Four-cell stage mouse blastomeres have different developmental properties

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Accepted 29 November 2004

Development 132, 479-490
Published by The Company of Biologists 2005
doi:10.1242/dev.01602

Summary

Blastomeres of the early mouse embryo are thought to be equivalent in their developmental properties at least until the eight-cell stage. However, the experiments that have led to this conclusion could not have taken into account either the spatial origin of individual blastomeres or the spatial allocation and fate of their progeny. We have therefore readdressed this issue having defined cell lineages in mouse embryos undergoing different patterns of cleavage in their second division cycle. This has enabled us to identify a major group of embryos in which we can predict not only the spatial origin of each given four-cell blastomeres, but also which region of the blastocyst is most likely to be occupied by its progeny. We show that a pattern of second cleavage divisions in which a meridional division is followed by one that is equatorial or oblique allows us to identify blastomeres that differ in their fate and in their developmental properties both from each other and from their cousins. We find that one of these four-cell stage blastomeres that inherits some vegetal membrane marked in the previous cleavage cycle tends to contribute to mural trophectoderm. The progeny of its sister tend to donate cells to part of the ICM lining the blastocyst cavity and its associated trophectoderm. Chimaeras made entirely of these equatorially or obliquely derived blastomeres show developmental abnormalities in both late preimplantation and early postimplantation development. By contrast, chimaeras made from four-cell stage blastomeres from early meridional divisions develop normally. The developmental defects of chimaeras made from the most vegetal blastomeres that result from later second cleavages are the most severe and following transplantation into foster mothers they fail to develop to term. However, when such individual four-cell blastomeres are surrounded by blastomeres from random positions, they are able to contribute to all embryonic lineages. In conclusion, this study shows that while all four-cell blastomeres can have full developmental potential, they differ in their individual developmental properties according to their origin in the embryo from as early as the four-cell stage.

Key words: Cleavage pattern, Cell fate, Chimaeras, Mouse embryo

Introduction

A number of studies have shown that the orientation of the first cleavage division in the majority of mouse embryos allows the prediction of the orientation of the future embryonic-abembryonic axis of the blastocyst (Piotrowska and Zernicka-Goetz, 2001; Gardner, 2001; Piotrowska et al., 2001; Fujimori et al., 2003; Zernicka-Goetz, 2002). More recently we have shown that for this to be the case, the relative spatial arrangement of the four-cell blastomeres, an outcome of the orientations of the second cleavages, is important (Piotrowska and Zernicka-Goetz, 2002). This study also clarified the relationship between the order of blastomere division from the two- to four-cell stage, and the polarity of this embryonic-abembryonic axis (Piotrowska and Zernicka-Goetz, 2002). Although the orientations of the second cleavages do not appear to be predetermined, in the great majority of embryos (80%), one of the second cleavages is meridional (M) and the other is rather equatorial or oblique (E) with respect to the second polar body. When the first two-cell blastomere to divide does so meridionally (ME embryos), the orientation of the first cleavage is predictive of the orientation and polarity of the embryonic-abembryonic axis. Thus, in embryos dividing in this way, the progeny of the earlier meridionally dividing cell contribute predominantly to the embryonic part of the blastocyst. By contrast, when the earlier of the second cleavage divisions occurs equatorially or obliquely and is followed by a meridional division (EM embryos), the orientation of the first cleavage is predictive of the orientation of the embryonic-abembryonic axis but not its polarity. In such cases, the earlier equatorially dividing cell has an approximately equal chance of contributing its progeny to either the embryonic part or the abembryonic part

of the blastocyst. In the less common situation (20% of embryos) when two-cell blastomeres undergo either sequential meridional or equatorial/oblique divisions, a relationship between the first cleavage and the embryonic-abembryonic axis is not observed.

Since in a major group of embryos (ME embryos) the progeny of individual four-cell blastomeres tends to follow different fates, the question arises as to whether they are equivalent to each other. This could happen, for example, as a consequence of their spatial relationship per se or because they inherit different properties when they divide. Indeed it has been proposed that an equatorial division of the two-cell blastomere might partition 'animal' and 'vegetal' components between daughter cells, whereas a meridional division would not (Gardner and Davies, 2003). Irrespective of whether such partitioning takes place, can cells have different developmental properties that reflect their positions in the embryo as early as the four-cell stage?

In the mouse, the only individual blastomeres recorded to undergo normal development to term have been from the two-cell stage embryo (Tarkowski, 1959; Tsunoda and McLaren, 1983; Papaioannou et al., 1989). Thus far, it has not been possible to demonstrate whether all individual blastomeres at the four-cell stage have this capability. Individual four-cell blastomeres will form miniature blastocysts (Tarkowski and Wroblewska, 1967; Rossant, 1976), but there is only one reported case of such a blastocyst ever giving rise to a postimplantation embryo (Rossant, 1976). This can be attributed to the difficulty of a four-cell blastomere to generate sufficient cells as the blastocyst forms to allow some to be enclosed and develop as inner cell mass (ICM) precursors. Thus, to determine whether single blastomeres have a full developmental capacity, they have been aggregated with other, 'carrier', blastomeres. Such studies provided evidence that four-cell blastomeres can retain the ability to form ICM and trophectoderm lineages (Hillman et al., 1972; Kelly, 1977). They also showed that individual four-cell blastomeres, when aggregated with carrier cells, were in some cases able to contribute exclusively to the resulting animals (Kelly, 1977). These experiments indicate the totipotency of at least some of the blastomeres at these early developmental stages. However, despite numerous efforts, quadruplet mice have never been produced from a single embryo (Tarkowski et al., 2001).

