both inducing tissues elicit a similar initial response but they later diverge. We characterise the initial common state by a variety of methods and show its similarity to ES cells, suggesting that these inductions may begin with a common "reprogramming" step. This initial state also shares many features in common with the border of the neural plate, suggesting that this region retains features of a "ground state".

Reserved for Publication Footnotes

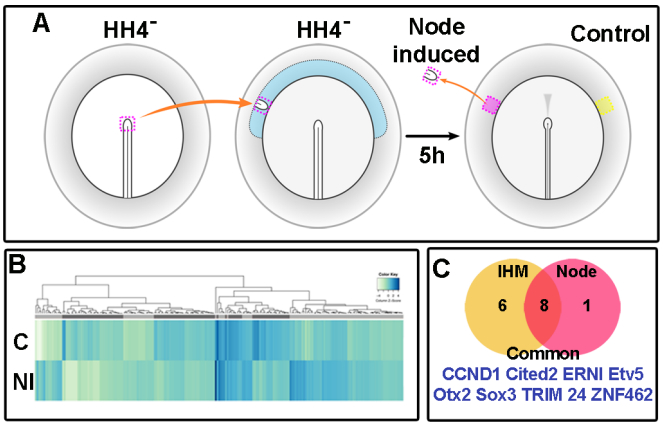

Fig. 1. Comparison of lHM and node grafts at 3 hours. A. Schematic of RNA-seq tissue collection for node induction. **B.** Heat map displaying differentially regulated genes between node-induced and uninduced controls after 5h. **C.** Venn diagram showing the overlap between lHM (yellow) and node (red) induced genes at the 3-hour time point, common genes in orange.

RESULTS

Analysis of genes regulated by an organizer graft reveals similarities between neural and placodal induction

Grafts of the "organizer", Hensen's node, can induce a complete, patterned nervous system in cells that are not fated to contribute to the neural plate. Previous studies have established that some genes (e.g. *ERNI* and *Sox3*) are induced in responding cells after less than 5h exposure to the graft (11). The same genes are expressed in a wide territory of the epiblast of the normal embryo before gastrulation begins, which led to the idea that in the normal embryo, the process of neural induction begins before gastrulation (11, 16). To gain a more comprehensive view of the transcriptional changes occurring at this time point we performed RNAseq from tissue exposed to organizer signals and compared this to the transcriptome of contralateral control epiblast from the same embryos. Hensen's node from a HH4$^-$ chick donor was grafted onto the area opaca epiblast of a host of the same stage. After 5h, the node was removed and the adjacent tissue ("induced epiblast") collected, along with the same region from the contralateral side ("control epiblast") (Fig. 1A). Fifty control and experimental tissues were collected, processed for RNAseq and analyzed for differentially expressed genes (1.5 fold change, $p<0.05$), which revealed a total of 2477 differentially expressed genes: 1166 upregulated and 1311 downregulated in the induced epiblast (Fig. 1B, Dataset S1).

We noticed that many transcripts upregulated in response to an organizer graft (252, of which 25 are transcription factors) were also identified in a recent screen for genes involved in sensory placode induction (grafts of lateral head mesoderm, lHM) (15). This suggests considerable similarity between the initial steps in neural induction by the node and PPR induction by the head mesoderm. To test this directly we designed a NanoString probe set containing known probes for neural, pre-placodal, neural plate border and non-neural ectoderm cells, as well as new genes identified in both screens (Dataset S2). Hensen's node from HH4$^-$ or lHM from HH5/6 donors was grafted into the area opaca of HH4$^-$ hosts; after 3 and 5h the adjacent epiblast and non-induced epiblast from the contralateral side were collected and processed for NanoString analysis. The node induces 9 genes after 3h and the lHM induces 14; eight of the transcripts are represented in both sets (Fig. S1, Dataset S3). Five hours after grafting, the responses to the two tissues start to diverge; 33% of the 123 induced genes are shared, and the lHM now induces many genes (51; 41%) that are not induced by the node (Dataset S3).

