Determination of embryonic polarity in a regulative system: evidence for endogenous inhibitors acting sequentially during primitive streak formation in the chick embryo

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Summary

Avian embryos have a remarkable capacity to regulate: when a pre-primitive streak stage embryo is cut into fragments, each fragment can spontaneously initiate formation of a complete embryonic axis. We investigate the signalling pathways that initiate primitive streak formation and the mechanisms that ensure that only a single axis normally forms. As reported previously, an ectopic primitive streak can be induced by misexpression of Vg1 in the marginal zone. We now show that Vg1 induces an inhibitor that travels across the embryo (3 mm distance) in less than 6 hours. We provide evidence that this inhibitor acts early in the cascade of events downstream of Vg1. We also show that FGF signalling is required for primitive streak formation, in cooperation with Nodal and Chordin. We suggest that three sequential inhibitory steps ensure that a single axis develops in the normal embryo: an early inhibitor that spreads throughout the embryo (which can be induced by Vg1), a second inhibition by Cerberus from the underlying hypoblast, and finally a late inhibition from Lefty emitted by the primitive streak itself.

Key words: Gastrulation, Embryonic axis, Regulative development, Vg1, FGF, Chordin, Nodal

Introduction

At early stages of development, the embryo needs to establish bilateral symmetry. In most invertebrates and in amphibians, the initial symmetry-breaking event occurs within the large egg contents by the localisation of maternal determinants that are inherited differentially by the daughter blastomeres, which thus acquire different fates. As a result, some blastomeres lose their ability to give rise to certain cell types. For example in amphibians after the third cleavage division, separation of dorsal and ventral halves leads to the dorsal half developing a partial axis, while the ventral half only gives rise to a ‘belly-piece’ (Bauchstück) comprising only ventral (blood, mesenchyme, epidermis) structures (Gerhart et al., 1989; Harland and Gerhart, 1997; Zernicka-Goetz, 2002).

By contrast, amniote embryos do not appear irreversibly to fix their polarity until much later, when gastrulation begins and the embryo may already contain many thousand cells. In the mouse, the inner cell mass can be dissociated and reaggregated, or made up from cells derived from different embryos, yet it will develop into a single normal embryo, although the extent to which the axis of polarity is specified by maternal cues or by the point of sperm entry has recently generated considerable controversy (reviewed by Gardner, 2002; Piotrowska and Zernicka-Goetz, 2002; Tam, 2002; Zernicka-Goetz, 2002; Gardner and Davies, 2003; Johnson, 2003). A more extreme case in mammals is seen in the armadillo, which normally produces four embryos from a single blastoderm (Enders, 2002). Likewise, chick embryos until the time of primitive streak formation (when they contain as many as 50,000 cells), can be cut into four pie-shaped fragments, all of which can spontaneously give rise to a complete, miniature embryo (Lutz, 1949; Spratt and Haas, 1960). Furthermore, composite embryos made up of equivalent pie-slices (for example the most anterior quadrant) derived from four or even more donor embryos will develop only a single axis, arising randomly from a margin of any one of these slices. Although this finding does not rule out a role for maternally inherited, differentially distributed components in biasing the initial polarity of amniote embryos, it certainly does rule out the concept that these components are true ‘determinants’ of cell fate, as no quadrant of the embryo, even at this advanced stage, possesses a unique ability for polarity determination or cells committed to particular fates, not shared by any other quadrant.

Surprisingly, the remarkable ability of the chick embryo to regulate has received little attention, as it is an excellent experimental system in which to study the molecular mechanisms underlying polarity determination in an extremely regulative system. The finding that any fragment of the chick embryo right up to the beginning of primitive streak formation can form a complete axis when isolated, while none of these regions other than the posterior part (containing Koller’s sickle and the marginal zone) do so in intact embryos, strongly suggests that the normal site of axis formation actively inhibits other regions from initiating the same process.

We provide evidence for an inhibitor operating before primitive streak formation. It is induced by Vg1 misexpression,
travels across the embryo (3 mm distance) at a speed of at least 500 μm/hour, and acts upstream of, or in parallel with, the signalling factors Nodal and Chordin. We argue that this inhibitor is distinct from the Nodal antagonist Cerberus, which is produced by the hypoblast. We also reveal a role for the FGF signalling pathway in initiation of the embryonic axis and suggest that this acts in cooperation with Nodal and Chordin.