In all previous experiments to analyse the developmental potency of blastomeres, chimaeras were constructed without reference to the origins of the donor cells. Knowledge of the ways in which individual four-cell blastomeres become arranged, on the one hand, and the blastocyst becomes populated with their progeny, on the other, has now allowed us to examine whether four-cell blastomeres are equivalent in their developmental abilities. To this end, we have generated chimaeras comprising four-cell blastomeres of single types that have not only defined spatial origins, but also 'preferred' developmental fate. Here, we report that such chimaeras differ in their developmental properties. We show that specific cells in the four-cell stage embryo have a reduced ability to develop successfully when aggregated with cells of a similar type. We describe the developmental defects shown by such embryos and discuss how these might arise.

Materials and methods

Embryo collection and culture

Two-cell stage embryos were collected from F1 (C57BL/6×CBA) females induced to superovulation by intraperitoneal injection of 7.5 IU of pregnant mares serum gondotrophin (PMSG, Intervet) followed 48 hours later by 7.5 IU of human chorionic gonadotrophin (hCG, Intervet) and then mated with transgenic males that express GFP tagged histone H2B (Hadjantonakis and Papaioannou, 2005). Two-cell embryos were collected 45 hours after hCG injection into M2 medium supplemented with 4 mg/ml BSA and cultured in vitro in drops of KSOM supplemented with amino acids and 4 mg/ml BSA, under paraffin oil in an atmosphere of 5% CO_2 in air at 37.5°C.

Non-invasive labelling of individual blastomeres at the two- and four-cell stage

All embryos were micromanipulated with Leica micromanipulators using a De Fonbrune suction-force pump and observed using an inverted (Leica) microscope with DIC optics. To mark blastomeres, DiD or DiI (Molecular Probes) was applied using a blunt-ended micromanipulation micropipette as previously (Piotrowska et al., 2001). The first step was to label one of the two-cell stage blastomeres with a red dye (between 45.5 and 47 hours after hCG). Ten two-cell stage embryos were placed in the manipulation chamber at the same time and ~80 two-cell embryos (recovered from four mice) that had a visible polar body between the blastomeres were labelled in each experiment. Approximately 95% of embryos survived the labelling procedure. As soon as the dye-labelling procedure was completed (up to 1.5 hours for 80 embryos), one or both two-cell stage blastomeres were labelled with beads (a step taking up to 2 hours for 80 embryos). Labelling of the vegetal pole of two-cell blastomeres with fluorescent beads was carried out as previously (Piotrowska and Zernicka-Goetz, 2001). In brief, green fluorescent beads (~2-3 μm in size) were first treated with 300 μg/ml phytohaemagglutin for 30 minutes. Only these beads, which settled down on the bottom of the dish were subsequently picked by 'sticking' them to the micromanipulation micropipette. They were subsequently inserted through a previous 'cut' in the zona, made by inserting and removing a micropipette, and deposited on the blastomere membrane. Labelled embryos were cultured in vitro in drops of KSOM medium and 4 mg/ml BSA, under paraffin oil in an atmosphere of 5% CO_2 in air at 37.5°C.

To monitor the order and orientation of the second cleavage divisions, and then at the four-cell stage to examine the arrangements of blastomeres, labelled embryos were cultured in the 5% CO_2 incubator at 37°C and after 1 hour of uninterrupted culture checked by fluorescent microscopy at intervals of 20-40 minutes over a period of 5 hours. If within this time both blastomeres had divided to the four-cell stage, the embryos were discarded. Those embryos in which the red labelled blastomere had divided first were collected into one drop of medium and those in which the unlabelled two-cell blastomere was first to divide into another. In some experiments (as specified in the Results), four-cell blastomeres located furthest away from the polar body were labelled with a blue dye. Embryos were finally analysed to reveal the positions of the progeny of the labelled cells at the blastocyst stage. In lineage tracing experiments blastocysts were observed when still alive using a BioRad confocal microscope taking optical sections every 7 μm. By examining all sections in each series, it was possible to determine the distribution of cells labelled by specific dyes in the embryonic (polar trophectoderm and deeper ICM cells) and abembryonic (mural trophectoderm) parts of the blastocyst. The boundary zone between these two parts was defined as a cell layer, approximately one cell deep and parallel to the 'roof' of the blastocoel cavity as previously (Piotrowska et al., 2001). After confocal sectioning, the zona pellucida was removed by a short treatment with Acid Tyrode's reagent and next each of the embryos was disassociated by treatment with 1% trypsin for 5 minutes

dispersing them by thorough pipetting to count the total number of cells in each embryo.