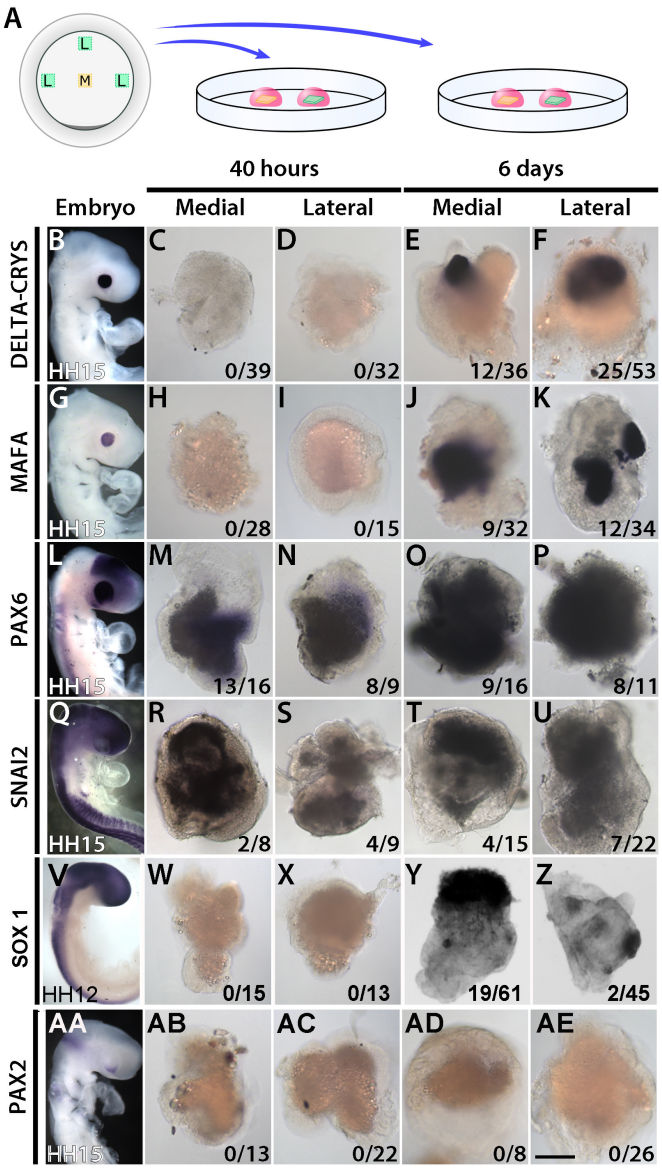

Fig. 2. Specification assay for the early epiblast. Explants were obtained from medial (M) and lateral (L) regions of pre-primitive streak epiblast and placed in culture. Expression of various markers was assessed after either 40h or 6 days. *MAFA* and δ-*crystallin* are lens markers, *Pax6* marks anterior neural and placodal regions as well as the eye, *Snail-2* is a marker for neural crest, *Sox1* is a neural marker and *Pax2* marks the otic vesicle. After 40h (see also Figure S6) the explants express neural plate border markers. By 6 days, about 1/3 of explants have differentiated into lens tissue (E,F,J,K), about a third as neural (O,P,Y,Z) and about a third as neural crest (T, U). No differentiation into otic placodes is detected (AD, AE). The number in each panel indicates the proportion of explants expressing the marker.

In summary, many early response genes induced by organizer and lHM grafts are identical. This reveals a striking similarity between the first steps in neural and pre-placodal induction and leads us to propose that before cells acquire their unique identity as central or peripheral nervous system they transit though a common transcriptional state that primes them for neural development.

The epiblast prior to gastrulation as a "pre-neural state"

If the above hypothesis is correct, genes identified in response to node- and lateral head mesoderm-derived signals should be expressed in precursors for both the central and the peripheral nervous system during normal development. To test this further

