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The Hypoblast of the Chick Embryo Positions the Primitive Streak by Antagonizing Nodal Signaling

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Summary

The hypoblast (equivalent to the mouse anterior visceral endoderm) of the chick embryo plays a role in regulating embryonic polarity. Surprisingly, hypoblast removal causes multiple embryonic axes to form, suggesting that it emits an inhibitor of axis formation. We show that Cerberus (a multifunctional antagonist of Nodal, Wnt, and BMP signaling) is produced by the hypoblast and inhibits primitive streak formation. This activity is mimicked by Cerberus-Short (CerS), which only inhibits Nodal. Nodal misexpression can initiate an ectopic primitive streak, but only when the hypoblast is removed. We propose that, during normal development, the primitive streak forms only when the hypoblast is displaced away from the posterior margin by the endoblast, which lacks Cerberus.

Introduction

A critical early event in embryonic development involves breaking the initially radial or spherical symmetry of the egg to establish the position from which the body axis will arise. Most of what we know about this is derived from studies in *Drosophila* and *Xenopus*. Both species rely on localization of maternal components to specific regions of the egg cytoplasm, such that they are differentially inherited by subsets of daughter cells, which thus acquire different fates. As a result, embryonic polarity and some cell fates are established very early in development. Despite having a very early bias in their polarity (Zernicka-Goetz, 2002), amniote embryos (reptiles, birds, and mammals) seem to have evolved a different, "regulative" strategy: many cell divisions take place before portions of the embryo lose their ability to form an embryonic axis (for review see Arendt and Nübler-Jung, 1999). In the chick embryo, any pie-shaped slice cut from an embryo at the "blastoderm" (20,000–60,000-cell) stage can generate a complete axis (Spratt and Haas, 1960). This regulative ability is retained up to the beginning of gastrulation, when the first axial structure (the primitive streak) appears. The finding that any portion of the blastoderm has the potential to generate an axis spontaneously raises an obvious question: what mechanisms ensure that only one primitive streak develops?

Before gastrulation, the chick blastoderm is a flat disc with two cell layers. The upper layer (epiblast) is an epithelium containing all the cells that will contribute to the later embryo. The lower layer contains a succession of cell populations (for review see Foley et al., 2000; Figure 1). A first component (hypoblast) forms by coalescence of cells probably derived by earlier polyingression from the epiblast; it expands in a posterior to anterior direction until it covers most of the blastoderm. Almost immediately, a second cell population (the "endoblast," or "sickle endoblast"; Vakaet, 1970; Stern, 1990; Callebaut et al., 1997) appears at the posterior edge and gradually spreads, displacing the original hypoblast anteriorly. Neither hypoblast nor endoblast contributes any cells to the embryo—both give rise only to extraembryonic tissues and, in many respects, resemble the visceral endoderm (VE) of the mouse (Thomas and Beddington, 1996; Beddington and Robertson, 1998), of which the hypoblast corresponds to the anterior part (AVE) (Foley et al., 2000). Immediately after the endoblast insinuates itself posteriorly, the primitive streak arises at this site.

Although this early lower layer does not contribute to the embryo, it influences the orientation of the embryonic axis (Waddington, 1932, 1933; Azar and Eyal-Giladi, 1981; Khaner, 1995; Callebaut et al., 1999; Foley et al., 2000), but the mechanisms are unknown. Many thought that it "induces" the primitive streak (Waddington, 1933; Azar and Eyal-Giladi, 1981), but this has been challenged by the finding that experimental rotation of the lower layer influences cell movements but does not change cell fates (Waddington, 1932; Khaner, 1995; Callebaut et al., 1999; Foley et al., 2000).

Here, we define a novel, inhibitory role of the hypoblast, which helps to reconcile earlier contradictory findings and provides a mechanism to prevent the formation of multiple embryos. Within the lower layer, the hypoblast component blocks primitive streak formation by emitting antagonists of the Nodal signaling pathway. We propose that the displacement of the hypoblast away from the posterior margin by the endoblast is a critical event in primitive streak formation, by removing Nodal antagonists from this site.

Results

The Hypoblast Is Marked by Expression of Antagonists of Signaling Pathways

Morphological (Vakaet, 1970), cell labeling (Stern, 1990), and molecular (Foley et al., 2000) evidence has revealed the existence of two distinct cell types within the lower (endodermal) layer of the preprimitive streak chick embryo: hypoblast and endoblast (see above). To identify novel signaling molecules distinguishing these cell types, we performed a differential screen between small groups of hypoblast and endoblast cells isolated from an embryo at stage 3 (Hamburger and Hamilton, 1951). Several new marker genes were identified (to be described elsewhere), but none encoded secreted proteins. The only hypoblast-specific secreted products identified thus far are antagonists of known pathways: Cerberus inhibits Wnt, BMP, and Nodal, Crescent is a

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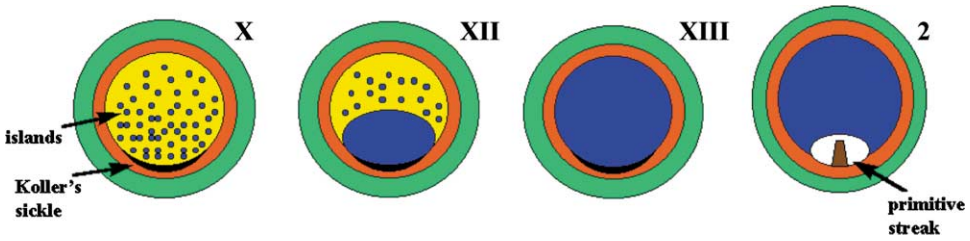

Figure 1. Hypoblast and Endoblast in Early Chick Embryos

Scheme of early stages of development. At stage X the embryo is essentially one-layer thick, except for small islands of hypoblast (blue) at its ventral surface. At stage XII, the hypoblast has formed a sheet that covers half of the area pellucida and, by stage XIII, covers the entire surface. The primitive streak (brown) appears at stage 2, when the hypoblast has started to become displaced anteriorly by the endoblast (white). Green, area opaca; red, marginal zone; yellow, area pellucida epiblast.