Making chimaeras from specific four-cell stage blastomeres

One blastomere was labelled at the late two-cell stage by fluorescent DiD as described above. Following the division from the two- to the 4-cell stage, all marked embryos were classified into groups according to whether the labelled or unlabelled blastomere first underwent the two- to four-cell stage division. Embryos were examined at the four-cell stage and those with tetrahedral morphology (three blastomeres gathered around the attached polar body and the fourth one more distal) were scored from the position of labelled and unlabeled blastomeres as being either ME or EM (see Results). The products of the meridional division (M-division) were not distinguished from each other and are referred to as m-blastomeres, but products of the oblique or rather more equatorial division (E-division) differed in their position, with one of the blastomeres always being more distal from the second polar body, a marker of the animal pole (Gardner, 1997). The blastomere most proximal to the polar body was termed e1 and the one most distal, e2. Control groups of chimeric embryos were generated from m- blastomeres. Our discovery that marked membrane in the vegetal position of the two-cell blastomere could be displaced in the course of the E-division, but not in the M-division, required labelling of the vegetal pole of two-cell stage blastomeres by attaching beads as described above. Thus, in the second set of experiments, we used only 'vegetally marked' or unmarked 'animal sisters' to make chimaeras. We do not use these terms to infer that all cellular components are partitioned respecting their true animal-vegetal origins. We refer to four-cell stage blastomeres carrying a bead arising from an E-division as e1+ or e2+, depending upon the position of the cell. Cells not marked with beads are termed e1– or e2–. Meridional chimaeras comprised completely separated individual m-blastomeres that were then associated. To control for any effect of beads being attached to the blastomere surface, we also generated chimaeras with meridionally dividing blastomeres that carried beads. These developed normally.

Before the individual blastomeres were isolated to generate chimaeras, the zona pellucida was digested with 0.5% pronase in Ringer's solution at 37.5°C for 15 minutes. When the zona pellucida started to become 'thin', we gently rinsed the embryos and transferred them to a drop of M2 medium in a glass chamber on the stage of an inverted Leica microscope. This step had to be carried out with great precision in order to avoid any change in the specific configuration of four-cell stage blastomeres. Individual blastomeres of known origins were delicately aspirated using a biopsy pipette and removed from embryo (see also Fig. 4). Four (or three as specified in the text) such blastomeres of a single cell-type from different embryos were placed together into a small depression made in the bottom of culture dish and cultured in KSOM alongside three 'helper' embryos enclosed in their zones of albino MF1 strain. We routinely added helper embryos from this different strain to our individual experimental chimaeras as we found that the embryos developed better when cultured in a group.

Assessing development of chimeric embryos comprised of a specific cell type

Chimaeras were made between 59-61 hours after hCG and observations carried out 45 or 55 hours later. When chimaeras and helper embryos reached the advanced morula or blastocyst stage they were transferred together to the same uterine horn of foster mothers as previously described (Hogan et al., 1994; Zernicka-Goetz, 1988). Prior to transferring, chimaeras were observed to monitor their phenotype. At this time, control chimaeras reached an advanced blastocyst stage but not all of the chimeric embryos developed to this extent (Tables 1 and 2). We monitored subsequent development, scoring results only if helper embryos developed to term. This was essential to control for the success of transfer of the embryos to foster mothers both in each experimental animal and in each experimental group of embryos.

In two additional series of experiments, we again generated three cell chimaeras from four-cell blastomeres of known individual types to examine further their pre- and postimplantation development. In the first set of experiments, we examined blastocysts arising from these groups by confocal microscopy to count the number of cells in each embryo. Some of these embryos were also subjected to in situ hybridisation to reveal the expression of *Oct4*, a marker whose expression becomes restricted to the ICM (Schöler et al., 1990). Embryos were fixed with 4% paraformaldehyde/PBS and the method used for whole-mount in situ hybridisation was as described by Saga et al. (Saga et al., 1996). In the second set of experiments, embryos that developed from all three groups of chimaeras were transferred to foster mothers to examine their early postimplantation development. Embryos were recovered at the egg cylinder stage (E6.5) as previously described (Weber et al., 1999). The morphology of the recovered embryos was analysed to assess their developmental progress and some of the chimeric embryos were fixed with 4% paraformaldehyde/PBS at 4°C and in situ hybridisation was performed as described by Perea-Gomez et al. (Perea-Gomez et al., 2004).

In a third additional series of experiments, we generated chimaeras of individual e2+ four-cell blastomeres by surrounding them with four other four-cell blastomeres from random positions. We thus selected individual e2+ cells from a line expressing GFP-H2B and aggregated these with four randomly selected non-labelled four-cell blastomeres of a wild-type strain. The chimaeras were cultured in vitro to the advanced morula/blastocyst stage, transferred to foster mothers and then recovered at E5.5 for examination by confocal microscopy.