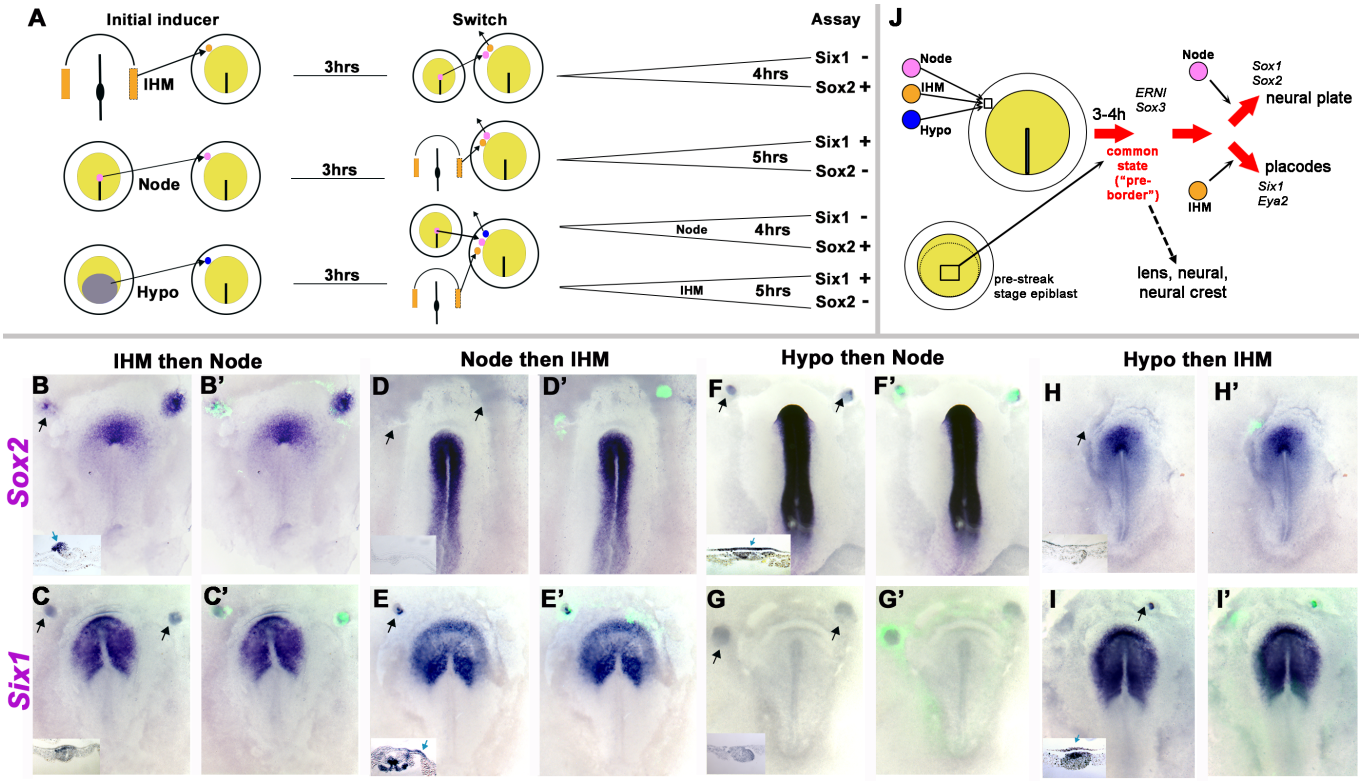

Fig. 3. Sequential transplantation demonstrates common state. A. Diagram of the experimental design. After a period of 3h following a graft of lHM, Hensen's node or hypoblast, the initial graft is removed and replaced by another inducing tissue (node or lHM); markers of neural plate (*Sox2*) or placode (*Six1*) are assessed after a further 4-5h incubation. **B, C.** lHM was grafted to a HH4$^-$ host for 3h and exchanged by a node for 4h (total time 7h); *Sox2* was induced in 4/6 cases (compared to 0/5 for 4h node only grafts; Node 7h: 4/5). *Six1* was not induced (0/8). **D, E.** Node was grafted to a HH4$^-$ host for 3h and exchanged for lHM for 5h (total 8h): *Six1* was induced (5/8; lHM-only grafts: 0/5 in 5h, 4/4 in 8h) (G), but *Sox2* was not (0/11). **F, G.** Hypoblast was grafted to a HH4$^-$ host for 3h, then replaced by a node for a further 4h (total time 7h), *Sox2* was induced in 7/12 (c.f.: 0/5 in 4h node grafts). **H, I.** Hypoblast was grafted to HH4$^-$ for 3h then exchanged for lHM for a further 5h (total time 8h); *Six1* was induced (10/14; 5h lHM alone: 0/4), but *Sox2* was not (0/7). Insets: sections at the level of the graft; cyan arrows indicate expression in host ectoderm. Panels designated with a prime (B'-I') show the fluorescence emitted by the graft-derived GFP$^+$ cells. *Sox2* was not induced in host embryos when the lHM was removed after 3h and cultured for 4h (0/6); *Six1* was not induced in host embryos when the lHM was removed after 3h and cultured for 5h (0/5). Black arrows indicate switch-grafts. **J.** Summary of the main findings. Grafts of either the node, lHM or hypoblast induce a "common state" (or "pre-border state") in host epiblast in 3-4h. When this tissue is isolated in culture for 6 days, it differentiates into lens, neural and neural crest. Among the characteristic genes for the common/pre-border state are *Sox3* and *ERNI*. Epiblast isolated from pre-primitive streak stage embryos shares a genetic signature with this state. When further exposed to node or to lHM, cells in the pre-border state can be directed to differentiate into neural plate (by the node) and express *Sox2*/*Sox1*, or into placodes (by lHM), expressing *Six1*/*Eya2*.

we analyzed the expression of 27 transcription factors common to both screens by whole mount in situ hybridization. Twenty transcripts are readily detectable (Fig. S2). At primitive streak stages (HH4$^-$/4), all are broadly expressed in the epiblast encompassing the future neural plate and its border, containing placode and neural crest precursors (15). At head fold stages (HH6-7), when the neural plate is clearly defined and PPR markers begin to be expressed in the adjacent ectoderm, only two transcripts are restricted to the neural plate, while 16 continue to be expressed in both tissues. Strikingly, all transcripts are already expressed at pre-primitive streak stages (Fig. S2, EGK XII-XIII) similar to *Sox3* and *ERNI* (11), suggesting that the transcriptional profile initially induced in response to neural- and PPR-inducing signals is akin to that of the young epiblast prior to gastrulation.