Wnt antagonist, and Dkk1 inhibits Wnt and BMP; no secreted proteins specific to the endoblast have yet been found (Piccolo et al., 1999; Belo et al., 2000; Marvin et al., 2001; Schneider and Mercola, 2001; Skromne and Stern, 2001).

Removal of the Hypoblast Causes Formation of Ectopic Primitive Streaks

The finding that the only known secreted proteins specific to the hypoblast are antagonists of known signaling pathways prompted us to investigate the effects of the removal of this cell layer on axis development. We were surprised to find that, after removal of the hypoblast sheet from stage XII–XIII embryos (Figures 1 and 2A) and incubation of the embryos for 15–20 hr, one or more ectopic primitive streaks appeared in random positions of the blastoderm (15/64 cases, 23%, versus less than 1% in unmanipulated embryos; Figures 2B–2E). No difference was observed ($p = 0.9$) in the incidence of ectopic axes between embryos operated at stage XII (11/46) or XIII (4/18). Up to three separate primitive streaks were seen in a single blastoderm (3/15 cases), each characterized by an accumulation of mesoderm cells, a primitive groove, and discernible Hensen's node. In some cases, however, one or more of the streaks regresses and only one axis develops further. By contrast, no ectopic axes formed when the hypoblast was removed and replaced in its original position ($n = 21$). The ectopic primitive streaks were correctly patterned, as revealed by the expression of *Brachyury* (a marker for the entire streak; Figures 2B–2D), *Vg1* (a marker for post-Nodal and middle streak; Figure 2E) and *Chordin* (a marker for the node; data not shown). These results suggest that the hypoblast somehow inhibits formation of the primitive streak.

Recently it was shown that Vg1, acting together with Wnts, can initiate a local cascade of gene activation, leading to formation of an ectopic primitive streak (Skromne and Stern, 2001, 2002). To assess which level of this cascade is antagonized by the hypoblast, we examined the expression of several genes in this pathway at shorter intervals (6–9 hr) after hypoblast removal. We did not observe ectopic expression of the most upstream component (*Vg1*) but detected ectopic expression of the more downstream components, *Chordin* and *Nodal* (Figures 2F and 2G), with a frequency similar to the observed incidence of ectopic streaks (8/26 cases, 31%). Together, these results suggest that the hypoblast inhibits primitive streak formation by antagonizing a step downstream of the initial induction by Vg1 plus Wnt.

Gain-of-Function Experiments Implicate Nodal

Which of the three known signaling pathways antagonized by the hypoblast (BMP, Wnt, or Nodal) accounts for the formation of ectopic primitive streaks following hypoblast removal? The hypoblast expresses Cerberus and Dkk1, two multifunctional secreted proteins that inhbit BMPs and other signals (Glinka et al., 1998; Piccolo et al., 1999). BMP inhibition by Chordin (but not Noggin) has been shown to be sufficient to initiate formation of a primitive streak (Streit et al., 1998; Streit and Stern, 1999). Moreover, misexpression of either BMP4 or BMP7 inhibits primitive streak formation (Streit et al., 1998); it is therefore very unlikely that hypoblast removal acts through this pathway.

Dkk1, Crescent, and Cerberus (Foley et al., 2000), expressed by the hypoblast, all antagonize the Wnt pathway. We therefore tested the ability of Wnt to induce a primitive streak by misexpressing Wnt1 in the presence (Skromne and Stern, 2001) or absence (this study; $n = 16$) of the lower layer. Virtually no ectopic streaks were seen when the hypoblast was present (1/24). When the hypoblast was removed, 2/16 (13%) embryos had an ectopic streak in addition to the normal axis, but none of these streaks arose from the site of implantation (data not shown).

Finally, Cerberus is also an antagonist of Nodal signaling (Piccolo et al., 1999; Belo et al., 2000). To investigate whether this pathway is responsible for the effects of hypoblast removal, we placed a pellet of Nodal-transfected COS cells into the lateral area pellucida of stage XII embryos either in the presence or absence (Figure 3A) of the hypoblast. When the hypoblast was present, Nodal did not induce ectopic primitive streaks or ectopic expression of the streak marker *Brachyury* (0/20; data not shown). Likewise, implantation of control, mock-transfected COS cells in the absence of the hypoblast had no effect (0/31; Figure 3D). By contrast, when the hypoblast was removed, Nodal induced an ectopic primitive streak arising from the site of misexpression in 18/66 cases (27%, $p < 0.001$) (Figures 3B–3C). This ectopic primitive streak expressed *Brachyury* throughout its length (Figure 3B) and the organizer marker *Chordin* at its tip (Figures 3C and 3E–3F), and histological analysis