Results

Developmental success of four-cell stage blastomeres reflects their spatial origins and arrangement

The position of the second polar body at the two- and four-cell stages has been used as a marker of the animal pole. When the daughter cells are separated by a plane parallel to the animal-vegetal axis and with the polar body located between them, the division is described as meridional (M-division). When the daughter cells are separated by a plane perpendicular, or oblique, to the animal-vegetal axis and the polar body is located closer to one daughter than the other, the division is described as equatorial/oblique (E division). Our previous study has shown that when the earlier of the second cleavage divisions is an M-division and the later one an E-division (ME embryos) this tends to predict the polarity of embryonic-abembryonic axis in relation to the boundary generated by the first cleavage (Fig. 1A) (Piotrowska-Nitsche and Zernicka-Goetz, 2005). When the earlier of the second cleavage divisions is an E-division (EM embryos), the orientation of the axis still bears a relationship to the first cleavage but its polarity appears to be random (Fig. 1B). Thus, ME embryos provide us with blastomeres whose origins and fate are well defined in the great majority of cases.

We specifically wished to test the developmental properties of the four-cell blastomeres resulting from later E-divisions and occupying positions either proximal to or distal to the polar body (the e1 or e2 cells, respectively, of ME embryos). In most ME embryos (82%), these two cells appeared to contribute predominantly to blastocyst lineages that develop primarily into the extra-embryonic tissues (Piotrowska-Nitsche and

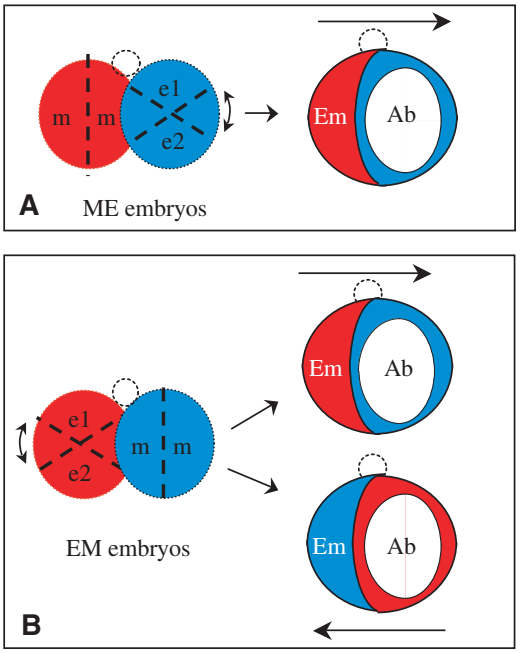
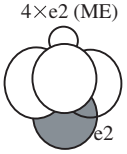
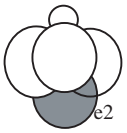
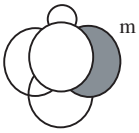
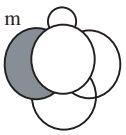


Fig. 1. A schematic representation of the origins of blastocyst pattern in the first two cleavages of the mouse embryo. Orientation of the first cleavage, and the order and orientation of the second cleavage divisions in both two-cell stage blastomeres affect the spatial pattern of allocation of their progeny at the blastocyst stage. Two groups of embryos are presented: ME, in which the earlier second cleavage is an M-division the later an E-division; and EM, in which the earlier second cleavage is an E-division and the later one an M-division. Taken together, both of these groups account for ~80% of all embryos in our studies (Piotrowska-Nitsche and Zernicka-Goetz, 2005). In both of these groups of embryos, there is a strong tendency for the one of the daughter cells to contribute most of its progeny to the future embryonic part and for the other to the future abembryonic part of the blastocyst. However, only in ME embryos does the early dividing blastomere preferentially contribute its progeny to the embryonic part. In EM embryos, the earlier dividing blastomere can equally often contribute to the embryonic or abembryonic part (hence two possible outcomes are presented).

Zernicka-Goetz, 2005). To this end, we first labelled one blastomere at random at the two-cell stage, assessed the order of blastomere divisions to the four-cell stage, and then selected ME embryos. To make chimaeras of e2 cells, we then combined four-cell blastomeres most distal from the second polar body from four such different embryos: 4×e2 chimaeras from ME embryos (Table 1). Such blastomeres could be derived from either labelled or unlabelled progeny of the two-cell blastomere. For comparison, we also made chimaeras from four four-cell blastomeres from the same positions in EM embryos: 4×e2 chimaera from EM embryos (Table 1). In such EM embryos the e2 cell has equal chance of contributing cells to either the embryonic or abembryonic parts of the blastocyst. We also generated two control groups of embryos that were aggregates of blastomeres resulting from meridional divisions: 4×m (ME) or 4×m (EM) (Table 1). In the first control group they were derived from different ME and in the second from different EM embryos. In all cases, chimaeras were cultured in

Table 1. Developmental success of chimeras made of e2 blastomeres

	Number transferred		Number of surviving pups		Total number (%)	
Group of chimeras	Chimeras	Helpers	Chimeras	Helpers	Chimeras	Helpers
4×e2 (ME) 	5 Bl/M	5Bl	0 (1 found dead)	4	8/27 (30%)	*30/32 (94%)*
	3 Bl	4Bl	1	4		
	8 Bl/M	5Bl	3	4		
	3BL/M	5Bl	2	5		
	3Bl	3Bl	1	3		
	1M	5Bl	0	5		
	2M and 2Bl	5Bl	1	5		
4×e2 (EM) 	3Bl/M	6Bl	1	5	11/24 (46%)	*35/37 (96%)*
	4Bl/M	4Bl	4	4		
	4Bl/M	4Bl	3	4		
	2Bl	6Bl	0	5		
	4Bl	5Bl	0	5		
	3Bl	3Bl	2	3		
	1Bl	5Bl	1	5		
	3Bl	4Bl	0	4		
4×m (EM) 	7 Bl/M	5Bl	4	5	18/26 (69%)	*22/22 (100%)*
	7Bl/M	5Bl	4	5		
	7Bl	4Bl	5	4		
	1M and 4Bl	8Bl	5	8		
4×m (ME) 	10Bl	4Bl	8	4	22/26 (85%)	*15/18 (83%)*
	6Bl	5Bl	5	3		
	3Bl	4Bl	3	4		
	7Bl	5Bl	6	4		