Materials and methods
Embryo culture, manipulation and in situ hybridisation
Fertile Brown Bovan Gold hens’ eggs (Henry Stewart, UK) were incubated for 1-18 hours to obtain embryos between stage X (Eyal-Giladi and Kochav, 1976) and stage 4+ HH (Hamburger and Hamilton, 1951). Embryo manipulation was performed in Tyrode’s solution (Stern and Ireland, 1981). Anterior halves were obtained by cutting embryos with a hair loop; the cut edge was then sealed by applying a strip of outer area opaca from the anterior region of another embryo. After manipulation, embryos were set up in modified New culture (New, 1955; Stern and Ireland, 1981). In situ hybridisation was carried out as previously described (Stern, 1998). The probes we used were chick Brachyury (Kispert et al., 1995a; Kispert et al., 1995b; Knezevic et al., 1997) (a gift from Dr B Herrmann), Chordin (Streit et al., 1998), Cerberus (Zhu et al., 1999), FGF8 (Streit and Stern, 1999a; Streit et al., 2000) (a gift from Dr J. C. Izpisúa-Belmonte), Nodal/cNR-1 (Levin et al., 1995), Vg1 (Shah et al., 1997), goosecoid (Izpisúa-Belmonte et al., 1993), Hex (Yatskievych et al., 1999; Foley et al., 2000), Crescent (Pfeffer et al., 1997) (a gift from Dr P. Pfeffer) and HNF3β (Ruiz i Altaba et al., 1995) (a gift from Dr A. Ruiz i Altaba).

FGF gain and loss of function
Heparin acrylic beads (Sigma) were incubated in 50 μg/ml FGF4 or FGF8b (R&D Systems) in PBS for 2 hours at 4°C. AG1X2-formate beads (100 μm) were coated with 250 μM SU5402 (Calbiochem) for 1-2 hours at room temperature. The beads were then washed in PBS before grafting.

Vg1, Wnt1, Chordin and Nodal misexpression
COS-7 cells were cultured in DMEM with 10% fetal calf serum. BVg1 (Shah et al., 1997), Chordin (Streit et al., 1998) or Nodal (Bertocchini and Stern, 2002) were transfected using Lipofectamine Plus (Gibco-BRL). After 24 hours, 1000 or 500 cells were allowed to aggregate into pellets in 20 μl hanging drops of medium. Mock-transfected cells were used as control. For experiments involving Vg1 grafted with SU5402-coated beads, 500-cell pellets were prepared each containing 250 Vg1-transfected cells and 250 mock-transfected cells. In all other experiments, 1000 cell pellets were used. For Wnt1 misexpression, we used a stable cell line (rat B1; a kind gift from Dr Jan Kitajewsky) as previously described (Joubin and Stern, 1999; Skromne and Stern, 2001; Skromne and Stern, 2002).

Assays
The experiments described here, like those of previous work in this field, rely on misexpression of secreted factors delivered by grafts of transfected cells. Although it is possible to deliver factors by electroproporation of expression constructs, we have not yet succeeded in doing this at pre-streak stages. Grafts of expressing cells have the advantage, however, that it is possible to titrate the number of cells grafted and thus adjust the levels of factor supplied. Although it is not possible to compare the absolute protein levels available to the embryo after such grafts, this method allows for internal comparison.

It is also worth pointing out that in this study, as in all other previous studies on embryos at these early stages of development, the results are ‘statistical’ – even those manipulations that give the strongest effects (such as Vg1 or Vg1+Wnt misexpression) produce ectopic streaks in only about 70% cases. The reasons for this are unknown, but one reason may be that there are many endogenous factors regulating axis development, as the present study reveals.

Results
Regulative properties of isolated anterior halves
During normal development, primitive streak formation is preceded by a cascade of gene activation (involving the secreted factors Vg1, Nodal, Wnt8C, FGF8 and Chordin and the transcription factors Brachyury, Tbx6L, goosecoid, Notl and HNF3β) adjacent to the region of the forming streak (Shah et al., 1997; Bachvarova et al., 1998; Skromne and Stern, 2001; Bertocchini and Stern, 2002; Skromne and Stern, 2002). The chick embryo has a remarkable ability to regulate, as any isolated pie-shaped piece can form an axis spontaneously, provided that the pieces are isolated before the primitive streak stage (Lutz, 1949; Spratt and Haas, 1960). This property provides us with an opportunity to investigate the precise order in which these genes are deployed as they initiate formation of the embryonic axis in its new location.