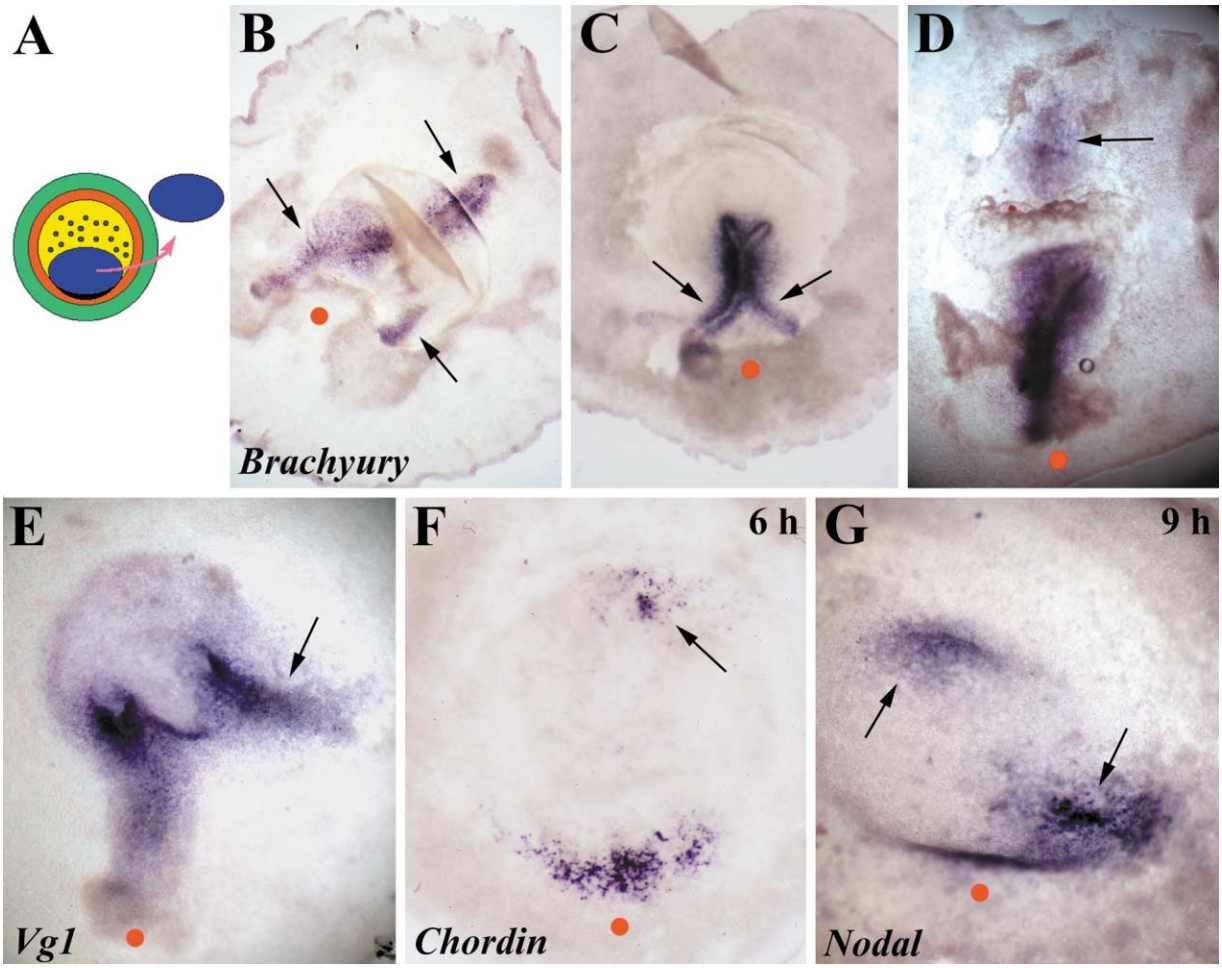

Figure 2. Removal of the Hypoblast Generates Multiple Primitive Streaks

(A) Diagram showing the operation.

(B–E) After incubation overnight (to stage 4), embryos were hybridized with *cBrachyury* (B–D) or *Vg1* (E). Arrows indicate ectopic streaks.

(F and G) Operated embryos cultured for a short period (6 hr in [F] and 9 hr in [G], still before streak formation) and probed for *Chordin* (F) or *Nodal* (G). Arrows indicate ectopic expression. The red spots mark the original posterior pole. Note that in many cases none of the primitive streaks arises from this site (B, C, and G).

revealed that a mesodermal layer had emerged laterally (Figure 3F). When the tip of the ectopic streak is grafted into the area opaca of a host embryo, it induces a full nervous system, including a head and expressing the neural marker *Sox2* ($n = 9$; Figure 3G). In all 18 cases the ectopic primitive streak arose immediately adjacent to the site of misexpression.

Taken together, these gain-of-function experiments indicate that the inhibitory effect of the hypoblast on primitive streak development could be due to antagonism of the Nodal signaling pathway.

Loss-of-Function Experiments Implicate Nodal in Primitive Streak Formation

At present it is difficult to perform loss-of-function experiments in chick embryos with genetics, and we therefore resorted to misexpression of the Nodal antagonist, Cerberus. This multifunctional antagonist inhibits BMP and Wnt signaling as well as Nodal (Piccolo et al., 1999; Belo et al., 2000). However, a truncated form of Cerberus, called Cerberus-Short (CerS), is a specific Nodal antagonist (Piccolo et al., 1999). To test the ability of Cerberus and CerS to inhibit Nodal signaling in this system, we compared the effects of misexpressing Nodal alone with those of Nodal together with either Cerberus or CerS. The hypoblast was removed from stage XII embryos, and a pellet of *Nodal*-transfected COS cells was implanted laterally on each side of the embryo. One of the two Nodal pellets was surrounded with four pellets of *Cerberus*- or *CerS*-transfected COS cells (Figure 3H). As expected from the experiments above, Nodal alone induced an ectopic primitive streak (13/69, 19%; Figures 3I and 3J, right side). Both Cerberus (0/42 with ectopic streaks from the site of implantation; Figure 3J, left side) and CerS (2/27, 7%; Figure 3I, left side) inhibited this induction ($p < 0.01$). As an additional control to rule out the possibility that the presence of additional cells inhibits the effects of Nodal, we surrounded the Nodal cells with four pellets of mock-transfected COS cells; an ectopic primitive streak arose from the implantation site in 6/37 (16%) cases, indistinguishable from the effects of Nodal alone. Together, these results show that