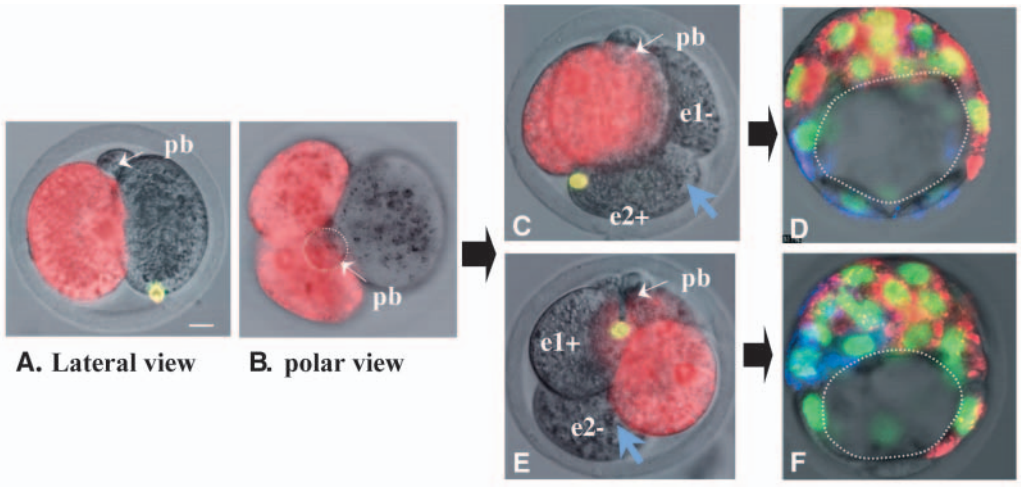


Fig. 2. Allocation to specific blastocyst regions of the progeny of blastomeres with vegetally marked membrane. (A) A lateral view of a two-cell stage embryo with one two-cell stage blastomere labelled with red dye and the other two-cell stage blastomere with a bead attached at its vegetal pole. pb, polar body. (B) Polar view of a three-cell stage embryo in which the blastomere labelled with dye has divided first through a meridional plane. (C) A four-cell stage embryo in which the dye labelled two-cell blastomere has divided meridionally and its sister two-cell blastomere undertakes an E-division. The bead remains in a 'vegetal' position (on the e2 cell), indicating that membrane has not been displaced during division. The cell carrying the bead (which we term e2+) is then labelled with a blue dye (blue arrow) and allowed to develop to the blastocyst stage. (D) Blastocyst derived from the embryo in C showing contribution of the blue labelled cells to mural trophectoderm. (E) A similar four-cell stage embryo to that shown in C in which the bead is now in a position proximal to the polar body (on the e1 cell) indicating that vegetal membrane has been displaced during division. The e2 cell that does not carry the bead (that we term e2–) is then labelled with a blue dye and allowed to develop to the blastocyst stage (F) Blastocyst derived from the embryo in E, showing contribution of the blue labelled cells to the boundary zone. Scale bar: 10 μm.

vitro to the advanced morula or blastocyst stage, and were then transferred (together with 'helper' embryos of the MF1 strain) to foster mothers. The proportion developing to live pups was then scored (Table 1).

We found that the developmental success of the group of chimaeras that were generated from the e2 blastomeres was significantly different. The two groups of control chimaeras, of m-blastomeres that had divided meridionally to four-cell stage, had a high probability of survival. Of these groups, between 85% (22/26) and 69% (18/26) developed to term, depending whether m-blastomere was a progeny of the earlier or later dividing two-cell blastomere. By contrast, the proportion of surviving progeny of the chimaeras of e2 blastomeres was reduced. This was most dramatic in chimaeras comprising e2 blastomeres taken from the later dividing two-cell blastomeres of ME embryos. The development of such chimaeras into viable pups was reduced to 30% (8/27). This was a statistically significant difference compared either with control chimaeras generated from later dividing m-blastomeres ($P<0.1$, χ^2 test 1 d.f. χ^2 is 2,846) or with chimaeras generated from earlier dividing m-blastomeres ($P<0.05$, χ^2 test 1 d.f. – χ^2 is 4,726). The survival to term of chimaeras comprising e2– blastomeres of EM embryos was also lower than the m-controls; however, as many as 46% (11/24) of embryos of this group gave rise to pups. This indicates that e2 blastomeres from ME embryos had significant differences from the other cells of the four-cell embryo.

