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Polarity of the mouse embryo is anticipated before implantation

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SUMMARY

In most species, the polarity of an embryo underlies the future body plan and is determined from that of the zygote. However, mammals are thought to be an exception to this; in the mouse, polarity is generally thought to develop significantly later, only after implantation. It has not been possible, however, to relate the polarity of the preimplantation mouse embryo to that of the later conceptus due to the lack of markers that endure long enough to follow lineages through implantation. To test whether early developmental events could provide cues that predict the axes of the postimplantation embryo, we have used the strategy of injecting mRNA encoding an enduring marker to trace the progeny of inner cell mass cells into the postimplantation visceral endoderm. This tissue, although it has an extraembryonic fate, plays a role in axis determination in adjacent embryonic tissue. We found that visceral endoderm cells that originated near the polar body (a marker of the blastocyst axis of symmetry) generally became distal as the egg cylinder formed, while those that originated opposite the polar body tended to become proximal. It follows that, in normal development, bilateral symmetry of the mouse blastocyst anticipates the polarity of the later conceptus. Moreover, our results show that transformation of the blastocyst axis of symmetry into the axes of the postimplantation conceptus involves asymmetric visceral endoderm cell movement. Therefore, even if the definitive axes of the mouse embryo become irreversibly established only after implantation, this polarity can be traced back to events before implantation.

Key words: Axis formation, Bilateral symmetry, Blastocyst, Cell lineage, Egg cylinder, Gastrulation, Mammal, MmGFP, Polarity, Proximodistal, Visceral endoderm

INTRODUCTION

In order to understand the mechanisms of spatial patterning in development, it is critical to determine when the polarity of an embryo is established. In most species, polarity of the whole organism can be traced back to the polarity of the egg. However, mammals are thought to be an exception to this model (reviewed by Davidson, 1991). The commonly held view is that the axes of the mouse embryo develop only after implantation in the uterus (reviewed by Davidson et al., 1999; Beddington and Robertson, 1999) so that the body plan of the adult would not relate to the polarity of the early preimplantation embryo. However, before implantation, the mouse blastocyst has been found to have an axis of bilateral symmetry, because the inner cell mass (ICM) is oval rather than circular in shape (Gardner, 1997). The later, implanting blastocyst has also been described as bilaterally symmetrical (Smith, 1980, 1985), although it has not been determined whether this relates either to the earlier axis of bilateral symmetry or to any axis of later stages. After implantation, the mouse embryo elongates into a cylindrical structure (the egg cylinder), which has generally been viewed as being radially symmetrical until 5.5 days of embryonic development (E5.5), when the anterior region of the definitive embryo becomes clearly distinguishable (reviewed by Beddington and Robertson, 1999). It has been hypothesised that the blastocyst axis of symmetry and the polarity of the egg cylinder might be related (Smith, 1980, 1985, Gardner, 1998), but it has not been feasible to test this due to the lack of an enduring cell lineage marker. Consequently there is no experimental information about whether the polarity of the egg cylinder originates before E5.5 (reviewed by Beddington and Robertson, 1998, 1999).

Resolution of this issue depends on cell lineage analysis of the intact embryo, but the rapid growth of the embryo after implantation (Snow, 1977) requires a lineage marker that is abundant and persistent to overcome the considerable dilution that will occur. We have described previously a modified form of green fluorescent protein (GFP), MmGFP (Zernicka-Goetz, 1999; Zernicka-Goetz et al., 1996, 1997) which can be detected at relatively low levels and is cell autonomous and stable. Moreover, it can be translated efficiently from a synthetic mRNA. In preliminary experiments, we found that single blastocyst ICM cells could be injected with this mRNA and that fluorescence from the protein could be detected in embryos allowed to develop in foster mothers for a further 3 days. We

therefore employed this marker to explore the establishment of polarity during mouse development.

We have chosen to trace the progeny of ICM cells into the visceral endoderm (VE), because several studies point to a role for this extraembryonic tissue in determining the axial organisation of the mouse embryo (reviewed by Beddington and Robertson, 1999; Tam and Behringer, 1998; Bielinska et al., 1999). The VE arises from the ICM, but later, at egg cylinder stages, it overlays both the ICM-derived epiblast (distal VE) and the trophectoderm-derived extraembryonic ectoderm (proximal VE). We found that VE descendants of ICM cells located initially near the polar body (a marker of the axis of blastocyst bilateral symmetry; Gardner, 1997) tended to become distributed distally as the egg cylinder forms, while those located opposite the polar body became proximal. Hence our results provide the first evidence that the polarity of the postimplantation mouse embryo can be traced back to events before implantation. We also show that the blastocyst axis of bilateral symmetry is converted into the proximodistal axis of the egg cylinder in a process involving asymmetric VE movement during peri-implantation embryonic growth.