Defining the transcriptional program for the "pre-neural state"

The above screens were performed in the extraembryonic epiblast of primitive streak stage embryos, which although competent to respond to neural inducing signals, normally never contributes to the central or peripheral nervous system. We therefore sought to define the transcriptional program for the normal pre-neural state using RNAseq. We reasoned that this program should comprise genes common to the pre-streak epiblast, the neural plate and the PPR, but exclude transcripts specific for the future epidermis, and largely contain *Sox3*-like genes. We performed RNAseq for medial and anterior pre-streak epiblast from EGK XII-XIII embryos, the HH6-7 neural plate and non-neural/non-placodal ectoderm as well as the anterior and posterior PPR from head fold stage embryos (HH6). Pair-wise comparisons (>1.5 fold-change) define genes enriched in each cell population (Dataset S1; all RNAseq results deposited in GEO under accession number GSE106346) with 505 transcripts being neural plate specific, 331 genes enriched in the aPPR and 266 transcripts in the pPPR (Fig. S3, Dataset S4).

To identify transcripts common to neural, anterior and posterior PPR, we compared each tissue to the non-neural/non-placodal ectoderm (fold-change cut-off of 1.5) (NNE in Fig. S4). 1128 transcripts are common to all three tissues but absent from the future epidermis (NNE); these common transcripts ("**N**eural - **A**nterior PPR – **P**osterior PPR": **NAP**; Fig. S4A-E) include 141 known or putative TFs (Fig. S4E). Anterior pre-streak epiblast specific mRNAs were subtracted from all pre-streak genes (Fig. S4F); the remaining transcripts were then intersected with genes induced in response to an organizer graft (see above; Fig. 1). This analysis reveals that the majority of genes induced by the node (1143/1167, including 155/158 TFs) are also expressed in the pre-streak epiblast (Fig. S4 H-I; "**P**re-**S**treak – **I**nduced": **PSI**). To identify *Sox3*-like transcripts as hallmark for the pre-neural state,

we intersected NAP and PSI genes (Fig. S4J-K). This uncovers 439 transcripts, all of which are also among the genes induced by the node (see Fig. 1, Dataset S1). We refer to this set of genes as "*Sox3*-like". These results support the hypothesis that, in response to neural and placode inducing signals, cells pass through a common, "pre-neural" state, which is similar to the epiblast prior to gastrulation.

Distinct regulatory modules define pre-neural, neural and PPR states

To uncover whether the common and divergent transcripts from the RNAseq datasets define distinct transcriptional states as cells transit from pre-neural to neural plate and PPR fate, we used an unbiased approach, GENIE3 (**Ge**ne **N**etwork **I**nference with **E**nsemble of trees) (17). Using a Random Forest machine-learning algorithm, GENIE3 infers a directed network that predicts functional links between genes. As input we used FPKM of all known and predicted transcription factors from each RNAseq dataset (FPKM≥10; 805 TFs), and generated a network in Cytoscape based on all interactions with an importance measure ≥0.005 (Fig. S5 A).

To identify modules within the network we organized its components according to their connectivity with each other using community clustering (18, 19). This approach generates three major clusters C1-C3 (Fig. S5 B-D, Fig. S6, Dataset S5): the majority of pre-neural (*Sox3*-like; P=1.23E-03 as assessed by hypergeometric testing) and PPR (*Six1*-like; P=4.59E-04) transcription factors are included in cluster C1 (Fig. S5 B), while cluster C3 (Fig. S5 D) is enriched for neural plate factors (*Sox2*-like; P=8.01E-09) and contains a few pre-neural genes. In contrast, cluster C2 does not show significant enrichment of any of the three TF classes (Fig. S5 C, Dataset S4). Only further sub-clustering segregates pre-neural (cluster C1A and C1B; P-values 5.3E-05 and 2.3E-04, respectively) and PPR genes (cluster C1C; P=1.02E-07) (Fig. S5 B). In conclusion, this analysis reveals that pre-neural and PPR genes are more interconnected with each other than with neural plate genes.

Early epiblast and newly-induced ectoderm have properties of the neural plate border

The above results uncover a set of *Sox3*-like, or "pre-neural" genes that are induced in peripheral epiblast within 3-5h in response to a graft of either Hensen's node (neural inducing) or the lHM (placode inducing). In normal embryos, these genes are initially expressed in a broad region of the pre-primitive-streak stage epiblast (Fig. S2). Could the pre-streak epiblast be specified as "pre-neural"? It was previously reported that when cultured in isolation, epiblast explants express neural plate border (20) or neural markers (21) (although the combination of *Sox2, Otx2* and *Pax6* expression reported in the latter study could also be interpreted as lens tissue).