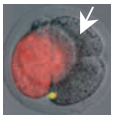
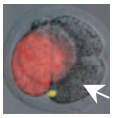
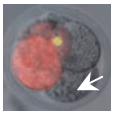
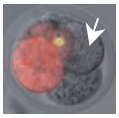
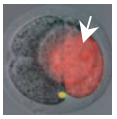
Progeny of blastomeres derived from later E-divisions of ME embryos are allocated to specific blastocyst regions

Our previous lineage tracing experiments showed that e2 blastomeres from ME embryos contributed most of their progeny to the abembryonic rather than the embryonic part of the blastocyst (23 out of 26 embryos) (Piotrowska-Nitsche and Zernicka-Goetz, 2005). This could either be as a clone of cells that comprised the mural trophectoderm or a clone contributing mainly to the boundary zone between the embryonic and abembryonic parts. We wondered whether these different fates might reflect alternative positioning of blastomeres at the four-cell stage.

One way that such a situation could arise would be if there had been displacement of all or part of the two-cell blastomere that undertakes an equatorial or oblique division. To test whether this could be the case, we labelled one two-cell stage blastomere with red dye and its sister with a fluorescent bead at a point opposite the second polar body (the 'vegetal' pole; Fig. 2A). We then selected those embryos in which the red cell divided first through the meridional plane (Fig. 2B) and then scored the position of the fluorescent bead when the other two-cell blastomere had undergone its E-division (Fig. 2C). We found that there were approximately equal proportions of embryos in which the e2 cell arising was labelled with the bead (68 blastomeres) or not (64 blastomeres) (Fig. 2C,E). Time-lapse observations indicated that following labelling, membrane marked by the bead could indeed either remain at the vegetal position or be displaced towards and even beyond the position of cleavage (see Movies 1 and 2 in the supplementary material). From this point onwards, we will continue to refer to the four-cell blastomeres arising from the later second cleavage in ME embryos as e1 and e2, reflecting whether they are located proximal or distal to the second polar body. However, we will adopt an additional + or – symbol to reflect whether or not they inherit a bead that was placed at a 'vegetal' position in their parental two-cell stage blastomere.

To examine whether such displacement of 'vegetally' marked membrane could relate to developmental fate of blastomeres, we carried out a similar bead-labelling experiment but subsequently labelled the e2 cell with blue dye irrespective of whether it was marked with a bead. Interestingly, we found that progeny of those e2 cells labelled with beads (e2+) populated predominantly the mural trophectoderm (Fig. 2D; Fig. 3A-D): 88% of labelled cells populated mural trophectoderm and 12% populated the

Table 2. Developmental success of chimeras made of e1– and e2–, e1+ and e2+ blastomeres

	Number transferred		Number of pups born		Total number (%)	
Group of chimeras	Chimeras	Helpers	Chimeras	Carriers	Chimeras	Carriers
No displacement 3×e1– 	1TB	6Bl	0	6		
	3M	5Bl	1	5	6/22 (27%)	32/32 (100%)
	2M and 2Bl	5Bl	1	5		
	2M and 3Bl	6Bl	3	6		
	4M and 1TB	6Bl	0	6		
	1Bl and 3M	4Bl	1	4		
No displacement 3×e2+ 	1TB	6Bl	0	5		
	1Bl (small ICM) and 3M	6Bl	0	6	0/22 (0%)	32/34 (94%)
	4M	7Bl	0	6		
	2Bl and 3M	5Bl	0	5		
	4M and 1TB	6Bl	0	6		
	1TB and 2M	4Bl	0	4		
Displacement 3×e2- 	1Bl	7Bl	1	6	4/22 (18%)	34/36 (94%)
	1Bl and 2 M	6Bl	0	6		
	2Bl (small) and 2M	6Bl	0	6		
	2Bl and 3M	5Bl	0	5		
	1Bl and 4M	7Bl	1	6		
	3Bl and 1M	5Bl	2	5		
Displacement 3×e1+ 	1Bl	7Bl	0	7	0/22 (0%)	33/36 (92%)
	1Bl (small ICM) and 3M	7Bl	0	6		
	4M	5Bl	0	4		
	1Bl and 4M	5Bl	0	5		
	1TB and 4M	7Bl	0	6		
	3M	5Bl	0	5		
Control 3×m (EM) 	4Bl	5Bl	2	5	7/16 (44%)	12/12 (100%)
	5Bl	4Bl	3	4		
	7Bl	3Bl	2	3		
3×m (ME)	5Bl	4Bl	5	4	7/8 (88%)	8/8 (100%)
	3Bl	4Bl	2	4		

Chimeras were made as described in Fig. 4 from the indicated e blastomeres arising from the E-division of ME embryos (see main text for definition of these terms). Control chimeras were also constructed from m blastomeres defined as arising from either early or late meridional second cleavage divisions (m-e or m-l respectively). Embryos were allowed to develop to the late blastocyst stage before transfer into foster mothers with helper ' blastocysts. Each row of the table represents a single transfer experiment in which the number of chimeras and their apparent developmental stage is indicated alongside the number of 'helper' embryos. M indicates morula; Bl/M, on the border of the morula and blastocyst stages; Bl, blastocyst stage. The percent survival of chimeras and helpers is presented as a sum for each of the four experimental groups.

boundary zone (total number of blue cells is 43 for six blastocysts). Progeny of those e2 cells not labelled with beads (e2–) populated predominantly the boundary zone (Fig. 2E; Fig. 3E-H); 11% of labelled cells populated mural trophectoderm, 62% populated the boundary zone and 16% populated the embryonic part (total number of labelled cells is 50 for six blastocysts). Thus, these results indicate that the allocation of e2 blastomere progeny might somehow relate to whether some components associated with the 'vegetal'-most part of its two-cell stage parent have been displaced or not.