MATERIALS AND METHODS

Cell lineage tracing using MmGFP

Inner cells of early blastocysts (C57BL/6 × CBA mated inter se) were injected as previously described for cleavage-stage mouse embryo (Zernicka-Goetz et al., 1997) with modifications for the smaller size of the target cells. Briefly, pulled needles were back-filled with in vitro transcribed MmGFP mRNA (0.5-1.5 μg/μl in water) then introduced into inner cells using negative capacitance. The mRNA was then pressure-injected using a Transjector apparatus (Eppendorf). Embryos at the early and expanding blastocyst stages were observed by differential interference contrast microscopy during rotation to identify the location of the polar body (PB), which was present in approximately 50% of blastocysts. The PB was evident as a small protrusion from the surface of the embryo (early blastocysts), or as a small, round object flattened between the embryo and the zona pellucida (expanding blastocysts). In embryos selected for injection (>75% of PB-containing blastocysts), the PB was located near the junction of ICM and mural trophectoderm, aligned with the greater diameter of the blastocyst, as described by Gardner (1997). A single ICM cell located either in close proximity to the PB (N/PB), or on the opposite side, away from the PB (A/PB) was selected for injection. Injected embryos were evaluated using an inverted fluorescence microscope (Nikon) between 1 and 4 hours after injection for the presence of GFP fluorescence. Typically one cell was labelled (N/PB or A/PB). When there was a second labelled cell in the ICM (<5% of embryos), it was often joined to the other labelled cell by a cytoplasmic bridge, indicating that the two cells were sisters from a prior cell division (Pedersen et al., 1986). The second cell was sometimes located away from the target site nearer to the middle of the ICM, reflecting movement of the two cells away from each other following division. Although an inner cell was targeted, the occasional embryo (7%) had a cell labelled in trophectoderm as the microinjection needle passed through it. GFP-positive embryos were pooled for transfer to the uteri of E2.5 pseudopregnant mice according to the location of injection (N/PB or A/PB).

Confocal analysis of MmGFP-labelled clones in postimplantation embryos

Embryos were dissected at E5.5-5.75 or E6.5-6.75, counting noon of the plug day of the pseudopregnant recipient as E0.5. They were cleaned of parietal yolk sac, consisting of trophoblast giant cells and parietal endoderm, then gently compressed between two coverslips. Each embryo underwent optical sectioning by confocal microscopy (Biorad 1024 attached to a Nikon Eclipse 800 microscope) and was scanned every 5-10 μm with 30-100% laser power to analyse the distribution of GFP-labelled cells. The use of this high laser power to detect GFP signal precluded further development of embryos in vitro to follow movement of labelled cells in real time. The regional distribution of GFP-labelled descendants of the injected cell was visualized by flattening the Z series of the confocal images onto a single image. The resulting image was then sketched onto a template, demarcating individual subclones with respect to the landmarks of the egg cylinder. These are the base, where ectoplacental cone, parietal endoderm and visceral extraembryonic endoderm intersect; the tip of the cylinder; and the extraembryonic/embryonic boundary, where there is a slight indentation approximately midway between the base and tip. The anteroposterior orientation of early primitive streak stage embryos was based on the slightly thicker posterior epiblast, which was often obscured by accumulating mesoderm, and by the more prominent indentation in the anterior endodermal outline (Lawson and Pedersen, 1987).

Quantitative analysis of MmGFP-labelled visceral endoderm cells

The number of GFP-positive VE cells in extraembryonic and embryonic regions was counted on the flattened Z-series images for each embryo. We used two approaches for estimating the expected number of VE cells that would be generated by labeling one or two progenitor cells. In the first approach, we estimated the fraction of the VE population expected to be represented by GFP-labelled cells. Assuming that the founder population for the VE layer has six to eight cells at the late blastocyst stage (Enders et al., 1978) when there are two to four GFP-labelled cells, then GFP-labelled descendants could comprise 25-67% of the total primitive endoderm cells. Estimates of the number of cells present in VE range from 250 cells at E6.5 to 430 at E7.0 (Snow, 1977), leading to the expectation that between 63 and 288 labelled cells might descend from the injected progenitors. In the second approach, the increase in number of labelled cells (N) expected between the blastocyst and primitive streak stage was estimated by the equation $N=N(0)\times e^{ln2\times t/T}$ where $N(0)$ is the number of labeled progenitor cells, t (elapsed time) = 72 hours and T (population doubling time for VE) = 10.5 hours. If we assume that there is a constant rate of expansion in the VE population, that all cells are dividing and that no cells are lost owing to cell death, this approach leads to an estimate of between 115 and 230 labelled cells, depending on whether one or two progenitors were marked. Accordingly, neither the injection procedure itself nor the GFP mRNA or protein significantly perturbed the proliferation of the labelled cells, because their number was within the predicted range (see Results).

For statistical analysis, initially, a univariate analysis was performed, using the Mann-Whitney U test, to assess whether there were any differences between the N/PB and A/PB series and between prestreak and streak stages in the percentage of total labelled VE cells located in the extraembryonic region. Then, a linear regression model was fitted to the entire dataset, with the series and stages included as independent variables. A comparison of the residuals versus the standard normal was used to validate the assumption of normality. A probability value of less than 0.05 was considered to indicate statistical significance.