To confirm at a molecular level the earlier findings (Lutz, 1949; Spratt and Haas, 1960) that embryos can regulate, we divided stage X-XIII (Eyal-Giladi and Kochav, 1976) chick embryos into anterior and posterior halves, and each half was grown separately in modified New culture (Fig. 1A) (New, 1955; Stern and Ireland, 1981). The posterior half (where the streak normally forms) develops an axis in 93/98 (95%) cases, within the same time course as normal uncut embryos. At each stage analysed, the anterior half forms an axis in almost 60% of cases [20/27 (74%) at stage X; 9/23 (39%) at stage XI; 17/28 (61%) at stage XII; and 11/20 (55%) at stage XIII]. The forming axis expresses markers of mesoderm (chick Brachyury) and organizer (Chordin) (Fig. 1B,C), and develops a lower layer composed of hypoblast (Crescent and FGF8 expression) and endoblast (Fig. 1D,J). Primitive streak formation in the anterior half is initiated with a delay of at least 8 hours compared to the posterior half and always arises from the most posterior part of the anterior half. A primitive streak (and Brachyury expression) is first visible after 15-18 hours. These findings confirm the observations of (Spratt and Haas, 1960) that isolated fragments of the early chick embryo can initiate primitive streak formation following a posterior-to-anterior gradient of ‘embryo-forming potentiality’.

Next, we analyzed the hierarchy of genes accompanying axis formation in the anterior half. Isolated anterior halves were grown and fixed at 3 hour intervals following the cut; at each time point we analysed the expression of signalling molecules previously implicated (Bertocchini and Stern, 2002; Skromne and Stern, 2002) in primitive streak formation (Vg1, Wnt8C, Nodal and FGF8) and of Brachyury, Chordin, Crescent, Goosecoid and HNF3β as markers of mesoderm, organizer, Koller’s sickle and hypoblast. The first signalling molecule expressed in the anterior half is Vg1, which appears after 9 hours’ incubation (Fig. 1E-H). Its expression is always found posteriorly within the fragment, at the left or the right margin of the new edge (Fig. 1G,H). After 12 hours, Nodal appears in a localized region adjacent to the margin (Fig. 1I), while Fgf8 is expressed only in the lower layer (Fig. 1J). Chordin, Brachyury and Wnt8C appear after 15-18 hours of incubation and are associated with the nascent streak (data not shown). This order of gene activation in the anterior half is similar to
the hierarchy of gene expression induced by ectopic expression of Vg1 in the anterior marginal zone of pre-streak embryos (Skromne and Stern, 2002). Interestingly, an ectopic Koller’s sickle is never apparent either by morphology or by expression of its characteristic markers (goosecoid, FGF8) (Fig. 1J).

**Vg1 induces an inhibitor that prevents multiple streak formation**

The above experiments confirm the finding that any region of the pre-primitive streak stage embryo has the ability to form an axis (Spratt and Haas, 1960). The fact that the anterior half of intact embryos never does so suggests the existence of endogenous inhibitory factors, from whose influence the anterior half is released upon cutting. For such a mechanism to work effectively to prevent multiple axes in normal development, the inhibitor should travel quickly across the embryo, which measures some 3 mm in diameter.

The existence of such an inhibitor can also be revealed by ectopic expression of Vg1 at various positions in the marginal zone. When misexpressed anteriorly, Vg1 induces an ectopic streak that co-exists with the endogenous axis, in more than 60% of cases (Seleiro et al., 1996; Shah et al., 1997; Skromne and Stern, 2001; Skromne and Stern, 2002). By contrast, when misexpressed in the lateral marginal zone at 90° from the posterior part (Fig. 2A), the newly induced primitive streak often inhibits the formation of the original axis [31/55 (56%) with only one axis arising from the Vg1 source; 5/55 (9%) with two axes, 10/55 (18%) with one axis arising half-way between the original site and the Vg1 pellet, 7/55 (13%) with only the original axis and 2/55 (4%) with no axis] (Fig. 2B). This result suggests that Vg1 induces an inhibitor that prevents the development of additional axes.

How fast does this inhibitor travel? To determine this, we grafted two Vg1 pellets on diametrically opposite lateral sides of the embryo (Fig. 2C). When grafted simultaneously (i=0), a primitive streak is induced by each pellet, resulting in embryos with double axes [16/28 (57%); Fig. 2D] (in these embryos the primitive streak from the original posterior part never forms). The same result is obtained when the second pellet is grafted 3 hours [t=3, 16/22 (72%)], 4 hours [t=4, 14/30 (47%)] or 5 hours [t=5, 14/23 (61%)] after the first (Fig. 2E). However, when the second Vg1 pellet was grafted 6 hours after the first, only 7/48 (15%) embryos developed two primitive streaks, while the majority (63%) formed a single axis arising from the first Vg1 pellet (χ² test, P<0.005 with respect to pellets grafted simultaneously; Fig. 2F). As we do not know how long it takes for Vg1 to induce expression of the inhibitor, this finding reveals that the putative inhibitor takes less than 6 hours to reach the opposite site of the embryo, some 3 mm away (>500 μm/hour).