To address the specification state of the early epiblast we cultured epiblast explants from pre-streak stage embryos and assessed the expression of markers for various ectodermal derivatives. We distinguished explants from the medial and lateral pre-streak epiblast, since they had previously been reported to have different properties (12, 21). After 40h culture (Fig. 2, Fig. S7), medial and lateral explants express the neural plate border markers *Dlx5*, *Gata3* and *ERNI*, the neural crest specifiers *Msx1* and *Pax7*, as well as the definitive neural crest marker *Snail2*, the pre-placodal markers *Six4* and *Eya2*, *Pax6*, which marks lens and olfactory progenitors, *Sox3*, expressed in both the neural plate and PPR, and the neural plate marker *Sox2* (Fig. 2, Fig. S7). In contrast, the "definitive" neural plate marker *Sox1*, mesodermal (*Tbx6*) and non-neural ectoderm transcripts (*Gata2*) are absent (Fig. 2, S7). No differences were seen between medial and lateral epiblast explants. In the developing embryo this combination of gene expression uniquely identifies the neural plate border, where precursors for the neural plate, neural crest and placodes overlap (22). These observations suggest that cells in the pre-neural epiblast at the pre-primitive streak stage are specified as neural plate border.

If this is indeed the case, after prolonged culture these explants should develop into lenses, the default fate of placode progenitors (23) as well as neural crest and central nervous system. Indeed, after 6 days a subset of pre-streak explants differentiates into lens-like structures expressing *Pax6*, *Mafa* and the lens differentiation marker δ-*crystallin* (Fig. 2 E,F,J,K), while another subset continues to express the neural crest cell marker *Snail2* (Fig. 2 T,U) and a third set of explants expresses the definitive neural marker *Sox1* (Fig. 2 Y,Z). In contrast, markers of other placodes like *Pax2* (otic) are never expressed (Fig. 2 AB-AE). Thus, like the PPR, the pre-streak epiblast has an autonomous tendency to develop into lens-like structures, while also generating other border derivatives like neural crest and neural plate cells. The presence of neural crest cells could account for why only a proportion of explants (about 1/3) express lens markers and also why this expression tends to be confined to a patch within the explants, since neural crest cells have been shown to inhibit lens development (23). Together these data suggest that prior to gastrulation, epiblast cells are specified as neural plate border. It is therefore more appropriate to refer to the "pre-neural" state as a "pre-border state" and we will use this designation henceforth.

Signals from the hypoblast induce the "pre-border state"

At pre-primitive-streak stages, before gastrulation, a layer of extraembryonic hypoblast cells underlies the epiblast (which forms the embryo proper). We have previously shown that grafts of the hypoblast transiently induce four "*Sox3*-like" genes: *ERNI*, *Sox3*, *Otx2* and *Cyp26A1* (24). Is the hypoblast sufficient to induce the full transcriptional profile of the "pre-border state"? To assess this we grafted hypoblast from pre-primitive streak stage donors into the peripheral area opaca of primitive streak stage hosts (HH4$^-$). After 5h the grafts were removed and the adjacent epiblast collected, along with the same tissue from the contralateral side. Transcriptional changes were assessed using NanoString. Many transcripts that are rapidly induced by the organizer and lateral head mesoderm are also significantly induced by the hypoblast (Fig. S8 and Dataset S3). These results suggest that the hypoblast may be responsible for priming the overlying epiblast by inducing a set of transcripts that characterizes a common "pre-border state".

Testing the hypothesis of a "common state"

If the "pre-border state" truly represents a common state that initiates the responses of epiblast to signals from different inducing tissues, it should be possible to replace any of these tissues by another for the early steps, the outcome of the induction being determined by the final inducing tissue. To test this, we first determined the minimum time required to induce placodal (*Six1*) and neural (*Sox2*) markers by the normal inducing tissue. We find that *Six1* induction by the lHM requires 8h (15), and induction of *Sox2* by Hensen's node requires 7h. Next, we grafted each tissue into the area opaca, replaced it after 3hours by the other tissue and cultured for a further 5h (for *Six1*) or 4h (for *Sox2*). We find that either tissue (lHM or node) can provide the initial signals, but it is the subsequent graft that determines whether PPR (*Six1*, induced by lHM) or neural plate (*Sox2*, induced by the node) is induced (Fig. 3 A-I).

Since the hypoblast also induces the pre-border genes, we tested whether 3h of hypoblast signaling is sufficient to prime cells to respond to neural and placode precursor inducing signals using the same experimental paradigm. The hypoblast alone does not induce neural or pre-placodal markers after any length of time. However, when 3h hypoblast signaling is followed by either a node (a further 4h) or the lHM (a further 5h), *Sox2* and *Six1* are induced, respectively. In the same experiment, replacing the

hypoblast with a node (5h) does not induce *Six1* and with the lHM (4h) does not induce *Sox2* (Fig. 3 A-I).

Together, our experiments indicate that the response of epiblast to induction by different signaling tissues first elicits a common set of responses irrespective of the inducing tissue, and that the final direction of differentiation is determined by the signals received in the following few hours. During normal development, the initial state characterizes the early (pre-primitive streak stage) epiblast, and may be induced by signals from the underlying hypoblast. These findings are summarized diagrammatically in Fig. 3J.