Cell lineage tracing of visceral endoderm cells using DiI

E5.5 embryos were labeled at their distal tip using the carbocyanine dye, CellTracker CM-DiI (DiI, Molecular Probes, Inc., Eugene, OR) as described by Thomas et al. (1998), except that DiI was dissolved in glycofurol (Sigma) as described by Denetclaw et al. (1997) and injection was performed using 0.25-1 second pressure pulses from a Picospritzer (General Valve Corporation, Fairfield, NJ). Labeled embryos were cultured in Dulbecco's medium (DMEM)

supplemented with 50% heat-inactivated, immediately centrifuged rat serum (Harlan, Indianapolis, IN) for ≤24 hours as described by Lawson et al. (1991), except that embryos were incubated in four-well plates (Nunc) containing 0.5 ml total medium under paraffin oil. Fluorescence was assessed at ≤E6.5, counting total time in utero and in culture.

RESULTS

MmGFP mRNA provides an enduring peri-implantation lineage tracer for visceral endoderm

To address the issue of whether the polarity of the embryo before implantation relates to the polarity of the postimplantation conceptus, we used a novel method involving injection of mRNA encoding the cell lineage marker, MmGFP. We used this marker to follow lineages of VE cells because of the role of this tissue in determining the axial organisation of the mouse embryo. We considered cells of the ICM adjacent to the blastocyst cavity of the 3.5- to 3.75-day-old embryo (E3.5-3.75) to be the most likely precursors for the VE and injected these cells with MmGFP mRNA to trace their fates. Approximately one half (53%, 491/923) of the injected embryos became distinctly labelled in one or two cells located in the ICM within 1 hour of incubation at 37°C.

GFP-positive blastocysts were transferred to the uteri of foster mothers and allowed to develop, in the first series of experiments, until early primitive streak stages (E6.5-E6.75) and, in a second series of experiments, until preprimitive streak stages (E5.5-5.75). Recovered embryos were optically sectioned by confocal microscopy to reveal the distribution and number of GFP-positive descendants of the labelled progenitor cells. In these two series, we obtained 58 (24%) positive embryos from 241 recovered after transfer to the uteri of foster mothers who became pregnant. In 13/58 embryos (22%), labelled cells were observed in the epiblast. The epiblast descendants were invariably distributed in a salt and pepper pattern (Fig. 1), with little or no coherent clonal growth, consistent with previous findings (Beddington et al., 1989; Lawson et al., 1991; Gardner and Cockroft, 1998). Because of this extensive cell mixing, descendants of ICM within the epiblast were uninformative about any axial relationship between blastocyst and gastrula stages. None of the positive embryos were labelled in trophectoderm. In a few embryos (3/58), marked cells were observed only in the parietal endoderm. However, in the majority of cases (42/58, 72%), the GFP-positive cells were located in the VE as extensive clones with a distribution that was non-random and therefore informative of the relationship between blastocyst and postimplantation polarity.

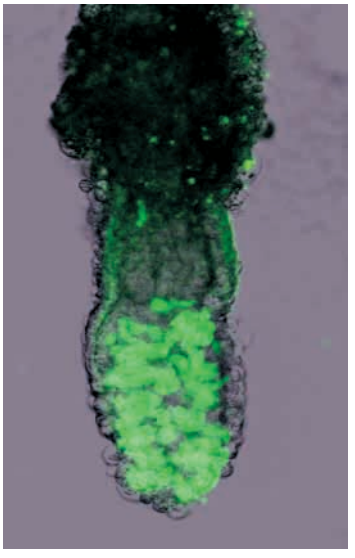

Fig. 1. Confocal image (combined fluorescence and transmitted light) showing the epiblast descendants of a single ICM cell injected with MmGFP RNA. Note the salt and pepper distribution of MmGFP-labelled cells (green) in the distal (embryonic, lower) portion of the egg cylinder.

Blastocyst polarity predicts polarity of the egg cylinder

As the second polar body marks one end of the axis of bilateral symmetry of the blastocyst, we chose to inject ICM cells either near the polar body (N/PB embryos; Fig. 2A) or on the opposite side of the embryo, away from the polar body (A/PB embryos, Fig. 2D). Analysis at the early primitive streak stage revealed that there were conspicuous differences between N/PB and A/PB embryos in the distribution of VE descendants along the egg cylinder. N/PB embryos tended to have either larger, coherent patches of cells located in the embryonic part of the egg cylinder, near the border with the extraembryonic region (Fig. 2B) or small, non-coherent patches of VE-labelled cells scattered mainly in the

Table 1. Distribution of GFP-labelled visceral endoderm cells at streak stage by region

		Extraembryonic region		Embryonic region	
Embryo	Total no. labelled cells	No. labelled cells	% of total labelled	No. labelled cells	% of total labelled
(A) Streak stage, N/PB series					
1	54	7	12.5	47	87.5
2	116	94	81	22	19
3	53	10	19	43	81
4	107	24	22	83	78
5	105	10	9.5	95	90.5
6	183	80	44	103	56
7	214	157	73	57	27
8	191	100	52	91	48
9	242	31	13	211	87
10	131	0	0	131	100
11	72	24	33	48	67
12	167	0	0	167	100
Mean	136	45	30	91	70
s.d.	63	51	27	56	27
s.e.m.	18	15	8	16	8
(B) Streak stage, A/PB series					
1	113	90	80	23	20
2	143	143	100	0	0
3	102	74	72.5	28	27.5
4	206	172	83	34	17
5	287	119	41.5	168	58.5
6	159	80	50	79	50
7	230	150	65	80	35
8	152	83	55	69	45
9	337	162	48	175	52
10	92	87	95	5	5
11	165	119	72	46	28
12	89	62	70	27	30
Mean	173	112	69	61	31
s.d.	79	38	18	58	18
s.e.m.	23	11	5	17	5