Recently, we reported that one mechanism contributing to ensure the formation of a single axis during normal development relies on the expression of the Nodal antagonist Cerberus by the hypoblast, which is then removed just prior to primitive streak formation by the appearance of the endoderm (which does not express Cerberus) (Bertocchini and Stern, 2002). Could Cerberus, or another molecule produced by the hypoblast, be the inhibitor revealed by the above experiments? To answer this, we analysed the expression pattern of hypoblast markers including Cerberus and Hex in time-course in embryos grafted with Vg1 pellets. We did not observe any increase in Cerberus expression around the second pellet (0/9 embryos). The hypoblast, marked by both Cerberus and Hex, is displaced from both sites as in the normal embryo when two streaks form (0-6 hours; Fig. 2G). When the pellets are grafted 6 hours apart, in no case did the hypoblast arising from the first...
pellet reach the second pellet after 6 hours (Fig. 2H). This finding suggests that Cerberus is not the inhibitory molecule induced by Vg1. Moreover, as hypoblast formation and movements similar to those of the normal embryo are seen in isolated anterior halves (Fig. 1D,J), this suggests that the inhibitor from which the anterior half is released by cutting is also distinct from Cerberus.

The primitive streak emits an inhibitor

In both experimental paradigms that reveal the existence of an inhibitor (isolated half-embryos and ectopic Vg1), additional axes can no longer be induced after the normal primitive streak appears (stage 2-3) (Spratt and Haas, 1960; Seleiro et al., 1996; Shah et al., 1997). Two different situations can explain this: either cells in the anterior marginal zone lose their competence to Vg1 at stage 2-3, or these cells are still competent but somehow inhibited. To distinguish between these possibilities, we grafted a Vg1 pellet in the anterior marginal zone of stage 2-3+ embryos, cultured either intact or after removal of the half of the embryo containing the primitive streak (Fig. 3A). In no case did an ectopic streak form in intact embryos (0/21 this study) (see also Shah et al., 1997); likewise, in no case did an axis develop when mock-transfected cells were grafted into anterior halves isolated at stage 2-3 (0/14). By contrast, 31% of isolated anterior halves implanted with a source of Vg1 developed a primitive streak arising from the pellet (8/26; P < 0.01; Fig. 3B). However the capacity to respond to Vg1 is lost at still later stages: anterior halves from older streak stage embryos (4+, 4, 4+), never form a streak after a Vg1 graft (0/22).

These results suggest that until the mid-primitive streak stage (stage 3), the primitive streak region emits an inhibitor which prevents axis formation in the anterior part of the embryo. By stage 4+, however, the embryo loses its competence for induction of an ectopic axis by Vg1.

**The inhibitor acts downstream of Vg1 but upstream of Nodal and Chordin**

Vg1 has been shown to require Wnt signalling to induce a primitive streak (Joubin and Stern, 1999; Skromne and Stern, 2001), raising the possibility that the inhibitor might work by antagonising Wnt signalling. To test this, we misexpressed Vg1 on the lateral margin, followed 6 hours later on the opposite side by another pellet of Vg1 together with a pellet of Wnt1-expressing cells. Only 3/34 (9%) embryos produced a primitive streak from the second implantation site, not significantly different from when a second Vg1 pellet was implanted alone (see above).

Vg1 misexpression in the anterior part of a pre-streak embryo induces a cascade of gene activation: *Nodal* is induced after 6 hours, followed by *Chordin* after 9 hours (Skromne and Stern, 2002). As both Nodal and Chordin have been implicated in primitive streak formation (Streit et al., 1998; Streit and Stern, 1999b; Bertocchini and Stern, 2002), it is important to determine whether the inhibitor acts by antagonising these factors. To this end, we grafted a Vg1 pellet on one side (stage X-XII), followed 6 hours later by either Vg1 together with Nodal or Vg1 with Chordin on the opposite side (Fig. 4A). If the inhibition takes place downstream of Chordin activation, co-expression of Chordin should not be sufficient to induce an ectopic axis. If the inhibitor works between Nodal and Chordin, or upstream of Nodal, misexpression of Chordin in the first case, or either Chordin or Nodal in the second case, should be enough to bypass the inhibitory step. Grafting a second Vg1 pellet together with either factor induces an ectopic primitive streak (18/27 (67%) with Vg1+Chordin;
Inhibitors of primitive streak formation in the chick