DISCUSSION

Our results lead us to propose that tissues (like Hensen's node and the lateral head mesoderm lHM) that induce diverse fates such as neural plate (CNS), neural crest and placodes (PNS) at the gastrula (primitive streak) stage initiate their action (around 3h) by eliciting a common, "pre-border" state, which only then diverges to follow distinct pathways depending on the inducing tissue. During normal development, this initial common state is displayed by the epiblast of the pre-primitive streak stage embryo and is also induced by the hypoblast (equivalent to the mouse anterior visceral endoderm) (25, 26). The specification state of this early epiblast is so similar to border/placodal precursors that when cultured in the absence of added signals, it will develop into a lens, the "default" state of pre-placodal cells (23).

The epiblast of early mouse embryos can generate stem cells ("embryonic stem cells", or ESCs) when cultured: the cells acquire the ability to self-renew *in vitro* and when allowed to differentiate, can give rise to all cell types (pluripotency). Could the "pre-border state" of pre-primitive streak chick epiblast and peripheral epiblast exposed to an inducing tissue for a short time be equivalent to pluripotency? To explore this we compared the genes in our PSI, *Sox3*-like, NP- and PPR-enriched TF lists to ESCAPE databases (27). This unbiased comparison shows that PSI and *Sox3*-like TFs are enriched for genes characteristic of mouse ESCs (embryonic stem line Bruce4 p13 and embryonic_stem_line_V26_2_p16), whereas TFs enriched in NP, PPR and NNE transcriptomes are not (Fig. S9). This suggests that the "pre-border state" shared by pre-primitive streak stage epiblast and later peripheral epiblast starting to respond to signals from different inducing tissues may resemble ESCs.

These conclusions support recent findings (28) that the animal pole of *Xenopus* embryos at the blastula stage is made up of cells that are "pluripotent" in that they not only contribute to neural crest and other neural border derivatives (see (20)) but can also give rise to mesoderm. Accordingly, the transcriptome of these animal pole cells includes genes normally associated with pluripotency, such as *PouV*, *Vent3*, *Sox3*, *Id3* and others (28). These findings are particularly interesting because to date it has not been possible to generate truly self-renewing pluripotent cells from any early amphibian or fish embryo. This is probably because the first 10 cell divisions of fish and amphibian blastomeres do not have G_1 or G_2 phases, there is no (or negligible) zygotic gene expression, and these cell divisions are not accompanied by cell growth (cells become smaller at each division): these properties do not allow true self-renewal (see (26) for discussion). Therefore, the common program shared by neural, neural crest and placodal lineages, manifested by the pre-gastrula stage ectoderm, precedes the evolutionary divergence of amniotes and anamniotes.

Experiments mainly in *Xenopus* suggested that the "default" state of cells in the animal pole of the blastula stage embryo is neural and that this is inhibited by endogenous BMP signals (29-32). However BMP inhibition alone is not sufficient to induce a neural fate either in chick epiblast or *Xenopus* ectodermal cells that lie remote from the neural plate (14, 20, 33). It can only expand the neural plate territory when BMP inhibited cells are in contact with the neural plate border. Our results, together with those of others (28) suggest that the "default" state of the early epiblast/ectoderm of both *Xenopus* and chick is in fact a "border-like state" and that explants of this tissue have a strong tendency to differentiate into neural crest and placode (lens) derivatives.

As ectodermal cells acquire neural identity they transit through successive states, characterized by specific sets of genes. The order in which these genes are induced by an organizer graft closely mimics the chronology of their expression during normal neural development (11, 16, 34-37). Likewise, a cascade of states has been suggested to accompany placodal induction (15), reminiscent of the successive states originally proposed by Jacobson to lead to lens and other inductions (38). Are the same signals responsible during normal development? When do these pathways diverge and what determines the outcome of inductive interactions? It was believed long ago that the specificity of inductive interactions depend largely on the responding tissue (6, 7), a view also supported by some grafting experiments, for example the finding that a graft of Hensen's node (Spemann organizer) or of the floor plate of the neural tube to the anterior limb bud can induce digit duplication (4, 39). Our results suggest that at least for early-occurring inductive interactions, the specificity of the induction caused in one particular tissue is determined at least in part by the inducing tissue: although an initial response is the same, the inducing tissue then directs the subsequent and final outcome. A likely initial signal is a member of the FGF family or other ligands that activate this pathway (2, 9, 24, 40), but the appropriate signaling tissues must also produce specific signals to account for the divergence into distinct pathways and fates that follow the initial, common state. A challenge for the future is to determine what these signals are, and how they are interpreted by responding cells.

ABBREVIATED Materials and Methods

Detailed Materials and Methods are described in Supplementary Information.