Chimaeras of specific four-cell stage blastomeres have dramatically reduced developmental capability

This additional knowledge that some components of sister four-cell blastomeres could be found in different positions in ME embryos led us to re-examine the developmental capabilities of these specific cells. To this end, we once again labelled one two-cell blastomere with red dye and then both with a fluorescent bead at their 'vegetal' poles, and allowed them to develop to the four-cell stage selecting the ME class of embryos. We then separated each of the four-cell blastomeres as shown in Fig. 4. This allowed us to construct four types of chimaeras that comprised three e1 or three of e2 cells, depending upon whether they carried a bead or not (Fig. 4H,I). In these experiments, we combined three rather than four blastomeres together, because the number of steps required to obtain such specific blastomeres (e1+, e2–, e1–, e2+) led to the experimental material becoming limiting: we were able to generate only limited numbers of specific cell type chimaeras from any single experiments. We allowed these chimeric embryos to develop in vitro until the equivalent of the late blastocyst stage and then transferred them together with helper embryos to foster mothers to test their ability to develop to term (Table 2).

In multiple experiments that transfer groups of chimaeras to

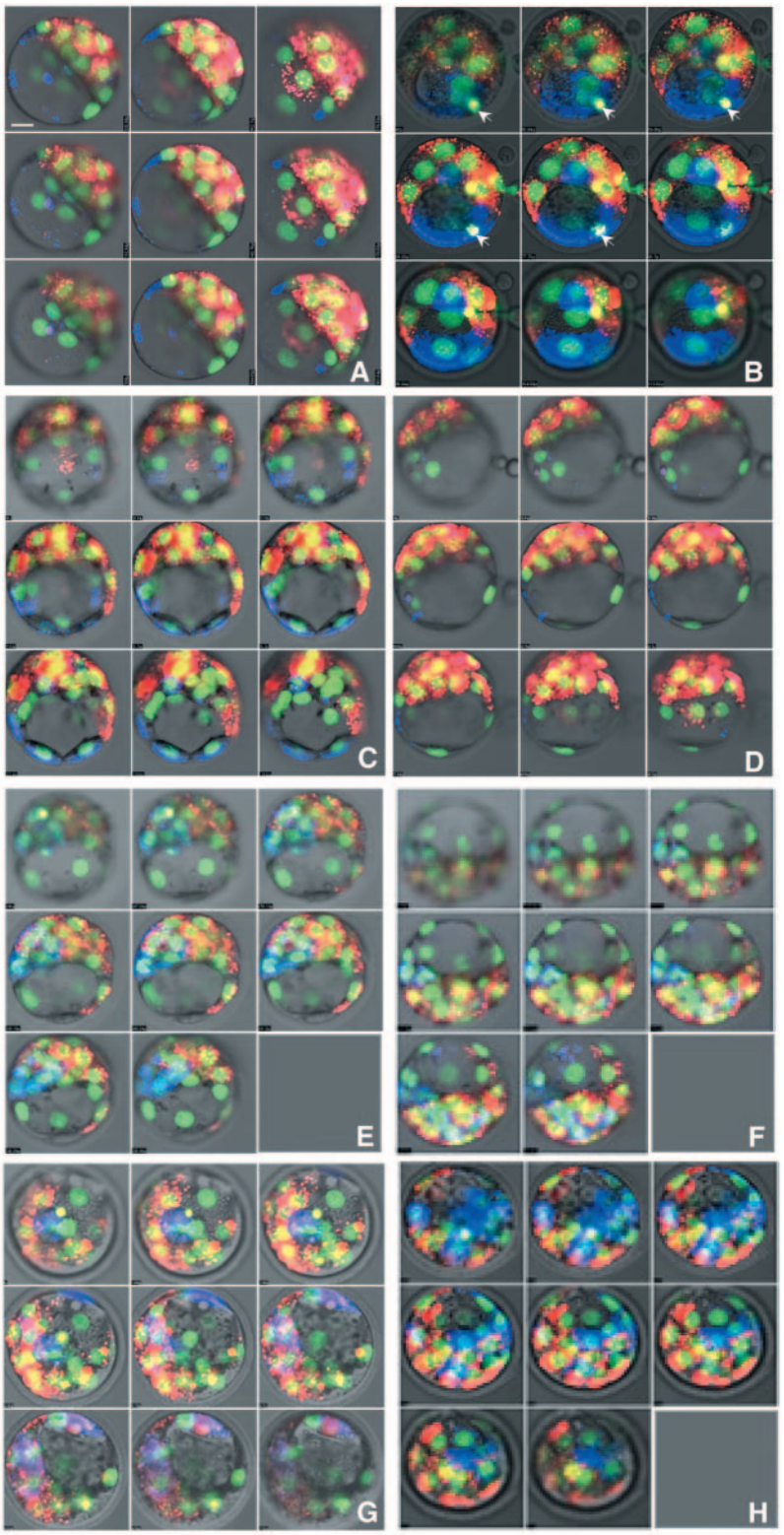


Fig. 3. Lineage tracing of e2+ (A-D) or e2– (E-H) four-cell blastomeres (marked in blue) to the blastocyst stage. (A-D) Series of optical sections of four blastocysts in which the e2 blastomere is labelled with blue dye. The red cells are progeny of one two-cell blastomere labelled with red dye that was first to divide to the four-cell stage. All cells have green nuclei due to the GFP-H2B transgenic marker. In all cases, the vegetal membrane of the labelled four-cell blastomere had not undergone displacement during its generation. In all four cases the blue labelled clone occupies predominantly the mural trophectoderm and does not contribute to ICM. The one exception is the embryo in B, in which a single blue cell was found in the superficial layer of the ICM. The arrowhead in B indicates a fluorescent bead that is retained in the development of this embryo. The bead, which was attached to the vegetal part of the two-cell stage blastomere still remains attached to a blue cell and so continues as a vegetal marker. (E-H) Series of optical sections of four blastocysts in which a vegetally labelled e-blastomere was found in the e1 position and the e2 cell was labelled with blue dye. In all four cases, the blue-labelled clone occupies predominantly the more superficial cells of ICM and also adjacent polar and mural trophectoderm. Scale bar: 18 μm.

