Distinct modes of floor plate induction in the chick embryo

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Summary

To begin to reconcile models of floor plate formation in the vertebrate neural tube, we have performed experiments aimed at understanding the development of the early floor plate in the chick embryo. Using real-time analyses of cell behaviour, we provide evidence that the principal contributor to the early neural midline, the future anterior floor plate, exists as a separate population of floor plate precursor cells in the epiblast of the gastrula stage embryo, and does not share a lineage with axial mesoderm. Analysis of the tissue interactions associated with differentiation of these cells to a floor plate fate reveals a role for the nascent prechordal mesoderm, indicating that more than one inductive event is associated with floor plate formation along the length of the neuraxis. We show that *Nr1*, a chick *nodal* homologue, is expressed in the nascent prechordal mesoderm and we provide evidence that Nodal signalling can cooperate with Shh to induce the epiblast precursors to a floor-plate fate. These results indicate that a shared lineage with axial mesoderm cells is not a pre-requisite for floor plate differentiation and suggest parallels between the development of the floor plate in amniote and anamniote embryos.

Movies available online

Key words: Floor plate, Prechordal mesoderm, Chick, Nodal, Sonic hedgehog

Introduction

The floor plate of the vertebrate embryo develops at the ventral midline of the neural tube. It acts as an embryonic organiser and plays an essential role both in the generation of specific neuronal subtypes along the dorsoventral axis of the brain and spinal cord and in the guidance of axons (Giger and Kolodkin, 2001; Jessell, 2000).

Although the role of the floor plate in patterning the neural tube is well accepted, its ontogeny has been a controversial subject (Le Douarin and Halpern, 2000; Placzek et al., 2000). The prevailing view, for which there is extensive evidence in the chick, is that axial mesodermal notochord cells are the source of an instructive inducing signal that mediates floor plate differentiation in medial cells of the overlying neural plate (Jessell, 2000; Placzek et al., 2000). A large body of evidence, moreover, suggests that the secreted signalling molecule Sonic hedgehog (Shh) mediates the ability of notochord to induce floor plate differentiation. Shh is expressed in the node and the notochord prior to floor-plate differentiation. Gain-of-function experiments show that Shh can induce the ectopic differentiation of floor-plate cells in the neural plate in vitro (Marti et al., 1995; Roelink et al., 1994), while blockade of Shh in the notochord eliminates its ability to induce floor plate cells (Ericson et al., 1996). In support of studies in the chick, mutations in the Shh gene, and in components of the Shh signalling pathway, in mouse, block ventral midline differentiation (Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998; Wijgerde et al., 2002).

Several recent studies, however, have questioned this model of floor plate induction. In particular, analysis of chick-quail chimaeras in which quail cells from the chordoneural hinge (CNH), a derivative of the Node, are grafted into chick embryos, have led to the proposal that medial floor plate cells are derived from a population of precursors that are initially situated in the Node, can segregate into either notochord or floor plate, and are already pre-specified within this region (Le Douarin and Halpern, 2000; Teillet et al., 1998). In this model, floor-plate cells thus derive from pre-specified cells that intercalate from the node into the neural midline.

A further challenge to the paradigm of notochord/Shh-mediated floor plate induction arises through observations of zebrafish embryos. Floor-plate cells persist in embryos in which notochord precursors are surgically ablated, demonstrating that a normally developed notochord is not a pre-requisite for floor plate differentiation in this species (Shih and Fraser, 1995). Analyses of zebrafish mutant embryos further supports this contention. Mutations in both *no tail* (*ntl*) and *floating head* (*flh*) affect notochord formation (Amacher and Kimmel, 1998; Halpern et al., 1993; Schulte Merker et al., 1994; Talbot et al., 1995). Despite this, in *ntl* mutant embryos the most medial set of floor plate cells are present,

and even expanded (Halpern et al., 1997; Odenthal et al., 1996; Odenthal et al., 2000). *flh* mutants likewise contain patches of cells at the ventral midline that express medial floor-plate markers (Halpern et al., 1995; Halpern et al., 1997; Schier et al., 1997). Moreover, while there is compelling evidence for a requirement for Shh signalling in the induction of floor plate character in amniotes, in zebrafish embryos Hh signalling appears crucial to the induction of lateral floor plate cells, but is not required for the differentiation of medial floor-plate cells (Chen et al., 2001; Etheridge et al., 2001; Odenthal et al., 2000; Schauerte et al., 1998; Varga et al., 2001). Instead, the TGFβ superfamily member Nodal appears essential for medial floor plate induction. Mutations in the zebrafish nodal-related gene *cyclops* (*ndr2*) and *one-eyed pinhead* (*oep*), an obligate co-factor for Nodal signal transduction (Gritsman et al., 1999) both cause loss of the medial floor plate throughout the length of the neural tube (Hatta, 1992; Hatta et al., 1991; Krauss et al., 1993; Rebagliati et al., 1998; Sampath et al., 1998; Schier et al., 1997; Shinya et al., 1999; Strahle et al., 1997; Zhang et al., 1998). Intriguingly, while analysis of the requirement for Nodal signalling in zebrafish has suggested that medial floor plate specification occurs early in development and within the organiser region, evidence from the analysis of *oep* mutants suggests that it is nevertheless the result of an inductive interaction (Gritsman et al., 1999; Strahle et al., 1997).

These extensive analyses of floor plate development appear to point towards very disparate models of floor plate formation in distinct species. However, a caveat, and possible explanation for the varied conclusions of these studies is their failure to analyse floor plate development at equivalent stages of embryogenesis. To perform a more direct comparative analysis, we have examined the differentiation of floor-plate cells in the early chick embryo, over the period of gastrulation/early neural plate formation. Our studies show that many medial floor plate cells that form at this time do not derive from Hensen's node itself. Instead, they derive from a region of the prenodal epiblast that lies anterior to Hensen's node, previously shown to contribute to the floor plate, and designated 'area a' (Garcia-Martinez et al., 1993; Schoenwolf et al., 1989; Schoenwolf and Sheard, 1990). Real-time lineage analyses reveal that 'area a'-derived cells exist as a separate population of floor-plate precursors and do not contribute progeny to Hensen's node, arguing that the shared origins with notochord cells are not a requirement for floor-plate development along the length of the neuraxis. Our studies show that 'area a' floor-plate precursors are induced by rapid signalling events mediated by the early forming prechordal mesendoderm. Together, our evidence shows that floor-plate cells along the neuraxis are induced to differentiate and argues against a requirement for pre-specification within the organiser.

In addition, we observe that *Shh* and *Nr1* are co-expressed in the nascent prechordal mesoderm at the time of 'area a' differentiation, suggesting that Nodal signalling may play a role in amniote floor plate induction. In support of this, we find that Nodal and Shh can co-operate to induce floor plate character in 'area a' cells in vitro. The data presented in this study thus indicate that different signalling events mediate early and late floor plate induction in the chick and support the development of an integrated model of floor plate differentiation in both amniote and anamniote embryos.

Materials and methods

Cell lineage analysis

Focal injections of fluorescent lipophilic dyes designed to label fewer than 50 cells were made by controlled pressure injection into live embryos either in ovo or in New culture. During New culture, embryos were explanted into L15 medium at HH (Hamburger and Hamilton, 1951) stage 4 and injected with a solution of DiI and/or DiD, 5 mg/ml in 100% ethanol (Molecular Probes). Focal injections were made using a picospritzer II microinjection system (General Valve). Following dye injection, embryos were replaced onto their vitelline membrane and prepared for New culture according to established techniques (New, 1955; Stern and Ireland, 1981).

For time-lapse analysis of cell movement, embryos were then cultured in plastic culture dishes over thin albumen in a culture dish in which the central plastic area had been replaced with a thin glass coverslip to facilitate visualisation. Embryos were visualised using an inverted confocal microscope as previously described (Kulesa and Fraser, 1998). The microscope was surrounded with an insulating chamber maintained at 38°C for the duration of the time-lapse experiment. Single confocal images were taken at 5 or 10 minute intervals for the duration of the analysis.

Tissue dissection and explant culture

All embryos were staged and dissected in cold L15 medium (Gibco-BRL). 'Area a' explants were prepared from HH stage 4 embryos by making two parallel cuts either side of and anterior to Hensen's node, followed by two cuts at right angles to remove a square of tissue from the region anterior to Hensen's node. The epiblast layer was then isolated from underlying tissue with dispase (1 mg/ml). Explants of 'area a'-derived tissue at HH stages 4+, 5 and 6 were isolated by taking an equivalent area of tissue just anterior to Hensen's node. In all cases, explant culture was performed in collagen gels according to published techniques (Placzek and Dale, 1999).