14/29 (48%) with Vg1+Nodal] (Fig. 4B,C), suggesting that the inhibition takes place downstream of Vg1 but upstream of Nodal and Chordin. However, when either Nodal or Chordin is grafted alone 6 hours after the first Vg1 pellet on the opposite side, no induction takes place (0/33 with Nodal, 1/34 with Chordin). This latter result suggests that in addition to Nodal and Chordin, Vg1 must induce another factor necessary to bypass the inhibitory step and allow an ectopic axis to form.

**FGF signalling cooperates with Vg1 to induce the primitive streak**

FGF8 is expressed in Koller’s sickle and in the hypoblast of pre-streak stage embryos (Mitrani et al., 1990; Foley et al., 2000; Streit et al., 2000; Chapman et al., 2002) (see also Fig. 1J). To analyse the involvement of FGF signalling in primitive streak induction, we grafted FGF8b- or FGF4-coated beads in the anterior marginal zone or area pellucida of young embryos. Both FGF8b and FGF4 can induce an ectopic axis that includes mesoderm and organizer cells (Fig. 5A,B), but FGF4 is more potent than FGF8b [40/51 (78%) and 4/35 (11%), respectively; control embryos, 0/22]. The competence to FGF wanes at later stages: anterior misexpression of FGF4 at stage 3/3+ produced an ectopic streak expressing Brachyury in 7/25 intact embryos (28%) (Fig. 5C), and in 3/7 (43%) isolated anterior halves (not shown). Together, these results implicate FGF signalling in primitive streak induction. Cells are competent to respond even at the primitive streak stage, but the effect is also subordinate to the inhibitor(s) produced by the primitive streak.

As a further test for a role of FGFs in axis formation, we used the FGF-receptor inhibitor SU5402 (Mohammadi et al., 1997; Streit et al., 2000). When applied to the posterior area pellucida (Fig. 5D), SU5402 either blocks streak formation completely [3/25 (12%); Fig. 5F] or causes a lateral shift in its position [13/25 (52%), Fig. 5G when compared with 4/17 (23%) in control embryos, Fig. 5E; \( \chi^2 \) test: \( P=0.011 \)].

Does FGF signalling overcome the Vg1-induced inhibitor? We grafted Vg1 in the lateral marginal zone and FGF4 in the opposite side 6 hours later (Fig. 6A); 14/26 (54%) embryos developed a double axis, one from each side (Fig. 6B), and 4/26 (15%) developed a streak only from the FGF4 side. This indicates that the inhibition initiated by Vg1 does not affect the response to FGF. The same result was obtained with FGF8b [which again was less potent; 4/40 (10%) with two axes; not shown].

This finding raises two possibilities: either the mechanisms of action of Vg1 and FGF are completely independent, or they act together. To distinguish between them, we misexpressed Vg1 in the anterior marginal zone together with SU5402 in the adjacent area pellucida (Fig. 6C,D). Only 6/24 (25%) cases generated an ectopic primitive streak, when compared with 16/18 (88%) of controls (Vg1 with a control bead; not shown) (\( \chi^2 \) test: \( P<0.005 \)). This result, together with the finding that SU5402 inhibits endogenous primitive streak formation, suggests that induction of the primitive streak by Vg1 requires FGF signalling, and therefore that the two mechanisms synergise.

Does FGF synergise with Vg1, or with one or both of the downstream components Nodal and Chordin? We addressed this using both loss of FGF function (SU5402) and gain-of-function (misexpression) approaches. For the former, we misexpressed Vg1 in the anterior marginal zone, together with a SU5402-soaked bead and either Chordin or Nodal in the...
adjacent area pellucida. An ectopic primitive streak developed in 18/39 (46%) embryos when Nodal was used and in 27/44 (61%) with Chordin, while 4/17 (24%) embryos grafted with Vg1+SU5402+mock-transfected cells developed a primitive streak (P<0.05 for Chordin, P≈0.2 for Nodal). This result suggests that Chordin can induce an axis in the absence of FGF signalling, and Nodal has a weaker effect.