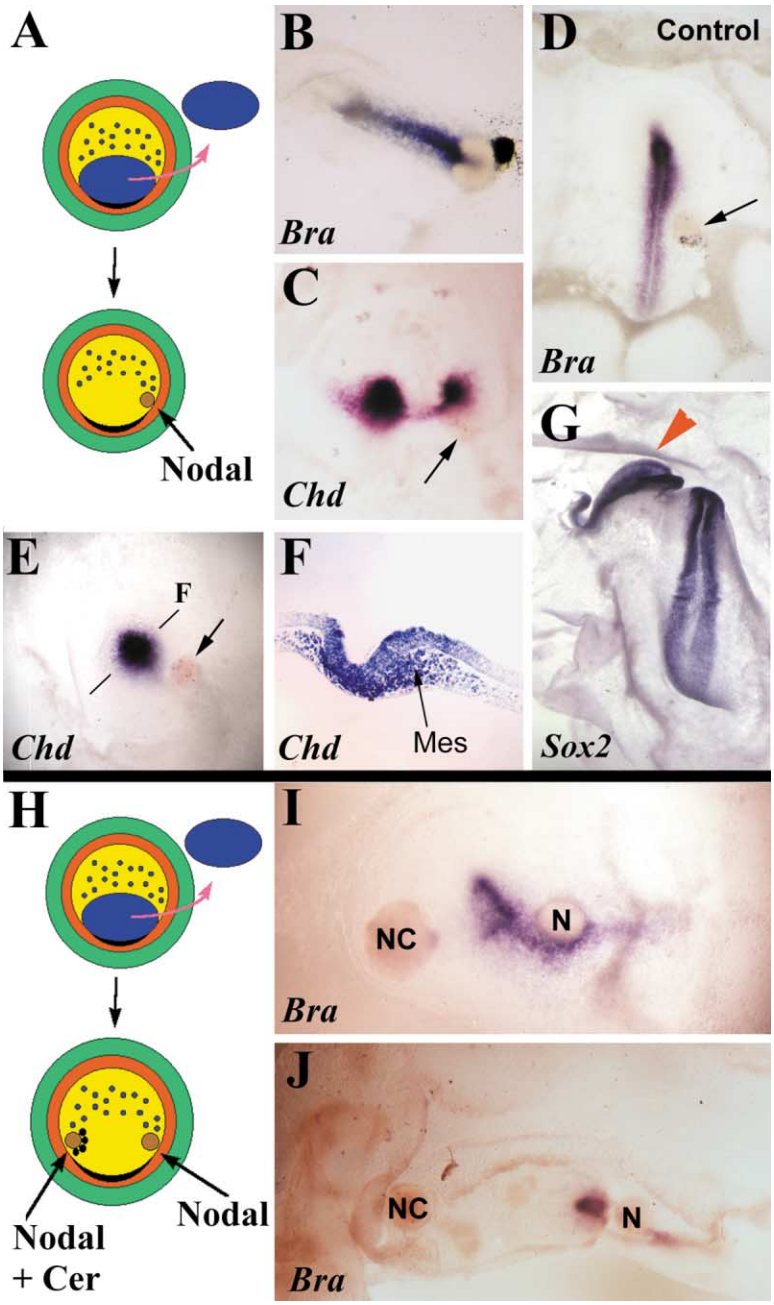

Figure 3. Nodal Induces and Cerberus Inhibits Ectopic Primitive Streak Formation

(A) The hypoblast was removed at stage XII, and *Nodal*-transfected COS cells grafted in the lateral area pellucida.

(B and C) Examples of the primitive streak induced by Nodal. Ectopic streaks arise either instead of (B), or together with (C), the original axis. Embryos were hybridized with *cBrachyury* (B and D) or *Chordin* (C, E, and F).

(D) Control embryo (hypoblast removed) grafted with mock-transfected cells.

(E–G) The ectopic primitive streak is patterned correctly. It expresses *Chordin* at its tip (C, E, and F), and, when this tip is transplanted to a host embryo, it induces a complete ectopic nervous system (red arrowhead) expressing the neural marker *Sox2* (G). Arrows point to the grafts. (F) shows mesoderm emerging from the ectopic streak in a section through the *Chordin*-expressing node of the embryo in (E).

(H) Two Nodal cell pellets were grafted after hypoblast removal, one on each side. Grafts of Cerberus or CerS cells were placed around one of the Nodal pellets (left in the diagram).

(I–J) Nodal induced a primitive streak only in the absence of CerS (I) or Cerberus (J) (right). Embryos hybridized with *cBrachyury*. N, Nodal ; NC, Nodal plus Cerberus or CerS.

Cerberus and CerS can act as effective antagonists of Nodal signaling.

If the hypoblast acts during normal development by antagonizing Nodal signaling until its removal by the endoblast, then it should be possible either to block or to displace the formation of the normal primitive streak by misexpression of Nodal antagonists at the site of normal streak formation. To test this, we implanted three to five pellets of *Cerberus*- or *CerS*-transfected COS cells at the posterior edge of the area pellucida at stage XIII, immediately before the endoblast starts to form at this site (Figure 4A). Because of the movements of the lower layer, some of these pellets moved away from the site of implantation (35/84)—these embryos were not considered in the analysis. In the embryos where the pellets remained in position, the primitive streak was either displaced laterally (radially), away from its normal site, arising from a region not immediately adjacent to the pellets (29/53 cases, 55%; Figures 4B and 4C) or failed to form altogether (9/53 cases, 17%). Cerberus caused displaced streaks in 15/32 (47%) cases and failure of streak development in 8/32 (25%) cases (total, 72% cases with effects on polarity). CerS was also effective, with 14/21 (67%) cases of displacement and no streak formation in 1/21 (5%) cases (total, 71% with affected polarity). Mock-transfected pellets had no effect (0/18, $p < 0.001$) (Figure 4D), demonstrating that the pellets do not impose a mechanical block on primitive streak formation.

Together, these experiments provide evidence that

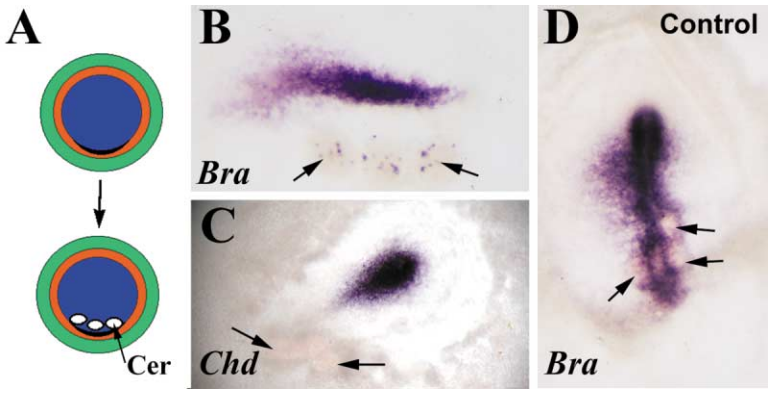

Figure 4. Cerberus Inhibits the Embryo's Own Primitive Streak

(A) *Cerberus*-transfected cells were grafted in the posterior area pellucida of a stage XIII embryo, where the streak normally forms.