Nodal protein, produced by transient transfection of 293T cells with pcDNA3-mNodal (containing the coding sequence for mouse Nodal) was concentrated tenfold using Centri-plus columns (Amicon) and then diluted 1:10 in explant culture medium. Human Shh-N protein (Biogen) was added to the tissue culture medium at the concentrations indicated.

For tissue recombination experiments, HH stage 4+ prechordal mesendoderm was identified by morphology. Explants were prepared by making cuts either side of Hensen's node and at the anterior and posterior limits of the prechordal mesendoderm using sharpened tungsten needles prior to separation of the tissues using 1 mg/ml dispase. Intermediate neural plate tissue from E9.5 rat embryos was isolated as previously described (Placzek et al., 1993). Prechordal mesendoderm was placed in contact with either 'area a' or rat neural plate explants in collagen gels and cultured as previously described (Placzek and Dale, 1999).

In vivo grafting of notochord and prechordal mesoderm

In vivo grafting experiments were performed as previously described (Placzek et al., 1990). Briefly, a small incision was made between the open neural groove and adjacent presomitic mesoderm in the caudal region of HH stage 10 chick embryos in ovo. Explants of notochord taken from the caudal region of HH stage 10 embryos or nascent prechordal mesendoderm from HH stage 4+ embryos were inserted into the incision adjacent to the neural plate at an intermediate position, in between basal and alar plates. After operations were performed eggs were resealed and incubated until HH stage 19-21 prior to fixation and analysis by immunohistochemistry.

Prechordal mesoderm ablations

HH stage 4, 4+ or 5- embryos were prepared for New culture, leaving the ventral surface of the embryo exposed. Removal of the prechordal mesendoderm was performed by making a shallow cut through the

endodermal and mesodermal layers just anterior to Hensen's node and then scraping away the mesendoderm anterior to it. Following operation, embryos were prepared for New culture as described (New, 1955; Stern and Ireland, 1981) and allowed to develop prior to fixation and further analysis.

Immunohistochemistry

Embryos and explants were analysed by immunohistochemistry according to standard techniques (Placzek et al., 1993). The following antibodies were used (dilutions in parentheses): 68.5E1, anti-Shh mAb (1:50) (Ericson et al., 1996); 4C7, anti-HNF3β mAb (1:40) (Ruiz i Altaba et al., 1995); anti-Sox2 pAb (1:500) (Pevny et al., 1998); anti-Lim1/2 (1:50); and anti-Not 1 (1:50). Appropriate secondary antibodies (Jackson Immunoresearch) were conjugated to Cy3.

In situ hybridisation

Embryos and explants were processed for in situ hybridisation as described previously (Vesque et al., 2000). The following template DNAs were to generate a digoxigenin labelled antisense RNA probes: plasmid pCM21 containing a cDNA encoding chick Netrin 1 was linearised with *Eco*RI and transcribed with T7 polymerase; plasmid pcvhh containing a cDNA encoding chick sonic hedgehog was linearised with *Sal*I and transcribed with SP6 polymerase; plasmid pcp7 containing a cDNA encoding HNF3β was linearised with *Hin*dIII and transcribed with SP6 polymerase; plasmid pcGsc containing a cDNA encoding chick Goosecoid was linearised with *Eco*RI and transcribed with SP6 polymerase (Vesque et al., 2000).

Results

'Area a' cells populate the midline of the developing neural tube

In vivo time-lapse confocal microscopy was performed to analyse the migration of 'area a' epiblast cells during gastrulation over the period HH stages 4 to 8. Focal injections of DiI were made into HH stage 4 embryos. Two sites were labelled; 'area a', just anterior to Hensen's node and as a reference point, a region of midline epiblast ~ 200μm more anteriorly (Fig. 1A,C; see Movie 1 at http://dev.biologists.org/supplemental/).

At the beginning of analysis, a few cells from 'area a' had already moved away from their original position (Fig. 1C, white arrowhead). Over the next 9 hours, many 'area a' cells moved posteriorly, along the axis of the embryo (Fig. 1D-J), and a smaller number of cells moved anteriorly (Fig. 1B-G, arrow in Fig. 1B). Very occasionally, cells moved laterally but with time moved back towards the midline to join the main, axial stream of cells (arrows, Fig. 1C-F). During their migration, 'area a'-derived cells extended long filamentous processes polarised in the direction of their movement (arrowhead in Fig. 1B).

To confirm that 'area a' cells colonise the midline, we examined whether 'area a'-derived cells express the ventral midline cell marker Shh. DiI was injected into 'area a' cells in vivo at HH stage 4, and embryos developed in ovo until HH stage 8. Examination of sections confirmed that DiI-labelled cells were confined to the ventral midline floor plate and revealed that they populated medial-most floor plate cells (Fig. 1K-M). 'Area a'-labelled cells were never detected within axial mesoderm. These analyses also indicated that 'area a' cells contributed largely to anterior ventral midline regions extending from the diencephalon, through the midbrain and hindbrain, with only sporadic labelling detected more posteriorly. Labelled cells were never detected anteriorly within the telencephalon (n=10).

These results demonstrate that cells from the midline, pre-nodal region of the chick epiblast rapidly populate the midline of the developing neural tube during gastrulation, extending long cellular processes as they migrate. In addition, they demonstrate that 'area a'-derived cells largely populate medial-most ventral midline cells that form in the anterior neural tube.

'Area a'- and Hensen's node-derived cells do not mix during early floor plate formation

We next addressed whether, in addition to populating the ventral midline, 'area a' cells contribute to Hensen's node to form a population of floor plate precursors within this structure.

Focal injections of DiI and DiD were made into the epiblast layer at HH stage 4 (n=5), and the embryos were followed for a 4 hour period, until they reached the equivalent of HH stage 6 (Fig. 2; see Movie 2 at http://dev.biologists.org/supplemental/). DiI was used to label cells in 'area a' (red labelling in Fig. 2). DiD was used to label cells in the superficial, epiblast layer of Hensen's node (blue labelling in Fig. 2); this also served to mark the position of Hensen's node. During time-lapse confocal imaging analyses, DiI-labelled cells from 'area a' were again observed to rapidly move both anteriorly and posteriorly to populate the midline of the embryo (Fig. 2B-H). However, at no point in this analysis were cells from 'area a' observed to colonise Hensen's node itself.

During the 4 hour period, Hensen's node regressed posteriorly (blue labelling in Fig. 2B-H). Simultaneously, a stream of Hensen's node-derived cells moved first laterally and then anteriorly to populate the midline. The rate at which this occurred was visibly greater than the rate of node regression, suggesting not only a depositing of cells by Hensen's node, but an active anterior movement of some of these node-derived cells. Focussing through the embryos during time-lapse analysis indicated that such DiD labelled cells populated only the mesodermal layer. To confirm this, single focal injections of DiI were made into the epiblast layer of Hensen's node at HH stage 4, and the embryos analysed at HH stage 6. Sectioning revealed that epiblast cells that leave Hensen's node over the period HH stage 4-6 populate the forming axial mesendoderm, and not the superficial neural layer (Fig. 2I; n=5). By contrast, when identically labelled embryos were allowed to develop beyond HH stage 6, labelled cells were detected in both notochord and floor plate (Fig. 2I, inset; n=3).

Together, these analyses indicate firstly that 'area a'-derived cells remain separate from Hensen's node as neurulation proceeds and do not contribute at this time to any population of floor plate precursors residing in Hensen's node. Second, it suggests that the principal contributor to the earliest forming, and most anterior ventral midline is 'area a' and not Hensen's node, with Node-derived cells only contributing to the later forming floor plate.