embryonic region of the egg cylinder (Fig. 2C). VE clones in A/PB embryos were generally extraembryonic, large and coherent with occasional small patches in the embryonic part (Fig. 2E,F). There were a few exceptions to these characteristic regional distributions and labeling patterns of VE cells (e.g. N/PB embryos 2 and 7; Table 1). These exceptions may have arisen from cases in which the polar body did not accurately represent the axis of blastocyst bilateral symmetry, due to extension or severance of the tether that connects the polar body to the embryo (Gardner, 1997). Alternatively, they could reflect the labelling of a more centrally located sister cell in addition to the injected inner cell. Nevertheless, the predominant outcome was for N/PB-derived VE cells to occupy the distal (embryonic) and for A/PB-derived cells to occupy the proximal (extraembryonic) portion of the egg cylinder.

To examine the significance of these observations, we quantified the distribution of labelled descendants of the injected cells to either the extraembryonic or the embryonic region of the egg cylinder. We found that N/PB-injected embryos had a mean of 136 labelled VE cells, of which only 1/3 were in the extraembryonic region of the egg cylinder, while A/PB-injected embryos had an average of 173 labelled VE cells of which 2/3 were in the extraembryonic region (Table 1). Statistical analysis of the number of labelled VE cells that were extraembryonic confirmed that N/PB descendants were distributed significantly more into the embryonic region than A/PB descendants (P=0.003; Mann-Whitney test, see Materials and Methods).

Thus our results show that the ICM of the early blastocyst has an axis of bilateral symmetry that predicts the spatial patterning of the postimplantation conceptus. Moreover, this axis has polarity, because cells located at opposite ends of the blastocyst axis have reciprocal developmental fates.

Transformation of blastocyst axis into proximodistal axis of the egg cylinder begins before E5.5

There are two possible explanations to account for the differences in proximodistal distribution of N/PB versus A/PB VE descendants. In the first view, the elongation of the egg cylinder is initially symmetric with respect to the two sides of the early embryo (N/PB and A/PB), but this is followed by a phase of asymmetric movement such that the N/PB descendants become localised distally, whereas A/PB descendants are moved into more proximal positions. Indeed, such a vectorial shift of the VE from the distal tip of the egg cylinder into an anterior position between E5.5 and 6.5 has been previously described (Thomas et al., 1998). However, an alternative view is that asymmetric proximodistal movement of the VE has already started before E 5.5. If this were the case, the asymmetric movement of VE cells would precede the distal-to-anterior shift.

To distinguish between these two alternatives, we performed a second series of experiments using younger embryos. Once again, we labelled progenitors of VE cells on either end of the blastocyst axis of symmetry but we now analysed the distribution of their progeny at the prestreak stage (E5.5). In the case of N/PB prestreak embryos, the coherent clones of labelled VE cells were present midway along the egg cylinder (Fig. 3A,B), and the non-coherent clones were scattered in small patches along the length of the egg cylinder. In contrast,