(B and C) Ectopic Cerberus expression makes the streak arise from a more lateral position (the apparent transverse orientation is a rotational deviation because the chick embryo is a disk).

(D) Control cells have no influence. Embryos hybridized with *cBrachyury* (B and D) or *Chordin* (C). Arrows indicate the grafts.

Nodal signaling regulates primitive streak formation in the chick embryo.

Nodal Signaling Is Required for Induction of a Primitive Streak by Vg1

It was previously shown that, provided that Wnt signaling is active, Vg1 can initiate formation of a primitive streak at any point around the circumference of the blastoderm (Seleiro et al., 1996; Shah et al., 1997; Skromne and Stern, 2001). A time-course analysis of gene expression after Vg1 misexpression revealed that *Nodal* is induced next to the implanted Vg1 source, but only in the area pellucida adjacent to the graft (Skromne and Stern, 2002). This suggests that Nodal signaling acts in the area pellucida, downstream of the initial induction by Vg1 plus Wnt in the marginal zone. To test this possibility directly, we explored whether Cerberus or CerS can inhibit the induction of a primitive streak by Vg1. *Vg1*-transfected COS cells were implanted in the marginal zone, and *Cerberus*- or *CerS*-transfected cells were placed either in the marginal zone together with the Vg1 pellet (Figure 5A, right) or in the area pellucida adjacent to the Vg1 cells (Figure 5A, left). Inhibition of Nodal signaling in the marginal zone by either antagonist did not affect induction of a primitive streak by Vg1 (11/21, 52%—a similar frequency to that seen with Vg1 alone, 38/62, 61%; Shah et al., 1997; Skromne and Stern, 2001) (Figure 5C). By contrast, inhibition of Nodal signaling in the area pellucida adjacent to the Vg1 source depressed the ability of Vg1 to initiate primitive streak formation (0/7 with CerS and 3/12 with Cerberus, $p < 0.05$; Figure 5B). These results suggest that, while Vg1 plus Wnt initiate a cascade of events from the marginal zone, Nodal signaling is required in the area pellucida adjacent to the marginal zone.

Together, our results strongly implicate Nodal signaling in primitive streak formation. It is required for formation of the normal primitive streak as well as for induction of an ectopic streak by Vg1. Since the hypoblast, but not the endoblast, secretes Nodal antagonists, we propose that the hypoblast may act by preventing primitive streak formation at early stages, until it is displaced by the endoblast.

Discussion

Hypoblast and Endoblast

Waddington's (1932, 1933) discovery that the early lower layer (which he mistakenly called "endoderm") of the chick embryo can control the polarity of the primitive streak was interpreted for a long time as indicating that the hypoblast induces the primitive streak (Waddington, 1933; Eyal-Giladi and Wolk, 1970; Azar and Eyal-Giladi, 1979, 1981, 1983; Mitrani et al., 1983, 1990). Although recent studies have challenged this interpretation (Khaner, 1995; Foley et al., 2000), our finding that removal of the lower layer causes ectopic primitive streaks to appear was still very surprising. It revealed that the hypoblast, rather than inducing a primitive streak, inhibits its formation.

The significance of this became more apparent when we considered the possible role of the endoblast, the second cellular component of the lower layer, which displaces the hypoblast away from the site of primitive streak formation, just before this structure appears (Vakaet, 1970; Stern and Ireland, 1981; Stern, 1990; Callebaut et al., 1997). We therefore investigated whether the expression of antagonists of known signaling pathways in the hypoblast and their absence from the endoblast could account for the finding that the hypoblast inhibits primitive streak formation. The paradoxical role of the hypoblast can be accounted for by postulating that, during normal development, the removal of hypoblast by the endoblast enables Nodal signaling at the margin of the embryo (Figure 6). This conclusion is greatly strengthened by the independent finding in Perea-Gomez (2002 [this issue of *Developmental Cell*]) that mouse embryos lacking two Nodal antagonists (Cerberus-like and Lefty1) also generate an ectopic primitive streak because both of these Nodal antagonists are normally expressed in the anterior visceral endoderm (AVE), the mouse equivalent of the hypoblast (Foley et al., 2000).

Roles of Nodal in Primitive Streak Formation and Mesendoderm Patterning

Compelling evidence implicates Nodal signaling in mesendoderm induction and patterning in mouse, *Xenopus* and zebrafish embryos (Zhou et al., 1993, Jones, 1995; Erter et al., 1998; Osada and Wright, 1999; Agius et al., 2000; Schier and Shen, 2000; Chen and Schier, 2001). Nodal appears to act as a true morphogen in this process, with higher levels of activity generating endoderm and dorsal (axial) mesoderm and lower levels specifying progressively more ventral (lateral) mesoderm (Agius et al., 2000; Chen and Schier, 2001; Episkopou et al., 2001;