'Area a' cells become progressively specified as floor plate following axial mesoderm formation

The majority of studies in amniotes showing the induction of floor plate by notochord have examined the differentiation of floor-plate cells that form in thoracic regions of the neuraxis (Artinger and Bronner-Fraser, 1993; Placzek et al., 2000; van

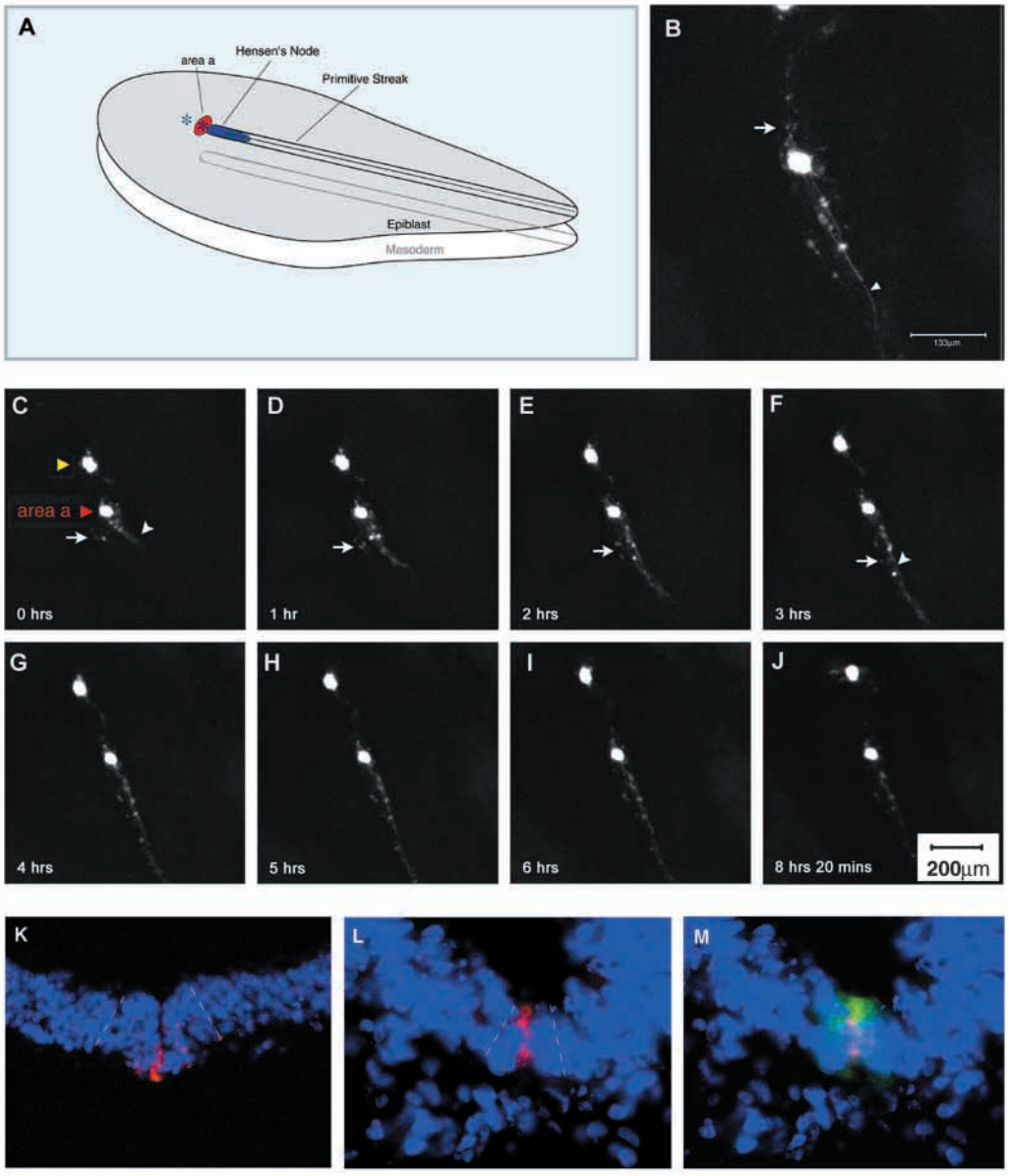

Fig. 1. 'Area a' cells populate the medial ventral midline. (A) Schematic of a HH stage 4 embryo, showing injection sites (asterisks) into 'area a' and a more anterior reference site. (B) High-magnification images of DiI-labelled cells taken after 8 hours 20 minutes. Midline cells extend long, polarised cell processes, in excess of 100 μm in length (arrowhead) as they colonise the ventral midline. Arrow indicates DiI-labelled cells that have migrated anteriorly. (C-J) Still images from time-lapse, confocal analysis. Each image represents a single confocal section of DiI-labelled cells (arrowhead in C shows migrating cells; arrows in C-J show laterally moving cells migrating back into midline). 'Area a' and anterior injection sites are indicated by red and yellow arrowheads, respectively (0 hours). The axis of the embryo is orientated such that the primitive streak lies towards the bottom right-hand corner of each panel. (K,L) Transverse sections of an 'area-a'-injected embryo, analysed at HH stage 8. DiI labelling (red) is detected in ventral midline cells of the midbrain (K) and hindbrain (L). (M) Immunolabelling of the section shown in L with anti-Shh antibody (green) reveals that DiI-labelled cells populate the medial-most part of the ventral midline. Broken lines indicate outline of Shh-expressing floor plate, as determined by analyses such as shown in M.

Straaten and Hekking, 1991; Yamada et al., 1991) and have not examined the induction of the anterior, 'area a'-derived population. We therefore next addressed the mechanisms by which 'area a'-derived cells differentiate into floor plate and examined whether these differ from those which generate floor plate in the posterior neural tube.

We first addressed the state of specification of 'area a'-derived cells as development proceeds. Explants of 'area a' (Fig. 3A), or its derivatives, were cultured and examined for expression of markers of floor plate and neural character. Despite being adjacent to Shh-expressing cells in Hensen's node (Fig. 3A, arrowhead), cells explanted from 'area a' at HH stage 4 do not express the floor-plate markers *Shh*, *Hnf3b*/*Foxa2* or *Netrin1* (Fig. 3B-D), indicating that at this stage they are not specified to become floor plate. Expression of floor plate markers was not observed at any time point analysed (20, 24, 36, 40 hours; $n>50$). Analysis of Sox2, a marker of undifferentiated neuroepithelial cells (Streit et al., 1997) indicates that 'area a' cells are specified as neural at the time of isolation (Fig. 3E). Thus, prior to the overt formation of axial mesoderm, floor plate precursors in 'area a' exist as committed neural precursors.

After HH stage 4, however, increasing numbers of 'area a'-derived cells express ventral midline characteristics. Explants isolated at HH stage 4+ contain a small number of cells which appear to co-express *Hnf3b*/*Foxa2* and *Shh* (20-40% of cells in all explants analysed, $n=10$; Fig. 3F,G). By HH stage 5 the proportion of cells in each explant co-expressing *Hnf3b*/*Foxa2* and *Shh* has increased to between 50 and 70% ($n=10$; Fig. 3H,I), and by HH stage 6 to between 60 and 90% ($n=10$; Fig. 3J,K). These cells do not express Lim1/2, *brachyury* or 3B9 (not shown), markers that label axial mesodermal cells, ruling out the possibility that 'area a' cells have differentiated to an axial mesoderm fate, and indicating instead that they are specified to ventral midline floor plate cells. Thus, over a timespan of less than 4 hours, the majority of 'area a'-derived cells become specified as floor plate.