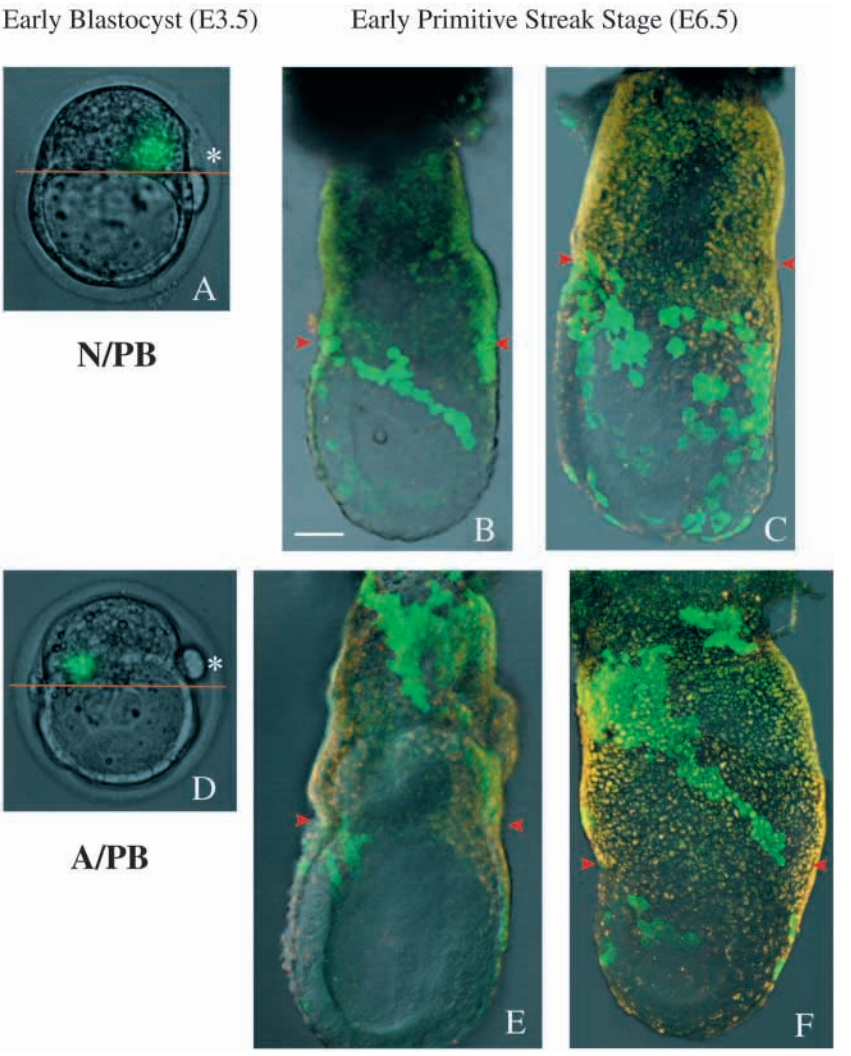

Fig. 2. Distribution of VE descendants of ICM cells injected with MmGFP mRNA near the polar body: N/PB (A-C) or away from the polar body: A/PB (D-F). (A,D) Image of the blastocyst after injection and expression of MmGFP (combined fluorescence and transmitted light). The diameter of the blastocyst is approx. 100 μm. Horizontal line indicates the orientation of the blastocyst axis of bilateral symmetry. Asterisks indicate location of the polar body. (B,C,E,F) Confocal image of early streak stage embryos (E6.5), anterior facing left, posterior facing right; bar, 50 μm. Arrowheads show boundaries between proximal (extraembryonic) and distal (embryonic) regions of egg cylinder. Embryo identities: B (embryo no. 3 from Table 1A) and C (embryo no. 10 in Table 1A); E (embryo no 1. Table 1B) and F (embryo no. 4 in Table 1B). MmGFP expressing cells are green and autofluorescent cells appear in orange/yellow.

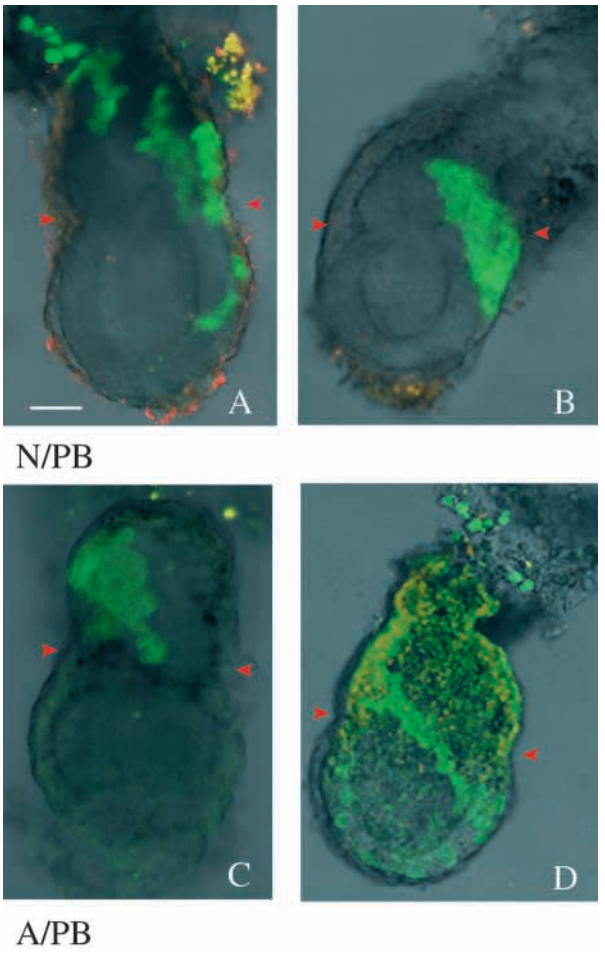

Fig. 3. Confocal images of representative prestreak embryos (E5.5 or E5.75) showing the distribution of VE descendants of ICM cells injected with MmGFP mRNA near the polar body (A, N/PB, embryo no. 3 in Table 2A; B, N/PB, embryo no. 7 in Table 2A) or away from the polar body (C, A/PB, embryo no. 5 in Table 2B; D, A/PB, embryo no. 3 in Table 2B). Arrowheads show boundaries between proximal (extraembryonic) and distal (embryonic) regions of egg cylinder; bar, 50 μm. E5.5 embryos are not oriented with respect to their anteroposterior axis. MmGFP-expressing cells are green and autofluorescent cells appear in orange/yellow.

A/PB prestreak embryos typically had coherent clones localised predominantly in the proximal (extraembryonic) region of the egg cylinder similar to early streak stage embryos (Fig. 3C,D compare Fig. 2E,F). This analysis revealed that VE descendants in N/PB embryos were already located more distally than the VE descendants in A/PB embryos at E5.5. These observations show that the transformation of the blastocyst axis involves processes that begin before E5.5, resulting in distal movement of N/PB-derived VE.