Hens' eggs were incubated and staged according to Hamburger and Hamilton (HH) (41) or Eyal-Giladi and Kochav (EGK) (42) for pre-primitive streak (pre-streak) stages, and cultured using a modified New culture method (43, 44). Induction assays were conducted as previously described. , Hensen's node was isolated from HH4$^-$ chick embryos (45) and hypoblast from EGK XII-XIII embryos (24). The lateral head mesoderm (lHM) at this stage corresponds to the lateral-most part of the head mesoderm, whose fate is to contribute to the heart – it does not include paraxial head mesoderm (see (13, 15) for details). Grafts were placed into the inner lateral area opaca of HH4- stage hosts (46, 47). In situ hybridization was performed as previously described (48, 49). Explant cultures were set up as described (20, 23).

Transcriptional responses to induction were assessed by RNA sequencing and using the NanoString nCounter® analysis system using a custom made probe set of 386 genes (Dataset S2). Experiments were performed in triplicate. All transcription factors from RNAseq with FPKM>10 were used for predicting gene regulatory interactions using Genie3 (17). Interactions with IM>0.005 were extracted and visualized with Cytoscape 3.2.0.

Acknowledgements We are grateful to Nidia De Oliveira, Rosalinda Guerra and Ewa Kolano for expert technical assistance, to Grace Lu for additional help with bioinformatics, and other members of the Stern and Streit groups for discussion. This study was funded by project grant BB/K007742/1 from the BBSRC to CDS and AS, NIH R01-DC011577 and R01-DE022065 to AS, Action on Hearing Loss 513:KCL:AS to AS, BBSRC and MRC studentships for KET and MJS respectively, Diana Trebble Studentship from the KCL Dental Institute for MH and ERC Advanced grant "GEMELLI" to CDS.

Author contributions KET and NM performed RNAseq for node induction and pre-streak embryos; KET and MT carried out in situ hybridization; KET and MH performed node and lHM grafts, NanoString experiments and analysis; MAFK and RSP analyzed the RNAseq data; RSP performed network

inference analysis; MH performed graft-switch experiments; MJS performed most explant experiments; ACS performed hypoblast graft experiment; MT and RR performed RNAseq for PPR and NNE, AS performed *Sox1* explant experiments, CDS and AS conceived the project, contributed to data interpretation and wrote the manuscript.