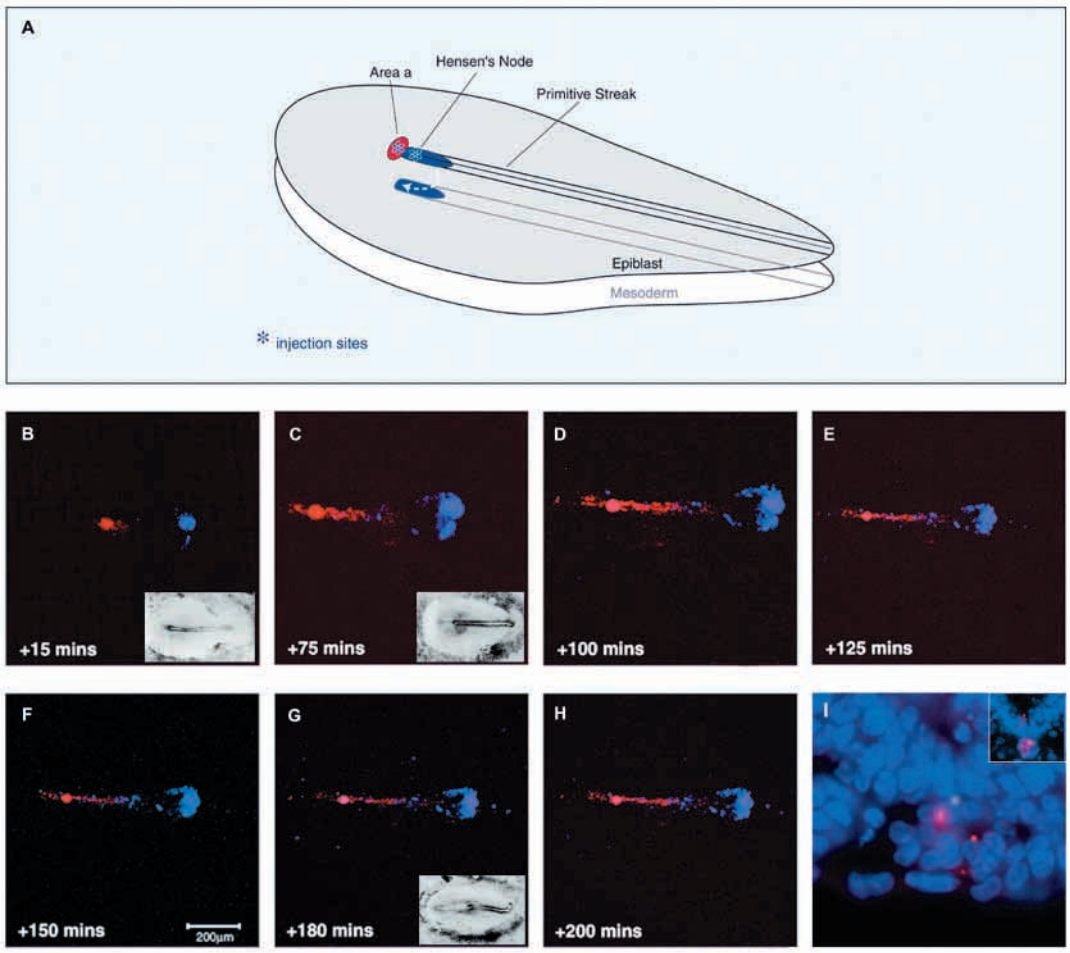

Fig. 2. 'Area a'- and Hensen's node-derived cells do not intermingle during gastrulation. (A) Schematic of a HH stage 4 embryo, showing injection sites (asterisks) into 'area a' (red) and Hensen's node (blue). Broken white arrow indicates the movement of Hensen's node cells into the deep mesodermal layer during axial mesoderm formation. (B-H) Still images from in vivo, time-lapse, confocal analysis of cell movements from 'area a' (red, DiI-labelled cells) and Hensen's node (blue, DiD-labelled cells) during ventral midline formation. All images are single confocal sections, orientated with anterior towards the left and posterior towards the right. (Insets) Embryonic stage of development after 15 minutes (HH st4+), 75 minutes (HH st5) and 180 minutes (HH stage 6). (I) Transverse section through a HH stage 6 embryo, after labelling Hensen's node epiblast cells at HH stage 4. DiI-filled cells label the notochord exclusively (red; blue shows DAPI labelling). (Inset) Transverse section through a HH stage 8 embryo, after labelling Hensen's node epiblast cells at HH stage 4. DiI-filled cells label notochord and floor plate. Scale bars: 133 μm in C,D; 200 μm in B,E-H.

Emerging mesendoderm rapidly induces 'area a' cells to a floor-plate fate

The specification of 'area a' cells to a floor-plate identity coincides with the onset of axial mesoderm formation, raising the possibility that the first emerging axial mesoderm cells are responsible for inducing 'area a' cells to a floor-plate fate. To test this, we removed the earliest forming axial mesendoderm, together with the deep layers of Hensen's node at HH stage 4 (Fig. 4A,B; *n*=8). Previous studies have indicated that these layers contribute to axial mesoderm and not to neural tissue (Selleck and Stern, 1991).

After 18 hours in culture, operated embryos appeared to be morphologically normal, although the anterior neuropore was rather pronounced and development was retarded, embryos only reaching HH stage 6 to 7 (Fig. 4D). In situ hybridisation revealed that expression of the early floor-plate markers *Hnf3b*/*Foxa2* and *Shh* could not be detected (Fig. 4D and not shown). Analysis of sectioned embryos revealed that the two halves of the neuroepithelium were conjoined (Fig. 4G, arrow). Sectioning confirmed that both prechordal mesoderm and notochord were absent in operated embryos (Fig. 4G arrowhead) and revealed that *Shh* expression was almost completely absent throughout the neuroepithelium (Fig. 4G, arrow), weak expression being detected on only individual cells on 2% of sections (not shown). Together, this analysis shows that ablation of the emerging mesendoderm leads to the lack of formation of axial mesoderm and the concomitant loss of floor-plate differentiation in 'area a'-derived cells.

We next determined whether 'area a'-derived floor plate cells require a prolonged period of contact with early emerging mesendoderm for their differentiation, by performing ablations at HH stage 4+. Embryos could be staged precisely, as when endoderm was removed at HH stage 4+, a fan of axial mesendoderm could be seen extending from Hensen's node (Fig. 4C, white arrowhead). After removal of this mesendoderm, and culture for 18 hours, embryos again appeared morphologically normal, although development was retarded to HH stage 7-8 (Fig. 4F; *n*=5). In situ analysis revealed that *Shh* was expressed within anterior ventral midline cells (Fig. 4F,I), although far fewer *Shh*-expressing cells were detected in the neural midline than were present in control embryo, with expression of *Shh* on these cells much weaker than on control floor-plate cells (compare Fig. 4F,I with Fig. 4E,H).

Together these analyses suggest that the early emerging mesendoderm is crucial for the normal differentiation of the anterior floor plate. In addition, they suggest that a short exposure to this mesendodermal population is sufficient to

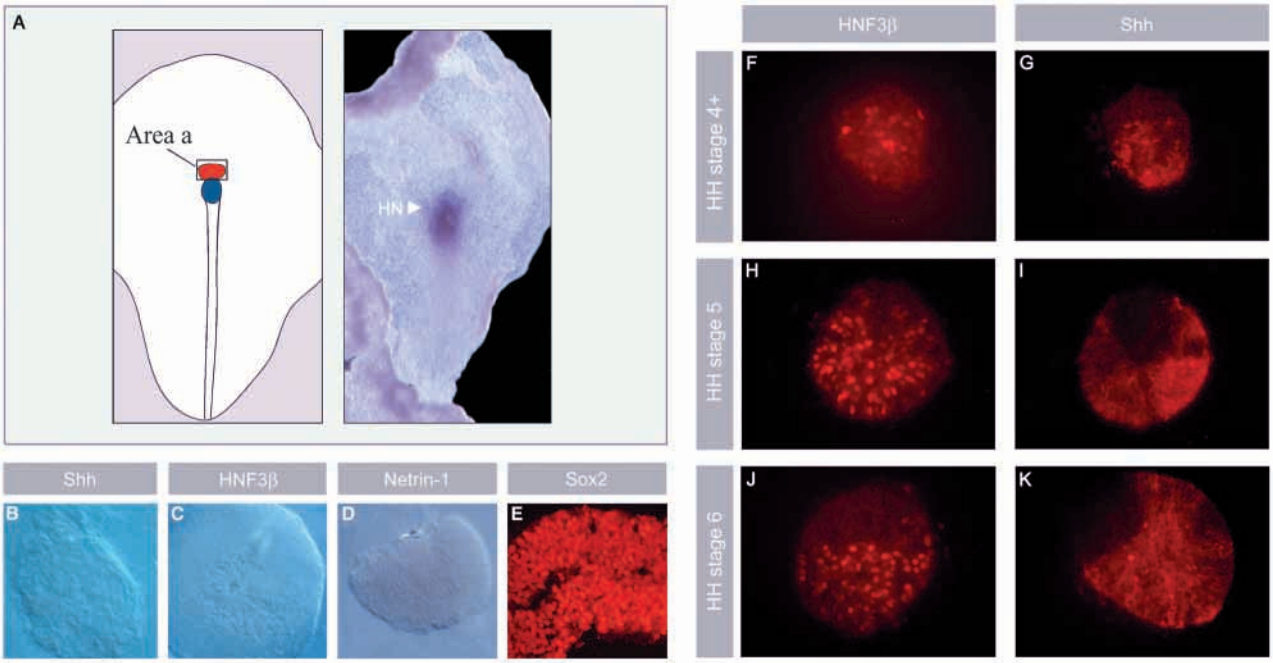

Fig. 3. Specification of 'area a' cells occurs after HH stage 4. (A) Left-hand panel: schematic of HH stage 4 embryo, showing 'area a' (red) and Hensen's node (blue). Box indicates 'area a' dissection. Right hand panel: whole-mount HH stage 4 embryo, showing *Shh* expression within Hensen's node (HN, white arrowhead) and anterior primitive streak. (B-E) Whole-mount views of HH stage 4 'area a' explants cultured for 40 hours and analysed for *Shh*, *Hnf3b*/*Foxa2*, *Netrin1* and *Sox2* expression. No expression of floor-plate markers is detected (B-D), but *Sox2* expression (E) reveals that 'area a' cells are specified as neural. (F-K) Midline neural explants isolated anterior to Hensen's node, in the region equivalent to 'area a' and cultured in isolation for 24 hours. Explants taken from embryos at HH stage 4+ (F,G), HH stage 5 (H,I) and HH stage 6 (J,K) were processed for immunohistochemistry to determine expression of the floor-plate markers HNF3β (F,H,J) and Shh (G,I,K). All panels show representative images of the results obtained and for each stage analysed show serial adjacent sections.