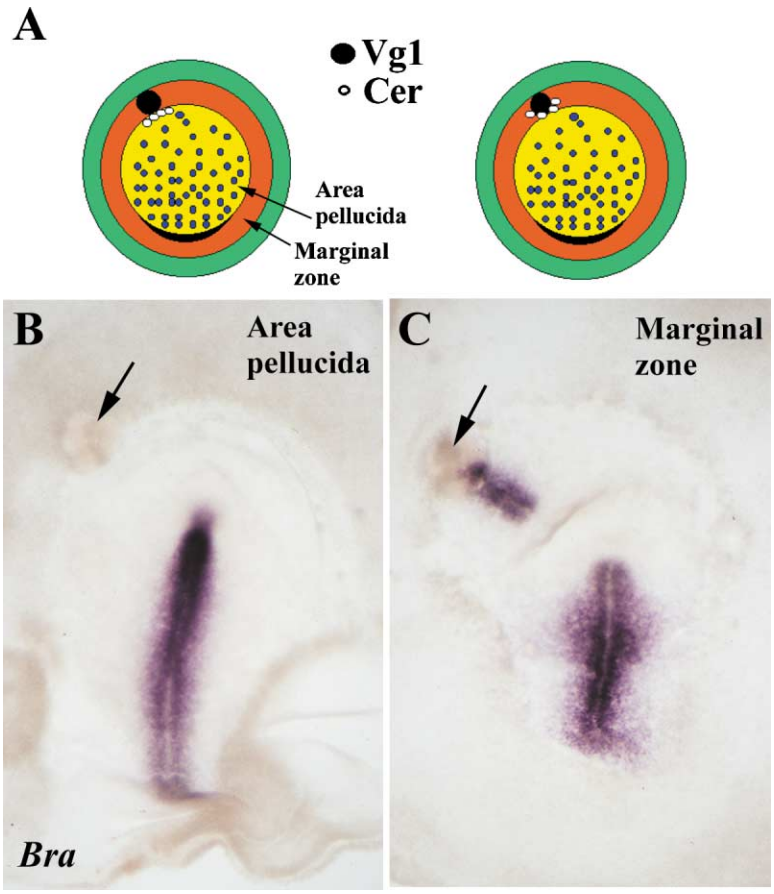

Figure 5. Cerberus Inhibition of Primitive Streak Induction by Vg1

(A) *Vg1*-transfected cells were grafted in the anterior marginal zone, and pellets of *Cerberus*- or *CerS*-transfected cells were placed either in the adjacent anterior area pellucida (left) or together with the Vg1 cells in the anterior marginal zone (right).

(B and C) CerS in the area pellucida prevents induction of an ectopic streak by Vg1 (B), while CerS in the marginal zone does not (C). Arrows point to grafts. Hybridization with *cBrachyury*.

Kodjabachian, 2001; Niederlander et al., 2001). In amniotes the primitive streak is the site of mesoderm and endoderm formation, wherein the most anterior parts (including Hensen's node) give rise to the most dorsal/axial mesoderm and to definitive endoderm, and progressively more posterior parts give rise to more ventral/lateral and extraembryonic mesoderm (Psychoyos and Stern, 1996; Kinder et al., 1999). It therefore seems likely that the initiation of active Nodal signaling that ensues after displacement of the hypoblast away from the site of primitive streak formation enables the process of mesoderm formation to begin but, at the same time, somehow generates gradients of Nodal activity that will eventually contribute to pattern descendants of the primitive streak, so that the anteroposterior axis of the streak will correspond to the axial-lateral axis of the mesoderm. At present we do not understand how this process takes place because the sites and timing of exposure of prospective primitive streak cells to Nodal signaling are complicated by the extensive cell movements that take place in this region at the time of streak formation (Vakaet, 1970; Foley et al., 2000).

Nodal signaling requires cofactors of the one-eyed pinhead/Cripto/EGF-CFC family (Zhang et al., 1998; Gritsman et al., 1999; Schier and Shen, 2000), is modulated by the nuclear factor Arkadia (Episkopou et al., 2001; Niederlander et al., 2001), and is inhibited by Cerberus (Piccolo et al., 1999; Agius et al., 2000) and antivin/Lefty proteins (Meno et al., 1998; Meno et al., 1999; Cheng et al., 2000; Agathon et al., 2001). In the chick, the expression patterns of all of these components are consistent with a role of Nodal in early development. *Nodal* is expressed at the most posterior edge of the area pellucida epiblast, adjacent to the posterior marginal zone (PMZ) (Lawson et al., 2001; Skromne and Stern, 2002; our unpublished data). The PMZ is the chick equivalent of the Nieuwkoop center of amphibians and expresses *Vg1* and *cWnt8C*, which synergize to induce *Nodal* (and eventually the primitive streak and organizer) in the neighboring area pellucida (Bachvarova et al., 1998; Skromne and Stern, 2001, 2002). *Cripto/CFC* is expressed throughout the epiblast at this stage (Colas and Schoenwolf, 2000; Schlange et al., 2001) (and *Arkadia* is likely to be as ubiquitous in chick as it is in mouse and *Xenopus*), suggesting that the regional distribution of Nodal activity is not primarily regulated by the availability of these cofactors. Of the antagonists, only *Cerberus* appears to be expressed early enough to play a role in the initiation of primitive streak development (Foley et al., 2000); *Lefty1* starts to be expressed in the young primitive streak itself (Ishimaru et al., 2000), and *Lefty2* has not yet been isolated. Together, these observations suggest that, in the chick, Nodal activity is primarily regulated by the expression of the Nodal in the epiblast and by the presence of its antagonist, *Cerberus*, in the hypoblast. It is worth noting, however, that chick *Lefty1* (Ishimaru et al., 2000) is expressed more like mouse *Lefty2* than the homonymous gene (Meno et al.,

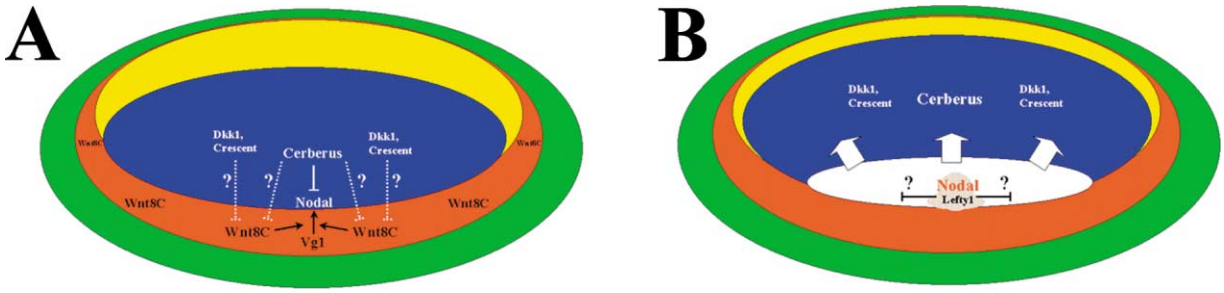

Figure 6. A Model of Primitive Streak Formation in the Chick

Summary of signaling interactions proposed to initiate primitive streak formation. Ventral views of embryos (posterior to the bottom) at stage XII (A) and 2 (B), showing area opaca (green), marginal zone (red), exposed epiblast (yellow), hypoblast (blue), and endoblast (white).