As a second approach, we misexpressed FGF8b together with either Chordin (Fig. 6G) or Nodal. For Chordin, 16/27 embryos (59%) grafted with FGF8b+Chordin in the area pellucida developed an ectopic axis (Fig. 6H), more frequently than either FGF8b [1/12 (8%)] or Chordin alone [3/15 (20%)]. When FGF8b+Chordin were misexpressed in the lateral side 6 hours after a Vg1 pellet in the opposite side (Fig. 6E), a second streak developed in 9/39 (24%) cases (Fig. 6F), more frequently than with either Chordin alone [1/34 (3%)] or FGF8b alone [4/40 (10%)]. For Nodal, however, the results were different. FGF8b+Nodal yielded an axis in 26/80 embryos (33%) (Fig. 4L), when compared with 0/20 with Nodal alone (Bertocchini and Stern, 2002) and 1/12 (8%) with FGF8b alone (P≈0.1); and misexpression of Nodal+FGF8b 6 hours after a Vg1 graft generated an ectopic axis in 6/39 embryos (15%), when compared with 0/33 with Nodal alone and 4/40 (10%; P>0.05) with FGF8b alone.

We suspected that the weak effect of Nodal in these experiments might be due to the expression of the Nodal antagonist Cerberus by the underlying hypoblast at the time of the graft (Bertocchini and Stern, 2002). Consistent with this, the same experiment performed after hypoblast removal led to an ectopic streak in a much higher proportion of cases (9/21; 43%), when compared with 0/24 for Nodal alone (P<0.001).

Taken together, these results suggest that FGF signalling is required for primitive streak formation and that it cooperates with Nodal and Chordin in this process.

Discussion

A fast-travelling inhibitor of embryonic axis development, distinct from Cerberus

The finding that fragments of chick embryos deprived of the normal site of primitive streak formation can spontaneously initiate formation of a complete embryonic axis (Lutz, 1949; Spratt and Haas, 1960) predicted the existence of an inhibitor emitted by the posterior end of the embryo. The experiments described here lend support to this hypothesis, and establish that the inhibition travels across the embryo (3 mm distance) in less than 6 hours.

Other recent work revealed that the hypoblast (an extra-embryonic tissue that underlies the embryonic epiblast, from which the embryo proper arises) emits Cerberus, which acts as an antagonist of Nodal and prevents premature and ectopic primitive streak formation (Bertocchini and Stern, 2002). It was proposed that just prior to primitive streak formation, the hypoblast (along with Cerberus expression) is displaced away from the posterior edge, which allows Nodal signalling to initiate the process of streak formation (Bertocchini and Stern, 2002). Comparable findings were made in mouse embryos lacking both cerberus and another Nodal antagonist, Leftib: in the absence of both antagonists, multiple primitive streaks develop (Perea-Gómez et al., 2002), confirming that release from an inhibitor of Nodal signalling is a conserved mechanism to ensure that only a single primitive streak forms in amniotes.

The inhibitor whose properties are revealed by the present experiments does not appear to be Cerberus, and is not associated with hypoblast movements. Although misexpression of Vg1 does coordinate the formation of a new lower layer (including both the coalescence of hypoblast cells into a layer and the induction of endoblast) from a new site, as revealed by the expression of hypoblast markers (goosecoid, Hex, Cerberus, crescent, HNF3β), the appearance of this layer does not correlate with the spreading of the fast travelling inhibitor described.
Other candidate inhibitors of primitive streak formation

Our experiments in the chick (Bertocchini and Stern, 2002) (this paper) are consistent with work from zebrafish (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Feldman et al., 2000; Shimizu et al., 2000; Sirotkin et al., 2000; Chen and Schier, 2001; Chen and Schier, 2002; Schier, 2003), *Xenopus* (Jones et al., 1995; Smith et al., 1995; Lustig et al., 1996; Agius et al., 2000; Kodjabachian, 2001) and mouse (Zhou et al., 1993; Conlon et al., 1994; Varlet et al., 1997; Perea-Gómez et al., 2002), implicating Nodal as an essential inducer of axial mesoderm and required for gastrulation. In addition to Cerberus, two antagonists of Nodal signalling have been identified in zebrafish, *Xenopus* and mouse: Leftb (Lefty1) and Ebf (Lefty2) (Meno et al., 1996; Chen et al., 1997; Chen et al., 1999; Chen and Schier, 2002).