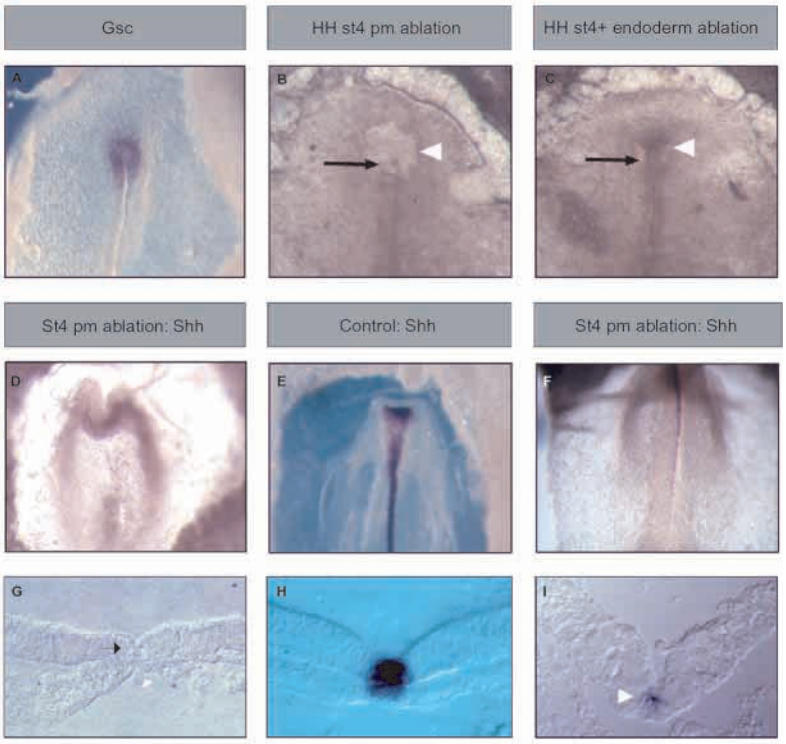

Fig. 4. Prechordal mesoderm induction of early-forming floor-plate cells. (A) Expression of *Gsc* in a HH stage 4 embryo. *Gsc*-expressing cells are ablated in B. (B) HH stage 4 embryo, after removal of both endoderm and prechordal mesoderm. 'Area a' cells remain intact, and are not deleted (white arrowhead). Black arrow indicates Hensen's node. (C) HH stage 4+ embryo, prepared for New culture. Removal of endoderm reveals that prechordal mesendoderm has begun to extend from the Node (white arrowhead). Black arrow points to Hensen's node. (D,G) Whole-mount view (D) and transverse section (G) of an 18 hour New cultured embryo, processed for *Shh* expression after prechordal mesoderm ablation at HH stage 4. Development is retarded, compared with control embryos (E,H), reaching HH stage 7. No expression of *Shh* can be detected on wholemounts (D). Sections reveal that the two halves of the neural plate are conjoined, although by only a few cells (black arrow in G) and that *Shh* cannot be detected in ventral midline cells (black arrow). Axial mesoderm cells are absent (white arrowhead, G). (E,H) Whole-mount view (E) and transverse section (H) of control embryos, processed for *Shh* expression. Expression of *Shh* is detected in both floor plate cells and axial mesoderm cells. (F,I) Whole-mount view (F) and transverse section (I) of an 18 hour New cultured embryo, processed for *Shh* expression after prechordal mesoderm ablation at HH stage 4+. Very weak expression of *Shh* is observed in the midline of operated embryos compared with controls, with fewer cells expressing *Shh* (white arrowhead in I; compare with H).

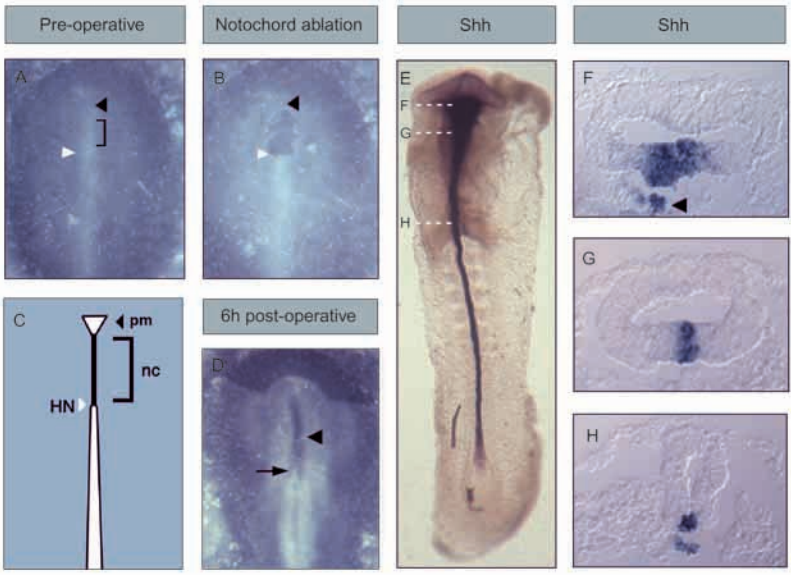

Fig. 5. Early floor-plate cells differentiate in the absence of notochord. (A,C) Phase-contrast micrograph (A) and schematic (C) of HH stage 5 embryo, prepared for New culture, showing Hensen's node (HN, white arrowhead), notochord (nc, black bracket) and prechordal mesoderm (pm, black arrowhead). (B) Phase-contrast micrograph of same embryo, after removal of notochord. Hensen's node and prechordal mesoderm remain intact (white and black arrowheads, respectively). (D) Same embryo, 6 hours after notochord removal. A small rod of notochord cells emerge from Hensen's node (black arrow), but do not extend along the majority of the axis. A visible gap is observed under most of the neuraxis (black arrowhead). (E) Same embryo, analysed 24 hours post-operatively. Analysis of *Shh* expression reveals normal expression throughout the axis. (F-H) Transverse sections taken through levels depicted in E. A normal complement of floor-plate cells differentiates throughout the neuraxis, expressing normal, high levels of *Shh*. In the diencephalon, prechordal mesoderm cells expressing *Shh* can be detected (arrowhead in F). Notochord cells are absent in anterior regions of the axis, extending from the midbrain into the hindbrain (see transverse section, G). Notochord cells reappear in the anterior spinal cord (H).

begin to induce the differentiation of 'area a' cells to a floor-plate identity.

Rescue of anterior floor plate differentiation by early exposure to prechordal mesoderm

The ablation of emerging mesendoderm at HH stage 4 and 4+ removes both prechordal mesendoderm and notochord progenitor cells. To distinguish which of these two cell types might be responsible for the induction of 'area a' cells to a floor plate fate, we performed ablations on slightly older, HH stage 5– embryos, and ablated only the early notochord, leaving anterior-most prechordal mesendoderm cells intact (Fig. 5A-D).

Analysis of operated embryos after 6 hours in culture revealed that notochord cells were not re-established; a clear gap was observed at the midline of the axis (Fig. 5D, arrowhead; *n*=3). In operated embryos analysed after 20 hours in culture, development appeared largely normal (Fig. 5E; *n*=6). Analysis of *Shh* expression in whole-mount preparations showed apparently normal expression in the midline along the length of the embryo. Analysis of sectioned embryos revealed that some prechordal mesendoderm remained intact (Fig. 5F, arrowhead). By contrast, the notochord was missing between diencephalic and spinal cord levels of the axis (Fig. 5G). Despite the lack of notochord, *Shh*-expressing cells differentiated throughout the neuraxis, including cells located between the diencephalon and hindbrain that derived from 'area a' (Fig. 5F,G). The levels of *Shh* expression, moreover, appeared to be similar to those in control embryos.

These analyses show that early exposure to the prechordal mesoderm can rescue anterior floor-plate cells and reveal that the notochord is not required for their differentiation.

HH stage 4+ prechordal mesoderm is a potent inducer of floor-plate character

Our studies indicate that prechordal mesoderm is required to rapidly induce 'area a' cells to a floor-plate fate. We next determined whether it is sufficient to induce their differentiation, by performing in vitro recombinations of HH stage 4+ prechordal mesendoderm with intermediate neural tissue from E9.5 rat embryos or with 'area a' from HH stage 4 chick embryos (Fig. 6A). Prechordal mesoderm induced mature floor-plate cells within rat neural plate, as assessed by the expression of the floor plate marker, FP3 (Fig. 6B) (Placzek et al., 1993). Similarly, when HH stage 4+ quail prechordal mesoderm was recombined with 'area a', expression of HNF3β and Shh, but not markers of axial mesoderm, were induced (Fig. 6C,D and not shown). Thus, HH stage 4+ prechordal mesendoderm is able to induce floor-plate cells in vitro.