(A) Early on, Vg1 (in the posterior marginal zone) and Wnt8C (throughout the marginal zone) cooperate to induce *Nodal* in the adjacent area pellucida epiblast. However, Nodal signaling is blocked by Cerberus, expressed by the hypoblast.

(B) The hypoblast is then displaced (wide arrows) away by the endoblast, freeing Nodal signaling, which then induces the primitive streak (gray) and expression of Lefty1, proposed to act as a feedback inhibitor of Nodal to prevent formation of additional streaks. Possible roles of Cerberus, Crescent, and Dkk1 as Wnt antagonists are also indicated.

1997; Perea-Gomez et al., 2002). This suggests either that the two genes have exchanged roles during evolution or, probably more likely, on the basis of phylogenetic trees of sequence similarities (Meno et al., 1997), that the gene currently known as chick *Lefty1* is actually *Lefty2*. Resolution of this question will have to await the isolation of the remaining *Lefty* gene in chick and analysis of its expression.

Lefty: A Later Phase of Nodal Inhibition?

Our finding that the hypoblast inhibits primitive streak formation until its removal by the endoblast provides a novel mechanism for ensuring that, in a regulative-type embryo, a single axis forms. However, after Nodal signaling is released from inhibition by the hypoblast and the primitive streak appears, the endoblast continues to push forward and to displace the hypoblast further anteriorly (see Figure 1A). Some mechanism must therefore exist to sense the presence of a streak posteriorly and prevent the appearance of other axes in an expanding region devoid of Nodal antagonists. An obvious possibility (Page et al., 2001) is that the streak itself produces some inhibitor(s). *cLefty1* (or mouse *Lefty2*) fulfills the criteria required for such an inhibitor, since it is expressed in the early primitive streak itself (Meno et al., 1997; Ishimaru et al., 2000) (Figure 6B). Moreover, *Lefty2* is induced by Nodal/Squint, and mice lacking functional *Lefty2* develop with a greatly expanded primitive streak and excessive mesoderm (Meno et al., 1999).

Upstream and Downstream of Nodal: Involvement of Other Pathways

Previous work has revealed that Vg1 will initiate primitive formation when misexpressed in the marginal zone, a region that also expresses Wnt8C (Seleiro et al., 1996; Shah et al., 1997; Skromne and Stern, 2001, 2002). In normal development, the expression of Vg1 and Wnt8C overlap in the posterior marginal zone, which has been shown to be functionally equivalent to the Nieuwkoop center of amphibians (Bachvarova et al., 1998) in that it will induce a primitive streak including the organizer, but without making a cellular contribution to these structures. Grafts of the marginal zone will only induce an ectopic axis until stages X–XI (Khaner et al., 1985; Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989; Bachvarova et al., 1998), suggesting that the role of the marginal zone is restricted to this very early stage of development. Misexpression of Vg1 in the marginal zone induces expression first of Vg1 itself within the marginal zone (3 hr) and then of a cascade of other signaling factors not in the marginal zone, but in the adjacent embryonic epiblast (area pellucida) (Skromne and Stern, 2002). Nodal is among these later components and is induced 6 hr after misexpression of Vg1. Removal of the hypoblast (this study) induces expression of *Nodal*, *Chordin*, and *Brachyury*, but not of *Vg1*. These findings place Nodal signaling downstream of the initial induction by Vg1 plus Wnt8C (Figure 6A).

Other factors appear to act downstream of Nodal. Chordin (Streit et al., 1998; Streit and Stern, 1999) and FGF (Skromne and C.D.S., unpublished data) misexpression can each initiate the formation of an ectopic primitive streak, but, in both cases, the induced streak is "ventral" in character and lacks an organizer. This, along with the finding (this study) that Nodal misexpression induces the dorsal/axial marker *Chordin*, suggests that the BMP and FGF pathways act downstream of, or in parallel with, Nodal. To establish the precise nature of the contribution of each of these signaling pathways to primitive streak formation will require experiments combining misexpression of one of the signaling molecules with inhibition of each of the other pathways in turn. These experiments are in progress in our laboratory.

Our results indicate that Nodal misexpression can initiate primitive streak formation in about 1/3 cases, a frequency somewhat lower than that obtained by misexpressing Vg1 (Shah et al., 1997; Skromne and Stern, 2001, 2002). This could suggest the involvement of other pathways required in conjunction with Nodal signaling to initiate primitive streak development. The most likely candidate is Wnt signaling. Support for the idea that the regulation of Wnt signaling is critical for primitive streak formation comes from several findings in mice. First, mutations in the *Axin* gene (an intracellular negative regulator of Wnt signaling; Zeng et al., 1997) cause the

embryos to develop multiple primitive streaks. Second, misexpression of chick cWnt8C in mouse embryos causes duplications of the primitive streak (Popperl et al., 1997). Third, mice with mutations in the *Wnt3* gene fail to form a primitive streak (Liu et al., 1999). In the chick, although Wnt signals have been shown to play a role in conjunction with Vg1 signaling from the posterior marginal zone (Skromne and Stern, 2001), this occurs at an early stage of development, before hypoblast formation (see above). It has been suggested that Vg1/activin signals and Wnt signals are integrated at the level of the promoter of organizer-specific genes, such as *goosecoid* (Watabe et al., 1995; Skromne and Stern, 2001). However, it is possible that Nodal can replace the related factors Vg1/activin in this interaction when Wnt signaling is active, consistent with the idea that increasing Nodal activity can generate progressively more dorsal (axial) mesoderm (Agius et al., 2000; Chen and Schier, 2001; Episkopou et al., 2001; Kodjabachian, 2001; Niederlander et al., 2001).