Could Lefty proteins be the early primitive streak inhibitors? This possibility is made likely by the finding that Lefty proteins act as long-range antagonists of Nodal (Squint) signalling without affecting short range Nodal (Cyclop) signals (Chen and Schier, 2002). These properties are exactly what would be required for an inhibitor emitted by the site of axis formation, as they would allow local Nodal signalling (primitive streak formation) while inhibiting this process remotely.

To date, only a single Lefty gene has been identified in the chick, named *Lefty1* (Ishimaru et al., 2000). However, this is more likely to be the orthologue of mouse *Ebf* (previously *Lefty2*), as it is expressed in the primitive streak itself but not at earlier stages (Meno et al., 1997; Meno et al., 1998; Ishimaru et al., 2000; Meno et al., 2001; Bertocchini and Stern, 2002). Despite several attempts, we have been unable to isolate another Lefty gene from the chick to test this. If a true orthologue of mouse *Lefty* does exist in the chick, its expression should reveal whether it fulfills the criteria for being the inhibitor described here. In the mouse, *Ebf* is expressed in the anterior visceral endoderm (AVE), which is equivalent to the chick hypoblast. If the putative chick orthologue is expressed only in the hypoblast, it could also not be the inhibitor whose identity we seek, because, as described above, the spreading of the hypoblast layer does not correlate in space or time with the inhibitor induced by Vg1 misexpression and is also too slow to ensure the formation of a single axis in bisected embryos.

Therefore, there appear to be at least two distinct inhibitors of primitive streak formation in the chick: an early, fast-travelling wave initiated by Vg1 or one of its targets in the marginal zone (for which the missing orthologue of mouse *Leftb* is a candidate); and a later antagonist of Nodal signalling (Cerberus), which accompanies the spreading of the hypoblast layer (Bertocchini and Stern, 2002). At later stages, we have also argued (Bertocchini and Stern, 2002) for a role of chick *Lefty1* (or the orthologue of mouse *Ebf*) from the primitive streak as a third mechanism to ensure that only a single primitive streak develops.

**Koller’s sickle, the posterior marginal zone and the amniote ‘Nieuwkoop centre’**

A previous study identified the posterior marginal zone (PMZ) of the avian embryo, the region that expresses Vg1, as the functional equivalent of the Nieuwkoop centre of amphibians (Bachvarova et al., 1998). This was based on the observation that grafts of the marginal zone that exclude the neighbouring Koller’s sickle can induce the formation of a complete embryonic axis without making a cellular contribution to the induced axis, the properties that initially defined the Nieuwkoop centre (see Nieuwkoop, 1969a; Nieuwkoop, 1969b; Lemaire et al., 1995; Harland and Gerhart, 1997). However, others have argued that Koller’s sickle is indispensable for axial development, and that this rather than the PMZ is the equivalent of the Nieuwkoop centre (Callebaut and Van Nuaten, 1994). Our present results argue strongly in favour of the former interpretation: both in embryo fragments and when an ectopic axis is initiated by misexpression of Vg1, the primitive streak appears without any sign of prior induction of a Koller’s sickle-like region. Furthermore, molecular markers for the sickle (*Chordin*, *FGF8*, *goosecoid*) are relatively late responses to either manipulation, and are not expressed until after the primitive streak can be seen both morphologically and by expression of the mesodermal marker *brachury* (this study) (Skromne and Stern, 2002).

Koller’s sickle has also been claimed to be the region through which the endoblast forms (Azar and Eyal-Giladi, 1979; Azar and Eyal-Giladi, 1983; Callebaut and Van Nuaten, 1994), which led to the original name of the latter as ‘sickle endoblast’ (Vakaet, 1970; Callebaut and Van Nuaten, 1994). In our experiments, an endoblast layer forms both in cut embryos and after misexpression of Vg1, but in the absence of a sickle. This finding is more consistent with the alternative explanation that the endoblast arises not from the sickle but from the adjacent yolky germ wall margin (Stern and Ireland, 1981; Stern, 1990). In conclusion, the present experiments suggest that Koller’s sickle is not essential either for the formation of the embryonic axis or for the development of endoblast in the lower layer.