To compare the inductive ability of the early prechordal mesoderm with that of the notochord in vivo, a standard assay of floor-plate induction was performed. Here, either notochord or prechordal mesendoderm were grafted adjacent to the forming neural tube in ovo (Fig. 6E). As previously described, notochord induced a discrete region of floor plate directly adjacent to the grafted tissue (Fig. 6G). By contrast, prechordal mesendoderm induced floor-plate character not only adjacent to the graft itself, but also in more dorsal regions of the neural tube on both the ipsilateral and contralateral sides (Fig. 6H). This result suggests that the nascent prechordal mesoderm is a more potent inducer of floor plate character than is notochord, and supports the idea that in vivo, early floor plate cells are induced by a rapid vertical induction mediated by underlying prechordal mesoderm.

Co-operation between Nodal and Shh signalling promotes floor plate differentiation in 'area a'-derived cells

Given the requirement for prechordal mesoderm in anterior floor plate induction, we assessed the early axial mesodermal expression of *Shh* and *Nodal*, the factors most strongly implicated in floor plate induction in amniote and anamniote embryos respectively. In situ hybridisation at HH stage 4 reveals that neither *Shh* or *Nr1* is expressed in tissues

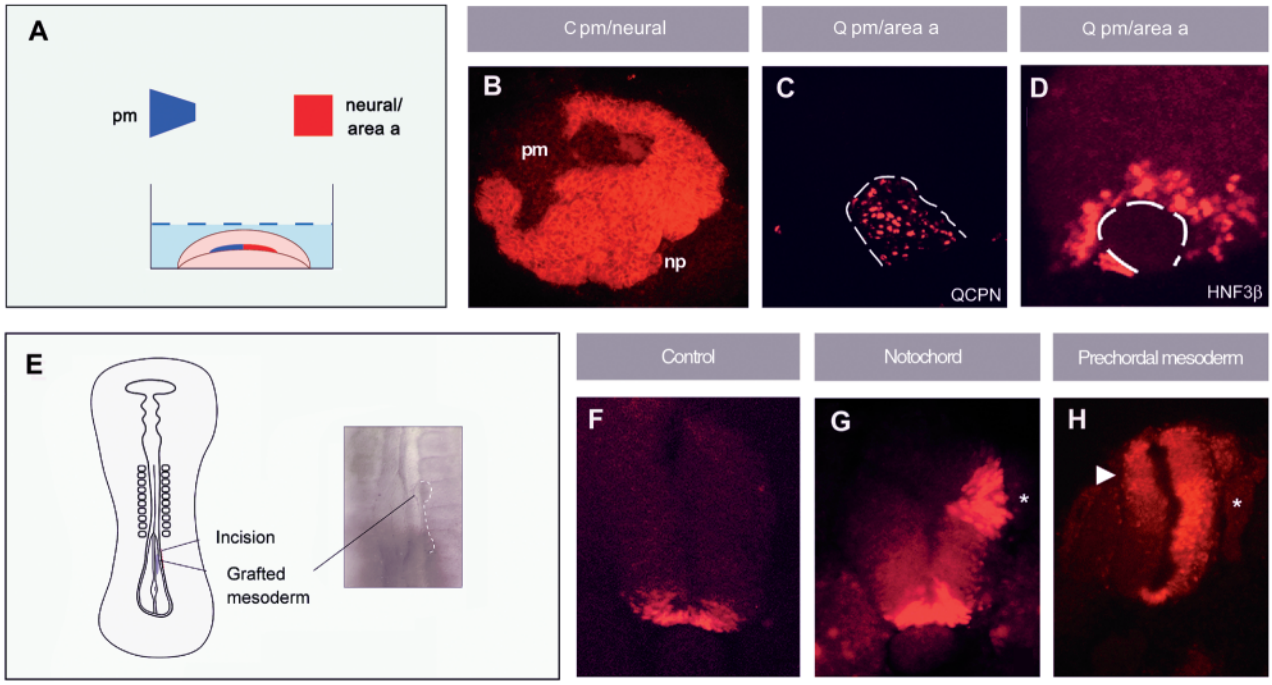

Fig. 6. HH stage 4+ prechordal mesendoderm is a potent inducer of floor-plate character in vivo. (A) Schematic, showing in vitro recombination experiments to determine the ability of HH stage 4+ prechordal mesoderm to induce floor-plate character. (B) Recombinate of HH stage 4+ chick prechordal mesoderm (Cpm) and E9.5 rat intermediate neural plate (np). After 48 hours in culture, rat neural plate expresses the floor-plate marker FP3, as assessed by immunohistochemistry. (C,D) Recombinate of HH stage 4+ quail prechordal mesoderm (Qpm) and HH stage 4 chick 'area a'. After 24 hours in culture the position of the prechordal mesoderm was analysed by immunohistochemistry with an antibody against the quail specific nuclear marker QCPN (C, prechordal mesoderm delineated by broken lines). Analysis of HNF3β expression by immunohistochemistry in an adjacent section reveals induction of floor plate cells in 'area a' tissue that immediately abuts a small region of prechordal mesoderm (D). (E) Schematic and whole-mount views (inset) of in vivo grafts of mesodermal tissue. Grafts were inserted between the neural plate and the adjacent pre-somitic mesoderm in the posterior neuropore region of HH stage 10 embryos. (F) Control embryo. HNF3β expression is restricted to the floor-plate region lying at the ventral midline of the neural tube. (G) Graft of a notochord next to the lateral wall of the neural tube (white asterisk in G) induces an ectopic floor plate as indicated by the localised expression of HNF3β directly adjacent to the graft. (H) Graft of a HH stage 4+ prechordal mesendoderm (white asterisk) induces floor-plate character throughout the ipsilateral neural tube and also on the contralateral side of the neural tube (white arrowhead in H).

underlying 'area a' at this stage of development (Fig. 7A,B). Coincident with the appearance of the nascent prechordal mesoderm at HH stage 4+, however, both *Shh* and *Nr1* are expressed in prechordal mesoderm cells as they pass beneath 'area a' (Fig. 7C,D). In notochord cells that follow immediately behind, expression of *Nr1* is completely absent while *Shh* is expressed only very weakly in a subset of cells (Fig. 7E,F). Thus, when 'area a' cells are being specified to a floor-plate fate, co-expression of *Shh* and *Nodal* is detected in the prechordal mesoderm cells lying directly underneath them. Subsequent to their transient exposure to *Shh/Nr1*-expressing prechordal mesoderm, 'area a'-derived cells themselves begin to express *Shh*, while underlying notochord cells express *Shh* at barely detectable levels. Given our observation that prechordal mesoderm can rapidly specify 'area a' cells to a floor-plate fate we therefore tested the ability of both Shh and Nodal to specify 'area a' cells to a floor plate fate in vitro.

HH stage 4 'area a' explants that do not express floor-plate markers if cultured alone (Fig. 3B-D; Fig. 7K,P,U,Z) were exposed to Shh, Nodal or a combination of the two signalling molecules. Expression of the ventral midline markers Shh (mRNA and protein), HNF3β and *Netrin1*, and of the axial mesoderm markers *brachyury* and 3B9 was assessed at different time points (12, 20, 40 hours). Neither *brachyury* nor 3B9 was induced (not shown). However, addition of Shh protein at high concentration to 'area a' explants was sufficient to induce all three ventral midline markers after 20 hours in culture (Fig. 7G,L,Q,V; 100%, n>40), while tenfold lower concentrations were insufficient to elicit this response (Fig. 7H,M,R,W; 0%, n>40). However, when explants were exposed to low concentrations of Shh together with Nodal protein, a strong induction of ventral midline markers was observed, again, after 20 hours in culture (Fig. 7J,O,T,Y; 100%, n>40). Although a weak induction of ventral midline markers was observed when Nodal protein was provided alone (Fig. 7I,N,S,X; 50%, n>40) the response to a combination of Shh and Nodal was notably robust, in many cases more so than the response to Shh alone. Induction in response to Nodal alone, or Nodal and Shh was first detected at the 20 hour time-point. To examine whether we could distinguish a differential induction of HNF3β and Shh in response to either Nodal or Nodal/Shh, a subset of explants were examined by double-labelling. HNF3β and Shh were induced to an identical extent (not shown). Taken together, these data are suggestive of a cooperation between Nodal and Shh signalling during the rapid