To examine the fate of N/PB-derived VE in greater detail, we again quantified the distribution of labelled descendants of the injected cells to either the extraembryonic or the embryonic region of the egg cylinder (Table 2). At the prestreak stage, embryos that resulted from N/PB injections had an average of 57 labelled VE cells, of which approximately half (56%) were in the extraembryonic region. This compared to an average of 60 cells in embryos at prestreak stages that resulted from A/PB

Table 2. Distribution of GFP-labelled visceral endoderm cells at preprimitive streak stage by region

		Extraembryonic region		Embryonic region	
Embryo	Total no. labelled cells	No. labelled cells	% of total labelled	No. labelled cells	% of total labelled
(A) Prestreak stage, N/PB series					
1	35	10	29	25	71
2	101	28	28	73	72
3	47	36	77	11	23
4	76	28	37	48	63
5	59	46	78	13	22
6	38	19	50	19	50
7	47	21	45	26	55
8	47	34	72	13	28
9	64	64	100	0	0
Mean	57	32	57	25	43
s.d.	21	16	25	22	25
s.e.m.	7	5	8	7	8
(B) Prestreak stage, A/PB series					
1	20	15	75	5	25
2	105	100	95	5	5
3	108	83	77	25	23
4	72	60	83	12	17
5	20	20	100	0	0
6	74	54	73	20	27
7	45	45	100	0	0
8	38	26	68	12	32
9	62	57	92	5	8
Mean	60	51	85	9	15
s.d.	33	28	12	9	12
s.e.m.	11	9	4	3	4

injections, of which the vast majority (85%) were extraembryonic (Table 2). Statistical analysis of the percentage of labelled VE cells that were extraembryonic confirmed that N/PB descendants were distributed significantly more into the embryonic region than A/PB descendants (P=0.034; Fig. 4). Moreover, the distribution of N/PB descendants into the embryonic region increased between the prestreak and streak stages (P=0.03). Therefore, the distal movement of N/PB-derived cells was already underway by 5.5 days of development and continued during the next day of egg cylinder growth. To determine whether this early distal movement of VE was specific to the N/PB-derived descendants, in a third series of injections, we marked VE progenitors located between A/PB and N/PB sites and examined their fate at E6.5. We found that the distribution of labelled cells was significantly different from N/PB (P=0.008) but similar to that of A/PB descendants (P=0.52): in 11 embryos examined, most of the progeny (65%; mean number of labelled cells per embryo, 182) were located in the extraembryonic region of the egg cylinder. Therefore, it appears that VE cells of N/PB origin have a distinct fate during egg cylinder growth.

How does the blastocyst axis of bilateral symmetry relate to anteroposterior axis of the embryo?

Our results revealed that the blastocyst axis predicts the proximodistal axis of the egg cylinder. However, it also appeared that this axis was related to the anteroposterior (AP) axis of the postimplantation embryo, because of the distribution pattern of labelled cells. First, coherent VE clones were typically arrayed in a distinctive diagonal orientation

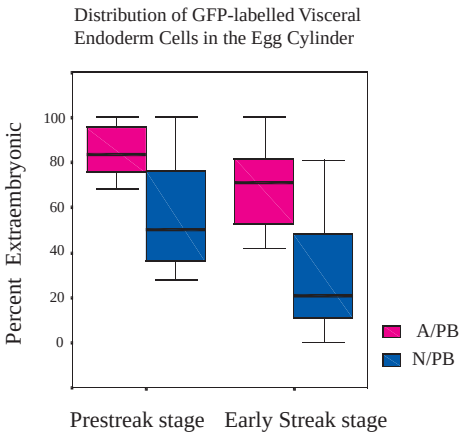

Fig. 4. Box-whisker plots showing VE-labelled cell distribution into the extraembryonic region of the egg cylinder after injection of ICM cells nearby the polar body (N/PB) or away from the polar body (A/PB). Data are from Tables 1 and 2. Bar indicates median; box and whiskers illustrate 25% to 75% of range and entire range of data, respectively.

(Fig. 2B,E,F). In the vast majority of embryos with coherent clones, involving both N/PB and A/PB series at E6.5 (15/19, 79%), the clones extended from anteroproximal to posterodistal regions, which could be clearly identified at this stage (23/24 E6.5 embryos) because the primitive streak was already forming at the posterior (see Material and Methods). This characteristic pattern of coherent VE cell clones resulted in the descendants of N/PB and A/PB cells becoming distributed into both anterior and posterior regions of the egg cylinder (Fig. 2B,E,F) and resulted in the distalmost portion of the coherent clones being consistently oriented towards the posterior of the embryo. Second, although the non-coherent N/PB-derived clones were also distributed across the entire embryonic region of the egg cylinder (Fig. 2B,C), this distribution could potentially be accounted for by the distal-to-anterior VE movement described previously by Beddington and colleagues (Thomas et al., 1998). To confirm whether such a movement also occurred in our embryos we followed lineages of labelled VE cells from E5.5 to E6.5 directly using in vitro embryo culture. Because the high laser power necessary to visualise clones of GFP-labelled cells precluded the further development of GFP-labelled embryos, we marked cells of E5.5 embryos using the carbocyanine dye, DiI, as described by Thomas et al. (1998). We marked the distal tip of E5.5 embryos with DiI and found that 31/34 (Table 3) cultured embryos exhibited a unidirectional movement of distal VE cells between E5.5 and 6.5, as described by Thomas et al. (1998). This VE movement, which transforms the proximodistal axis into the anteroposterior axis, could thus distribute the distal N/PB-derived VE cells into anterior portions of the embryonic region. Taken together, the asymmetric movements of VE in both extraembryonic and embryonic regions reveal a relationship between the blastocyst axis of bilateral symmetry and the AP axis of the gastrulating embryo. Our model for the visceral endoderm cell movements that occur during peri-implantation development is represented in Fig. 5.