**A role for FGF signalling in axis formation**

A large body of evidence has implicated Nodal signalling in mesoderm/endoderm induction (see above). Other experiments have established that the induction of mesoderm/endoderm by TGFb signals related to Nodal requires activation of the FGF pathway (Kimmelman and Kirschner, 1987; Cornell and Kimmelman, 1994; Le bonne and Whitman, 1994; Cornell et al., 1995). In the chick embryo, similar to early findings in amphibians, it was reported that FGF can act as a sufficient mesoderm inducer (Mitrani et al., 1990). The present results are consistent with the new widely accepted notion that FGFs act in synergy with Nodal-related signals in mesendoderm induction. Of all the factors implicated in primitive streak initiation to date (Vg1, Wnt8C, Nodal, Chordin, FGFs), only the latter can by itself overcome the effects of the inhibitor induced by Vg1, consistent with the idea that FGFs act through a parallel pathway with TGFb5, and synergise with them. We also show that, as in amphibians, FGF signalling is required for normal primitive streak formation and for induction of a streak by Vg1 and its targets, as both processes are completely blocked in the presence of the FGF inhibitor SU5402.

**A cascade of genes regulating axis development in the chick embryo**

Our findings, together with previous studies on the molecular bases of initiation of primitive streak formation in the chick embryo, allow us to propose a model (Fig. 7) for how the embryo initiates primitive streak formation and how it ensures
that only a single axis forms despite the widespread potential of any region of the embryo to do so. The earliest known molecular asymmetry preceding axis formation is the localised expression of chick \textit{Vg1} in the posterior marginal zone, in a region overlapping with expression of chick \textit{Wnt8C}. It has been shown that Wnt activity is required for \textit{Vg1} to induce an axis, and that chick \textit{Wnt8C} is expressed all around the margin of the embryo, suggesting that this factor defines the entire marginal zone as a unique region (Skromne and Stern, 2001). The convergence of \textit{Vg1} and Wnt activity then induces \textit{Nodal} in the adjacent epiblast of the area pellucida (Bertocchini and Stern, 2002; Skromne and Stern, 2002). However, \textit{Nodal} cannot act because it is covered by the hypoblast, which secretes Cerberus (Bertocchini and Stern, 2002): primitive streak formation is repressed until the removal of Cerberus that results from displacement of the hypoblast by endoblast tissue (which does not express \textit{Cerberus}). We now suggest that FGF8 (which is expressed both by the hypoblast and by Koller’s sickle cells) and/or other members of the FGF family synergise with \textit{Nodal} to initiate primitive streak formation at this time.

\textit{BMP} signalling also regulates primitive streak formation: its activity needs to be lowered for primitive streak formation to occur, which can be effected by Chordin but not by Noggin (Streit et al., 1998; Streit and Stern, 1999b). During normal development, \textit{Chordin} is expressed in Koller’s sickle, while BMP4 is present at a low level throughout the embryo (Wilson et al., 2001; Chapman et al., 2002). However, neither Koller’s sickle nor Chordin expression is seen in isolated anterior halves or after ectopic expression of \textit{Vg1} until after the appearance of an ectopic primitive streak, suggesting that Chordin is not required for the initiation of streak formation. This is supported by the observation that mouse embryos lacking Chordin function still develop a primitive streak and only die at much later stages (Bachiller et al., 2000). We propose that the most parsimonious mechanism to unify all of these results is that cells in the epiblast integrate signals that result in the activation (phosphorylation) of Smad1/5/8 (BMP targets) and Smad2/3 (Nodal/activin targets) by measuring the ratio between them (van Grunsven et al., 2002; Shi and Massague, 2003; Zwijsen et al., 2003). Provided that FGF signalling is also present, and that the ratio of Smad activation favours Smad2/3 over the BMP targets, primitive streak formation will occur.

At the same time, an antagonist of primitive streak formation other than Cerberus further ensures the formation of a single primitive streak by rapidly conveying to remote parts of the embryo the information that primitive streak development is already underway elsewhere. A crucial feature of this inhibitor, which remains to be identified, must therefore be either rapid propagation through the embryo without affecting cells near its site of production (therefore \textit{Lefty1} may be a candidate; see above). Our results also suggest that it is induced by \textit{Vg1} and acts between \textit{Vg1} and \textit{Nodal} in the genetic cascade.

An additional mechanism that remains to be understood is how the embryo positions \textit{Vg1} expression at the site where primitive streak formation will begin. Chick \textit{Vg1} is the earliest known gene to be expressed in anterior half-embryos in a manner that predicts where the axis will form and is also the only known marker for the PMZ. Anterior half-embryos, which only generate a single axis, positioned randomly on the left or right of the fragment, also possess only a single randomly located site of chick \textit{Vg1} expression. Whatever is responsible for this must therefore also travel rapidly across the embryo (and therefore may be identical to the fast inhibitor described), or be present as a pre-existing gradient (for which \textit{Wnt8C} is a...
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