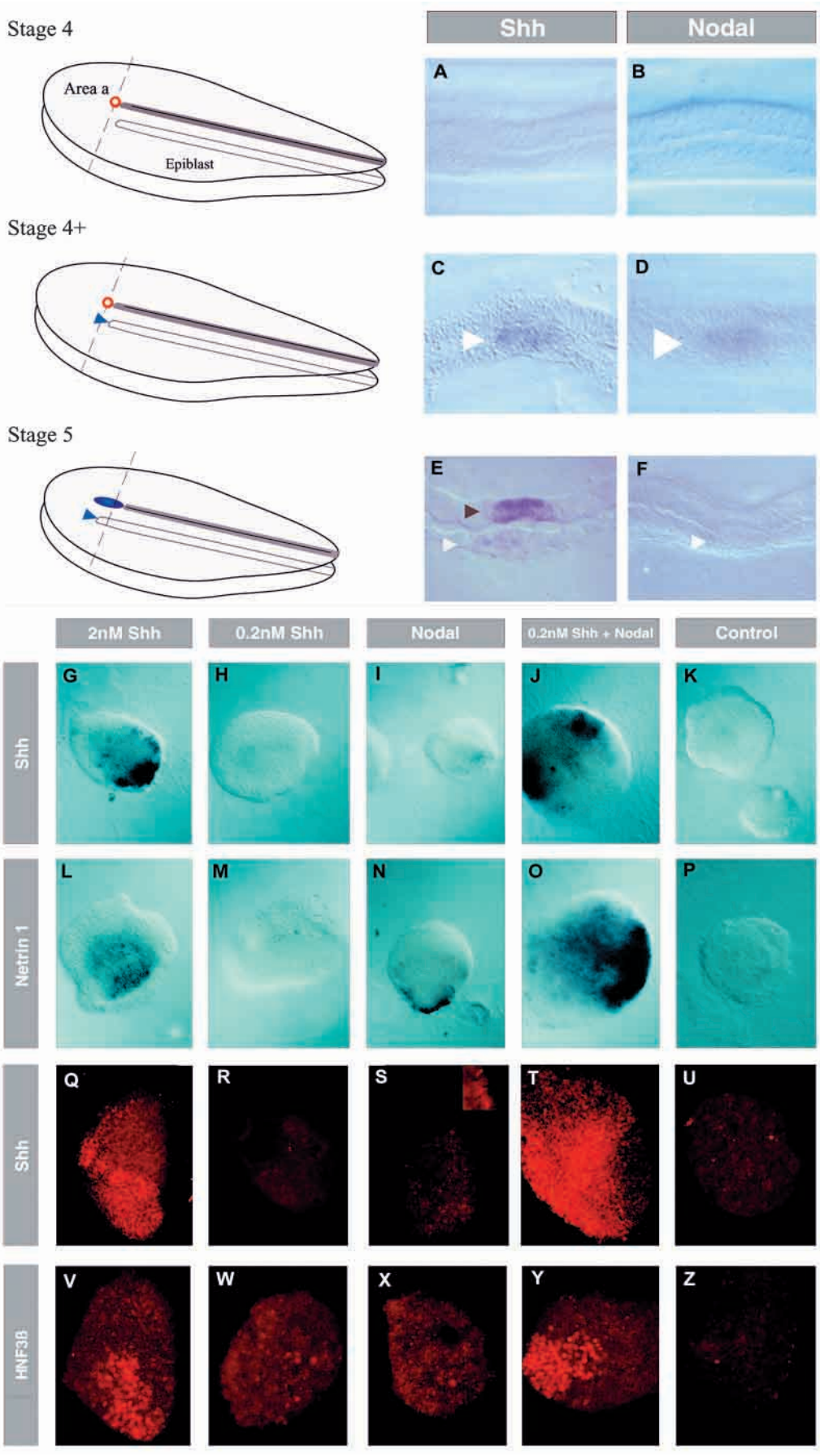

Fig. 7. Induction of floor-plate character in 'area a' cells through a cooperation between Shh and Nodal signalling. (A-F) *Shh* and *Nodal* are co-expressed in the HH stage 4+ prechordal mesoderm as it migrates underneath 'area a'. Sections of HH stage 4 (A,B), 4+ (C,D) and 5 (E,F) embryos processed for mRNA in situ hybridisation with probes against *Shh* (A,C,E) or the chick Nodal gene *Nr1* (B,D,E). The axial level of the sections, just anterior to Hensen's node, is indicated in the schematics to the left of each pair of images. At HH stage 4, no expression of either *Shh* (A) or *Nodal* (B) is observed in 'area a' or the underlying tissue. Approximately 1 hour later, at HH stage 4+, *Shh* (C) and *Nodal* (D) are co-expressed in the nascent prechordal mesoderm lying underneath 'area a' (white arrowheads in C and D). At HH stage 5, after the nascent prechordal mesoderm has passed underneath 'area a', no expression of either *Shh* or *Nodal* is seen in the axial mesoderm (white arrowheads in E,F) while 'area a'-derived cells of the neural midline now exhibit strong expression of *Shh* (black arrowhead in E). (G-Z) Nodal potentiates Shh signalling to induce floor plate character in 'area a' explants in vitro. (G,L,Q,V) Exposure to 2 nM Shh induces expression of Shh, *Netrin1* and HNF3β in 'area a' explants. (H,M,R,W) Exposure to 0.2 nM Shh is insufficient to induce floor-plate markers. (I,N,S,X) Exposure to Nodal alone can result in a very weak induction of ventral midline markers (I,N and inset in S), although sometimes elicits no ventral midline differentiation (S,X). (J,O,T,Y) Exposure of explants to a combination of 0.2 nM Shh and Nodal results in strong induction of ventral midline markers. (K,P,U,Z) Control medium does not elicit ventral midline differentiation.

induction of floor-plate character in ‘area a’ cells by nascent prechordal mesoderm at HH stage 4+.

Discussion

The floor plate is common to all vertebrate embryos, possibly with an evolutionary origin earlier in the chordate lineage (Corbo et al., 1997). The importance of this structure in the regulation of CNS patterning has made it the focus of a large number of studies. In recent years, studies into floor plate formation in different model organisms have revealed apparent differences in the mechanism of floor-plate formation. The studies suggest that floor-plate induction requires the presence of the notochord and Shh in amniote embryos, but that neither is required for the differentiation of medial floor plate cells in anamniote embryos, which instead are dependent on Nodal signalling. The studies we describe here go some way towards reconciling these different observations, suggesting that in the early chick embryo, the floor plate arises from at least two principal sources. Our experiments show that an epiblast-derived population of floor plate precursors, ‘area a’, primarily populate medial cells in the anterior ventral midline and suggest that this population is rapidly induced to a floor-plate fate early in gastrulation. Our studies suggest that the nascent prechordal mesoderm, and not the notochord, is responsible for the rapid vertical induction of floor-plate character in ‘area a’-derived cells, and suggest that ‘area a’-derived cells are left behind in a specified state as they extend along the ventral midline of the neural plate and are not reliant upon further inductive signals from the earliest forming notochord. Strikingly, in vitro experiments also suggest that, as in anamniotes, Nodal signalling may indeed play a role in floor plate induction in the chick embryo.

‘Area a’ and Hensen’s node-derived floor plate cells: discrete populations of ventral midline cells

Previous fate-mapping studies have shown that in the HH stage 4 chick embryo, epiblast cells in ‘area a’ contribute to the floor plate (Schoenwolf and Sheard, 1990). However, these studies did not analyse whether, prior to populating the midline, ‘area a’ cells might transiently populate Hensen’s node. Our in vivo time lapse analyses reveal no evidence for this possibility: we do not observe that ‘area a’ cells enter the node. Previous lineage analyses have shown that, prior to HH stage 4, Hensen’s node cells do not give rise to floor plate, suggesting in turn that ‘area a’-derived cells do not themselves migrate out of Hensen’s node (Selleck and Stern, 1991; Lopez-Sanchez et al., 2001). Together, these results suggest that ‘area a’ and Hensen’s node cells are distinct populations. Importantly, this separation demonstrates that a shared lineage between notochord and floor plate cells is not a prerequisite for floor plate differentiation.

Our real-time analysis of cell movement also reveals that, although floor plate precursors are actually present in the node at HH stage 4, they do not migrate out until HH stage 6 (see also Selleck and Stern, 1991; Lopez-Sanchez et al., 2001). Together these studies suggest the existence of two early populations of floor-plate precursors in the chick, one in the prenodal epiblast (‘area a’), which gives rise exclusively to cells of the neural midline, principally in anterior regions, and one in the epiblast layer of Hensen’s node, the descendants of which leave the Node only after HH stage 6 and are later found in the more posterior ventral midline. Our observation that cells in ‘area a’ form an earlier floor plate population than cells in the Node is supported by previous studies in the chick (Lopez-Sanchez et al., 2001) and raises the possibility that ‘area a’ cells, or their progenitors, exist as a specialised population prior to formation of the organiser. In support of this, even before primitive streak formation, the midline of the epiblast exhibits specialised properties, and itself undergoes powerful anterior extension movements (Kelly et al., 2002; Lawson and Schoenwolf, 2001).