DISCUSSION

The aim of our studies was to determine whether the polarity of the embryo before implantation bears any relationship to polarity of the postimplantation conceptus. Our results unexpectedly revealed that the blastocyst axis of bilateral symmetry predicts the proximodistal axis of the egg cylinder, as shown by the reciprocal patterns of N/PB and A/PB descendants in visceral endoderm. There were two possible explanations for the observed difference in patterns of visceral endoderm cells. Either the initial egg cylinder development would be symmetric on both sides of the embryo, and this would be followed by a phase of asymmetric growth between E5.5 and E6.5, as described by Thomas et al. (1998). Alternatively, the asymmetry could be initiated earlier during egg cylinder elongation, before E5.5. Our analysis of the distribution of labelled visceral endoderm cells showed that their asymmetric proximal-to-distal movement had already begun by E5.5, as the progeny of N/PB marked cells had already shifted by this stage, and that this movement continued between E5.5 and E6.5.

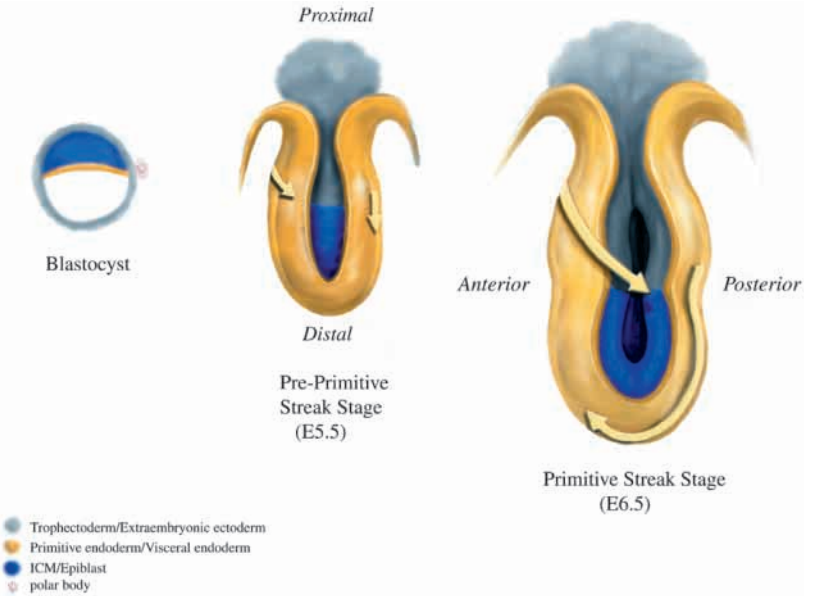

Fig. 5. Model showing the colonization of VE cells during egg cylinder formation by descendants of ICM cells near (N/PB) or away (A/PB) from the polar body. N/PB descendants move to more distal locations than A/PB descendants by the prestreak stage. Left drawing: blastocyst; asterisk indicates the axis of bilateral symmetry. Middle drawing: prestreak stage embryo. Right drawing: early primitive streak stage embryo. Cut-aways of VE (yellow) expose the underlying epiblast (blue) and extraembryonic ectoderm (grey) of the egg cylinder. Arrows represent the direction of VE cell movements during egg cylinder growth. The asymmetric movement of VE cells is already occurring by prestreak stages as N/PB descendants move more distally. This vectorial VE movement continues between prestreak and streak stages.

Table 3. Extent of asymmetric movement of distal visceral endoderm cells

No. of embryos cultured	No. developing normally	No. developing abnormally*	Distribution of embryos by angular displacement of DiI-labelled cells‡				
			None	11-30	31-50	51-70	71-90
44	34	10	3	10	7	11	3

*Abnormally developing embryos had one or a few DiI-labelled cells localized to the distal tip of a compact (not elongated) egg cylinder, an apparent consequence of having an intact Reichert's membrane. These embryos are excluded from the summary of distributions according to angular displacement.

‡The extent of unidirectional shift of the visual centre of DiI-labelled cells was determined as angular displacement (in degrees) with respect to the proximodistal axis, as measured from its junction with the boundary between embryonic and extraembryonic regions. Embryos with >10° movement were considered to have undergone a unidirectional shift.