1. Spemann H & Mangold H (1924) Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Roux' Arch EntwMech Org* 100:599-638.
2. Stern CD (2005) Neural induction: old problem, new findings, yet more questions. *Development* 132(9):2007-2021.
3. Saunders JW, Jr., Gasseling MT, & Cairns JM (1959) The differentiation of prospective thigh mesoderm grafted beneath the apical ectodermal ridge of the wing bud in the chick embryo. *Dev Biol* 1:281-301.
4. Hornbruch A & Wolpert L (1986) Positional signalling by Hensen's node when grafted to the chick limb bud. *J Embryol Exp Morphol* 94:257-265.
5. Anderson C & Stern CD (2016) Organizers in Development. *Curr Top Dev Biol* 117:435-454.
6. Holtfreter J (1938) Differenzierungspotenzen isolierter Teile der Urodelengastrula. *W. Roux Arch. Entwicklungsmech. Org.* 138:522-652.
7. Waddington CH (1940) *Organisers and Genes* (Cambridge University Press, London).
8. Bailey AP & Streit A (2006) Sensory organs: making and breaking the pre-placodal region. *Curr Top Dev Biol* 72:167-204.
9. Streit A (2004) Early development of the cranial sensory nervous system: from a common field to individual placodes. *Dev Biol* 276(1):1-15.
10. Bhattacharyya S, Bailey AP, Bronner-Fraser M, & Streit A (2004) Segregation of lens and olfactory precursors from a common territory: cell sorting and reciprocity of Dlx5 and Pax6 expression. *Dev Biol* 271(2):403-414.
11. Streit A, Berliner AJ, Papanayotou C, Sirulnik A, & Stern CD (2000) Initiation of neural induction by FGF signalling before gastrulation. *Nature* 406(6791):74-78.
12. Wilson SI, *et al.* (2001) The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. *Nature* 411(6835):325-330.
13. Litsiou A, Hanson S, & Streit A (2005) A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. *Development* 132(18):4051-4062.
14. Linker C & Stern CD (2004) Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. *Development* 131(22):5671-5681.
15. Hintze M, *et al.* (2017) Cell interactions, signals and transcriptional hierarchy governing placode progenitor induction. *Development* 144:2810-2823.
16. Pinho S, *et al.* (2011) Distinct steps of neural induction revealed by Asterix, Obelix and TrkC, genes induced by different signals from the organizer. *PLoS One* 6(4):e19157.
17. Huynh-Thu VA, Irrthum A, Wehenkel L, & Geurts P (2010) Inferring regulatory networks from expression data using tree-based methods. *PLoS One* 5(9): e12776.
18. Girvan M & Newman ME (2002) Community structure in social and biological networks. *Proc Natl Acad Sci U S A* 99(12):7821-7826.
19. Su G, Kuchinsky A, Morris JH, States DJ, & Meng F (2010) GLay: community structure analysis of biological networks. *Bioinformatics* 26(24):3135-3137.
20. Linker C, *et al.* (2009) Cell communication with the neural plate is required for induction of neural markers by BMP inhibition: evidence for homeogenetic induction and implications for Xenopus animal cap and chick explant assays. *Dev Biol* 327(2):478-486.
21. Wilson SI, Graziano E, Harland R, Jessell TM, & Edlund T (2000) An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. *Curr Biol* 10(8):421-429.
22. Grocott T, Tambalo M, & Streit A (2012) The peripheral sensory nervous system in the vertebrate head: a gene regulatory perspective. *Dev Biol* 370(1):3-23.
23. Bailey AP, Bhattacharyya S, Bronner-Fraser M, & Streit A (2006) Lens specification is the ground state of all sensory placodes, from which FGF promotes olfactory identity. *Dev Cell* 11(4):505-517.
24. Albazerchi A & Stern CD (2007) A role for the hypoblast (AVE) in the initiation of neural induction, independent of its ability to position the primitive streak. *Dev Biol* 301(2):489-503.
25. Foley AC, Skromne I, & Stern CD (2000) Reconciling different models of forebrain induction and patterning: a dual role for the hypoblast. *Development* 127(17):3839-3854.
26. Stern CD & Downs KM (2012) The hypoblast (visceral endoderm): an evo-devo perspective. *Development* 139(6):1059-1069.
27. Xu H, *et al.* (2013) ESCAPE: database for integrating high-content published data collected from human and mouse embryonic stem cells. *Database (Oxford)* 2013:bat045.
28. Buitrago-Delgado E, Nordin K, Rao A, Geary L, & LaBonne C (2015) Shared regulatory programs suggest retention of blastula-stage potential in neural crest cells. *Science* 348(6241):1332-1335.
29. Hemmati-Brivanlou A & Melton D (1997) Vertebrate neural induction. *Annu Rev Neurosci* 20:43-60.
30. Sasai Y, Lu B, Steinbeisser H, & De Robertis EM (1995) Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. *Nature* 376(6538):333-336.
31. Zimmerman LB, De Jesus-Escobar JM, & Harland RM (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86(4):599-606.
32. Wilson PA & Hemmati-Brivanlou A (1995) Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* 376(6538):331-333.
33. Streit A, *et al.* (1998) Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. *Development* 125(3):507-519.
34. Papanayotou C, *et al.* (2013) Calfacilitin is a calcium channel modulator essential for initiation of neural plate development. *Nat Commun* 4:1837.
35. Papanayotou C, *et al.* (2008) A mechanism regulating the onset of Sox2 expression in the embryonic neural plate. *PLoS Biol* 6(1):e2.
36. Sheng G, dos Reis M, & Stern CD (2003) Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. *Cell* 115(5):603-613.
37. Gibson A, Robinson N, Streit A, Sheng G, & Stern CD (2011) Regulation of programmed cell death during neural induction in the chick embryo. *Int J Dev Biol* 55(1):33-43.
38. Jacobson AG (1966) Inductive processes in embryonic development. *Science* 152(3718):25-34.
39. Wagner M, Thaller C, Jessell T, & Eichele G (1990) Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* 345(6278):819-822.
40. Streit A & Stern CD (1999) Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity. *Mech Dev* 82(1-2):51-66.
41. Hamburger V & Hamilton HL (1951) A series of normal stages in the development of the chick embryo. *Journal of Morphology* 88(1):49-92.
42. Eyal-Giladi H & Kochav S (1976) From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol* 49(2):321-337.
43. New DAT (1955) A new technique for the cultivation of the chick embryo in vitro. *J. Embryol. Exp. Morphol.* 3:326-331.
44. Stern CD & Ireland GW (1981) An integrated experimental study of endoderm formation in avian embryos. *Anat. Embryol* 163(3):245-263.
45. Storey KG, Crossley JM, De Robertis EM, Norris WE, & Stern CD (1992) Neural induction and regionalisation in the chick embryo. *Development* 114(3):729-741.
46. Stern CD (2008) Grafting Hensen's node. *Methods Mol. Biol* 461:265-276.
47. Streit A & Stern CD (2008) Operations on primitive streak stage avian embryos. *Methods Cell Biol* 87:3-17.
48. Stern CD (1998) Detection of multiple gene products simultaneously by in situ hybridization and immunohistochemistry in whole mounts of avian embryos. *Curr. Top. Dev. Biol* 36:223-243.
49. Streit A & Stern CD (2001) Combined whole-mount in situ hybridization and immunohistochemistry in avian embryos. *Methods* 23(4):339-344.