Prechordal mesoderm induces ‘area a’ cells

Our analyses show that ‘area a’ cells are induced to a floor-plate identity between HH stage 4 and 4+, and suggest that the nascent prechordal mesoderm mediates this induction. Ablation of the mesendoderm as it forms in the deep layers of Hensen’s node leads to the loss of the entire floor plate at early stages of development, including floor-plate cells that normally arise from ‘area a’. This ablation removes both prechordal mesoderm and notochord precursor cells; thus, in principle, either of these could contribute to the induction of ‘area a’ cells to a floor plate identity. However, a number of lines of evidence suggest that prechordal mesoderm, and not notochord cells, are responsible for ‘area a’ induction. First, anterior floor plate cells form normally in embryos in which prechordal mesoderm is present, but notochord is absent. The differentiation of these cells is dependent upon only a very short exposure to the prechordal mesoderm: in embryos in which prechordal mesoderm is eliminated after only a short exposure to ‘area a’ cells, early floor-plate cells still form, albeit fewer in number and expressing lower levels of Shh than normal. It is likely that homeogenetic lateral induction mediated by these early specified cells accounts for the complete rescue of the anterior floor plate in the absence of notochord signals (Placzek et al., 1993). Second, in an ectopic situation the prechordal mesendoderm can induce floor plate in neural tissue with marked potency, supporting the assertion that in vivo, prechordal mesoderm mediates the rapid induction of ‘area a’ cells. Finally, we find that the chick *nodal* homologue *Nr1* is co-expressed with *Shh* in the nascent prechordal mesoderm at the time at which this tissue is required for the rapid induction of floor plate character in ‘area a’ cells. By contrast, nascent notochord cells do not express *Nr1* and barely express *Shh*.

A role for Nodal signalling in chick floor plate induction: parallels between amniote and anamniote floor plate differentiation

Many lines of evidence have suggested that in zebrafish, Nodal signalling is required for medial floor plate formation early in development. Both *cyc* and *oep* mutant phenotypes include a loss of medial floor plate cells (Hatta, 1992; Hatta et al., 1991; Krauss et al., 1993; Rebagliati et al., 1998; Sampath et al., 1998; Schier et al., 1997; Shinya et al., 1999; Strahle et al., 1997; Zhang et al., 1998). Importantly, the cell-autonomous requirement for *oep* indicates that the formation of the medial floor plate occurs as the result of an inductive interaction (Gritsman et al., 1999; Strahle et al., 1997). In addition to loss of the medial floor plate, both *cyc* and *oep* have defects in prechordal plate formation, in the case of *oep* a complete loss of this tissue (Schier et al., 1997). This correlation may suggest

that the prechordal plate is in fact the source of a floor plate-inducing Nodal signal during gastrulation, a possibility supported by the fact that rescue of the floor-plate phenotype in *cyc* mutants requires the presence of wild-type cells within the prechordal plate (Sampath et al., 1998).

Our analyses provide a first indication that Nodal may play a role also in chick floor-plate induction: Nodal can cooperate with low levels of Shh to induce floor plate character in 'area a' cells. Studies of the zebrafish have suggested that Nodal can induce *Shh* expression within the neural tube, providing a potential mechanism of cooperation (Muller et al., 2000). Whether such a cooperation does in fact operate in vivo in the chick remains unclear, but is indicated through studies of mouse embryos: both *Shh*-null mice and mice that are conditionally mutant for *Smad2*, a downstream effector of Nodal signalling, lose *Shh*-expressing cells in the anterior neuraxis (Chiang et al., 1996; Heyer et al., 1999). Taken together, these data are suggestive of a cooperative role for Shh and Nodal signalling during floor plate formation in amniote embryos, potentially via Shh activation.

A dual model for floor-plate induction

Our studies, together with earlier work (Artinger and Bronner, 1993; Placzek et al., 2000; van Straaten and Hekking, 1991; Yamada et al., 1991) suggest that floor-plate cells are induced by two different mechanisms along the anteroposterior axis of the chick embryo. Floor-plate cells that derive from 'area a' arise during gastrulation and largely populate anterior regions of the neuraxis. Our studies show that 'area a'-derived floor plate cells are induced through a rapid interaction mediated by prechordal mesoderm that may involve a previously unrecognised role for Nodal signalling in an amniote embryo. By contrast, floor-plate cells that differentiate in the neurula stage embryo, and occupy posterior regions of the neuraxis, require a prolonged period of contact with underlying notochord (Fig. 8). Studies in both mouse and zebrafish embryos support the idea that distinct mechanisms operate to specify the floor plate in anterior and posterior regions. Distinct cis-acting regulatory sequences have been identified within the mouse Shh promoter that direct Shh expression to specific regions of the neural tube, supporting the view that multiple genes are involved in activating Shh transcription along the length of the CNS (Epstein et al., 1999). In zebrafish *flh* and *ntl/spt* double mutants, the anterior floor plate develops normally but the posterior floor plate is severely affected (Amacher et al., 2002; Halpern, 1995; Schier et al., 1997). Similarly, while *ntl* acts a partial suppressor of the *oep* or *cyc* phenotypes, rescue is observed only posteriorly (Halpern et al., 1997; Schier et al., 1997; Strahle et al., 1997).

An unresolved issue is whether the distinction between these two apparently different schemes is absolute. Intriguingly, studies have shown that posterior floor-plate cells eventually form even after notochord ablation or neural plate isolation in culture (Artinger and Bronner Fraser, 1993), raising the possibility that the induction mechanism leading to anterior floor-plate specification may in some way contribute to floor plate differentiation in the posterior neuraxis. Studies in zebrafish have likewise indicated an additive effect of floor-plate phenotypes: in *oep* mutants, a few floor-plate cells are present, while in *flh* mutants the floor plate seems normal anteriorly but scattered posteriorly. By contrast, double mutants show a complete absence, or very severe reduction in number, of floor plate cells (Schier et al., 1997; Strahle et al., 1997).

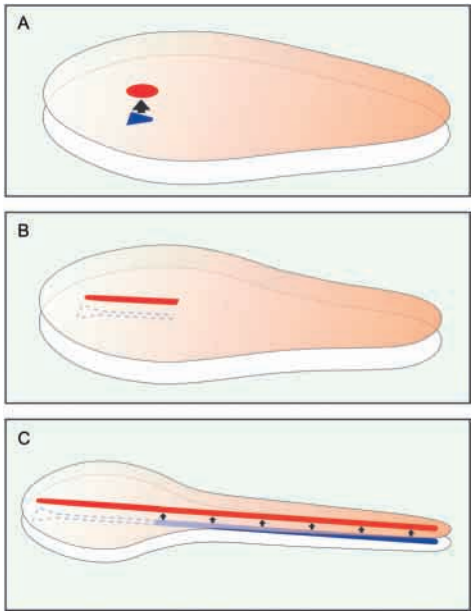

Fig. 8. Dual mode of floor-plate formation along the AP axis. (A) In the gastrulating chick embryo at HH stage 4+, prechordal mesoderm cells pass beneath 'area a', and mediate a potent induction of 'area a' cells to a floor-plate fate. (B) Once prechordal mesoderm cells have activated 'area a' cells, they migrate further forwards, but are not required for any further induction of floor-plate character. Floor-plate cells that have been induced in 'area a' migrate anteriorly and posteriorly. (C) In more posterior regions of the neuraxis, floor-plate cells require a prolonged period of contact with underlying notochord cells for their induction to a floor-plate fate.

Our observations raise the question of why the floor plate should arise in this dual manner. A likely explanation is that it occurs because of the different modes of cellular movements in the gastrula and neurula embryo. Early in development, the rapid morphogenetic movements associated with gastrulation and formation of the early neural tube mean that the registration of floor plate and underlying axial mesoderm is not stable (Dale et al., 1999; Woo and Fraser, 1995). By contrast, during neurulation, the ventral midline of the caudalmost neural tube is formed in register with the notochord, so that floor-plate cells arise through the interaction of two stably apposed tissues. It is likely, then, that the functional significance of rapid specification of 'area a' cells by the early prechordal mesoderm is to circumvent the requirement for prolonged exposure to a Shh-expressing notochord until such time as stable tissue interactions and Shh expression are re-established in more posterior regions of the embryo following gastrulation.

Finally, the early specification of a population of floor plate cells by signalling from the prechordal mesoderm suggests parallels with anamniote embryos. Our observations that 'area a'-derived cells occupy a medial position in the developing anterior floor plate, and that a floor plate is able to develop in the absence of notochord signalling, contingent upon early specification of 'area a' cells by the nascent prechordal

mesoderm suggest similarities between early floor-plate specification in amniotes and the generation of the medial floor plate in anamniote embryos. In addition, our observation that Nodal signalling may be responsible for mediating this rapid induction of a population of floor-plate cells indicates further parallels with the situation in anamniote embryos. Thus, our studies may go some way towards reconciling models of floor-plate formation in different vertebrate systems.

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