The mechanistic basis for such asymmetric cell movement is not clear, but it is possible that differential growth could be responsible. Because of the extensive cell mixing in the epiblast lineage, it was not possible to distinguish whether the visceral endoderm movement observed in our study occurred separately from the epiblast or in concert with epiblast asymmetric growth. The morphogenetic events of gastrulation beginning at E6.5 (thus, occurring after the endpoint of our studies) also consist of asymmetric movements in which epiblast cells converge posteriorly on the streak, and epiblast-derived embryonic endoderm displaces or replaces primitive endoderm-derived VE in an anterior direction (Lawson and Pedersen, 1987; Lawson et al., 1991). It is unclear, however, whether the movements of VE that accompany gastrulation are mechanistically related to the VE movements preceding gastrulation. It is also possible that the extraembryonic ectoderm undergoes asymmetric growth before gastrulation, contributing to the asymmetric VE movement. Whatever the mechanism, we now show that the vectorial VE movements relate to the inherent bilateral asymmetry documented in the blastocyst. Moreover, these movements begin prior to E5.5, thus considerably earlier than previously recognized, and well before formation of the primitive streak (Fig. 5). Therefore it follows that the axis of the preimplantation blastocyst and that of the postimplantation conceptus are related.

It has previously been reported that between E5.5 and 6.5 the proximodistal axis of the egg cylinder transforms into the anteroposterior axis of the definitive embryo (Thomas et al., 1998; reviewed by Beddington and Robertson, 1998, 1999), which is consistent with our findings. Although our aim was not specifically to relate the blastocyst axis of symmetry with the AP axis of the postimplantation embryo, the patterns of labelled VE cells show that such a correlation does exist. Most notably, the coherent VE clones consistently expanded to form diagonal lines such that, by the time the primitive streak formed, the most proximal cells were in an anterior position and the most distal cells occupied the posterior. Thus, the proximo-to-distal VE movement appears to occur predominantly at the future posterior aspect of the embryo. Moreover, the majority of VE descendants of ICM cells labelled between A/PB and N/PB came to occupy the extraembryonic part of the egg cylinder, behaving like cells of A/PB-injected and not like N/PB-injected embryos. It follows that visceral endoderm cells enveloping the embryonic part of the egg cylinder originate preferentially from the region of the ICM near the polar body. Hence these must be the cells that have undergone asymmetric proximal-to-distal movement between E5.5 and 6.5. These distally located cells move unidirectionally to occupy the anterior region, as has been described by Thomas et al. (1998) and confirmed in our experiments (Table 3). We propose that the unidirectional movement of distal VE to the anterior region is a particular aspect of the overall early asymmetric VE movements that we report here (Fig. 5). Comparable movements (polonaise movements) occur in the early development of the chick embryo (Pasteels, 1937; Bachvarova et al., 1998).

The unidirectional shift of VE cells observed by DiI labelling is accompanied by a movement in the expression pattern of *Hex*, an early gene implicated in axis definition (reviewed by Beddington and Robertson, 1999; Bielinska et al., 1999). Intriguingly, the anterior movement of *Hex*-expressing cells is blocked by a mutation in the *cripto* gene, whose expression is required for gastrulation (Ding et al., 1998; Xu et al., 1999). This mutation and several others (*nodal*, Iannaccone et al., 1992; *Brca1*, Ludwig et al., 1997; activin receptor Type I, Gu et al., 1998; *Xrcc1*, Tebbs et al., 1999), which disrupt gastrulation by diverse mechanisms all interfere with the thinning of embryonic VE that normally occurs between E5.5 and 6.5. Thus, it appears that the thinning of distal VE relates to its pregastrulation movement.

What is the earliest stage in development at which the mouse embryo shows polarity? Because the polarity of the blastocyst is related to the animal-vegetal polarity of the egg (Gardner, 1997), it follows that the polarity of the postimplantation conceptus can be traced back to the polarity of the egg. Could the animal-vegetal asymmetry of the egg ultimately be responsible for the asymmetric development of the egg cylinder? At its face value, this appears to be inconsistent with the finding that removal of either the animal or the vegetal pole of the egg is compatible with full-term development (Zernicka-Goetz, 1998). That study showed that if determinants of polarity are present in the animal and vegetal poles of the mouse egg, they are not absolutely required for development. Moreover, it has recently been shown that animal-vegetal polarity at the 2- and 8-cell stages is also non-essential for the successful development of mouse embryos into fertile adult mice (M. A. Ciemerych and M. Z.-G., unpublished data). This seems to suggest that establishment of the axis of bilateral symmetry of the blastocyst is independent of axial information in the egg, with the latter simply providing orientation cues during cleavage. However, an alternative possibility is that the highly regulative nature of early mouse embryos allows polarity to be re-established if intrinsic polarity derived from the egg is perturbed. Indeed, such regulative capabilities are not unique to mammals, nor are they limited to the earliest stages of development, as recently shown in chick embryos (Psychoyos and Stern, 1996; Bachvarova et al., 1998; Yuan and Schoenwolf, 1998; Joubin and Stern, 1999). Therefore, even if

the definitive axes of the mouse embryo become irreversibly established only after implantation, this polarity can be traced back to events before implantation. In this respect, studies aiming to understand how spatial patterning is established in the mammalian embryo need to address both the mechanisms that operate in normal, unperturbed development as well as those that can accommodate the effects of even extreme experimental perturbation. The conclusions reached on the basis of the work presented here emphasize the importance of studying cellular and molecular mechanisms of axis development before the embryo implants. This may also provide insight into how such mechanisms are capable of the extensive regulation shown by mammalian embryos. Moreover, our studies indicate that mammals after all may not be exceptional with respect to when their polarity develops, and suggest that they might share important features of this process with other vertebrates.

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