Inhibition by the Hypoblast and Regulative Ability

Classical embryo fragmentation experiments (Spratt and Haas, 1960) have suggested the existence of a "gradient of embryo-forming potential," highest posteriorly and lowest anteriorly. This could be due to a posterior to anterior gradient of a factor required for streak formation or to an inhibitory graded property in the reverse direction. Neither factor has ever been found. Our findings raise the intriguing possibility that there is indeed an inhibitory gradient, but this is a temporally as well as spatially graded property, conveyed by the hypoblast.

Our study and the accompanying paper (Perea-Gomez et al., 2002) provide a novel mechanism (Figure 6) to ensure that only a single axis forms during early amniote development and implicate tight spatial and temporal control of the distribution of Nodal antagonists in this process. In the chick, it appears that the site of primitive streak formation is initially specified by the overlapping activity of Vg1 and Wnt signals in the posterior marginal zone, which induce *Nodal* expression in the neighboring area pellucida epiblast. However, any point around the circumference of the embryo is capable of initiating formation of the primitive streak. This is prevented by the hypoblast, which, through the action of Cerberus, initially suppresses Nodal (and perhaps Wnt) signaling throughout the epiblast, until it is removed from the posterior end by the appearance of the endoblast, which does not express *Cerberus*. This exposes the posterior epiblast to Nodal signals required for induction of the mesendoderm, and the primitive streak appears. An important question remaining to be addressed concerns the mechanisms that cause the endoblast to form at its normal, posterior site.

Experimental Procedures

Embryos and In Situ Hybridization

Fertile hens' eggs were obtained from SPAFAS (White Leghorn) and Henry Stewart & Co. (UK) (Brown Bovan Gold). Eggs were incubated for 1–10 hr to stages X–XIII (Eyal-Giladi and Kochav, 1976). Embryos were set up in modified New culture (New, 1955; Stern and Ireland, 1981); hypoblast removal was performed with the embryo submerged in Pannett-Compton saline (Pannett and Compton, 1924). After marking the posterior area opaca with powdered carmine, we removed the hypoblast using insect pins. Operated embryos were incubated at 38°C in a humidified chamber for various periods of time and fixed. Whole-mount in situ hybridization was performed as described previously (Stern, 1998). The probes used were as follows: *cBrachyury* (Kispert et al., 1995; Knezevic et al., 1997), *Chordin* (Streit et al., 1998), *Nodal/cNR1* (Levin et al., 1995), *Cerberus* (Zhu et al., 1999), *Crescent* (Pfeffer et al., 1997), and *Hex/PRH* (Yatskievych et al., 1999).

DNA Constructs

Xenopus Cerberus-Short (kind gift from E. De Robertis) (Piccolo et al., 1999) was subcloned into XhoI/EcoRI sites of pcDNA 3.1(−)/*Myc*-His B. The construct was checked by sequencing.

To misexpress Nodal, we used a *Dorsalin-Myc-Nodal* expression construct. The original cDNA for *cNodal* (Levin et al., 1995) (kind gift of M. Kuehn) lacked 54 nucleotides at the 3′ end of the coding sequence (see GenBank Accession number AF486810). To correct this and to add a half-*Myc* tag at the 5′ of the cDNA, we used a forward primer (5′-CGGAATTGC*ATATCGAGGAGGACCTG*AAGGAGAGGCAGAGGCTC-3′) containing a half-*Myc* epitope (in italics) and the EcoRV restriction site (underlined) and two reverse primers (first, 5′-GCCGCACTCCTCGATGATCATATCCTCGTGGTGGCGGACGACGATCTCACCCTTCTCATAGATCAG-3′; second, 5′-CGACCGCTCGAGCGGCCATTATCACTCTTATCATCAGTTGCAGCCGCACTCCTCGATGATCATATC-3′). Two rounds of amplification were performed (10 and 20 cycles, respectively), with the same forward primer in both cases and the first and second reverse primers sequentially. The amplified fragment was then cloned into pGEM-T, excised with EcoRV and NotI sites, blunted, and cloned in the EcoRV site of pMT2.3-dorsalin-*Myc*1/2 (see Shah et al., 1997). The construct was checked by sequencing.

In both cases, the production of protein was controlled by immunohistochemistry on transfected COS cells and Western blot analysis on conditioned medium from transfected COS cells with anti-Myc antibody.

Cell Culture and Transfection

COS cells were grown in DMEM medium containing 10% newborn calf serum. *Vg1* (Shah et al., 1997), *Dorsalin-Myc-Nodal*, *Cerberus* (Zhu et al., 1999), and *Cerberus-Short* were transfected with Lipofectamine Plus (Gibco-BRL). After 24 hr, aliquots of 1000 or 500 cells were allowed to aggregate overnight in 20 μl hanging drops of medium. For Vg1 and Nodal experiments, one pellet of 1000 cells was used per graft, while three to five pellets were grafted for Cerberus or CerS experiment. When Cerberus or CerS was grafted together with Vg1 or Nodal, four pellets of 500 cells were used. As control, mock-transfected COS cells were used.

A rat B1-fibroblast-derived stable cell line secreting Wnt1 was grown in DMEM containing 7.5% newborn and 2.5% fetal calf serum. Pellets of 2500 cells were made for grafting as previously described (Joubin and Stern, 1999).

Differential Screen

Small groups of endoblast and hypoblast cells (<20 cells each) were dissected from a single chick embryo at stage 3 (Hamburger and Hamilton, 1951). cDNA was generated from each tissue, and libraries were prepared as previously described (Dulac and Axel, 1995). From 60 pairs, we selected 1 pair in which both samples displayed strong expression of β-*actin*, but the hypoblast sample had a very low level of *goosecoid* and *hex*, which are expressed in the hypoblast, but not in the endoblast. The endoblast library was plated at low density and differentially screened with radioactively labeled total cDNA from both hypoblast and endoblast cell populations. Differentially expressed cDNAs were sequenced and analyzed by in situ hybridization.

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