foster mothers, we found that of a total of 22 chimaeras derived from e2+ four-cell blastomeres, none was able to develop to term (Table 2). Similarly, none of the 22 chimaeras constructed from e1+ blastomeres could develop to term. By contrast, 27% (6/22) and 18% (4/22) of chimaeras derived from their respective e1– or e2– sister four-cell stage blastomeres from the same embryos were able to do so. The differences in survival between e2+ and e1– chimaeras was statistically significant ($P<0.05$, χ^2 test 1 d.f. – χ^2 is 4.726). Thus, it appears that chimaeras generated from blastomeres that inherit at the very least some components of the 'vegetal' membrane from the egg have dramatically reduced developmental survival in comparison with both their sisters and m-cell cousins. Both e1– and e2– chimaeras also appeared to be compromised in their developmental abilities in comparison to chimaeras generated from the m-blastomeres that are the products of the early meridional division (Table 2).

Chimaeras of like-blastomeres arising from the later E-division of ME embryos show defects during pre- and postimplantation development

We noted that a significant proportion of the chimaeras derived from the four categories of blastomere arising from the later E-division developed to an advanced morulae but did not reach the blastocyst stage at the same time as their m-cell-derived counterparts (Table 2). Some of such e1 and e2 chimaeras did not form blastocysts even when permitted an additional 10 hours of development. This suggested that developmental defects in at least some of these embryos may be already arising prior to implantation. Thus, we carried out an additional series of experiments to examine further their development prior to and postimplantation.

To this end, we again generated the chimaeras from four-cell blastomeres arising from the later E-division of ME embryos, noting first whether this division had resulted in the 'vegetal' membrane being displaced. We found that irrespective of such displacement, three blastomere chimaeras constructed from both e1+ or e2+ and e1– or e2– blastomeres from the equatorial division developed into morulae but showed a variety of

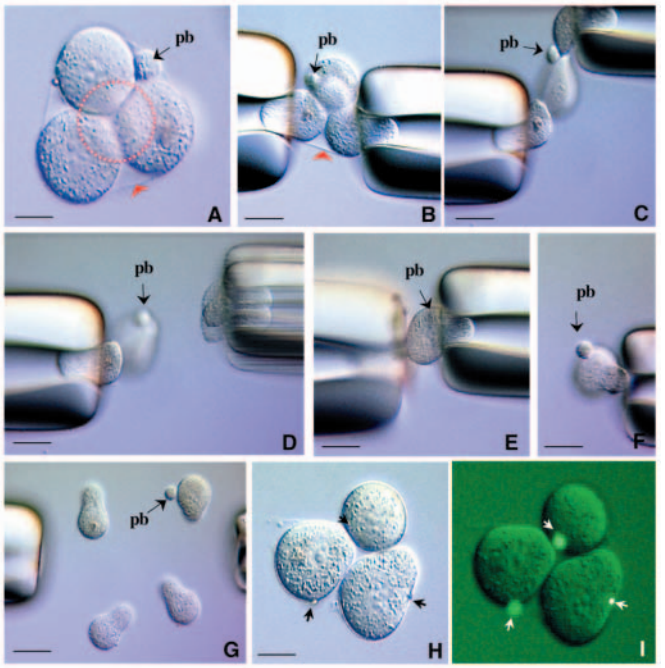


Fig. 4. Construction of chimaeras of individual four-cell stage blastomere types. ME embryos were selected in which the later dividing two-cell blastomere had been labelled at its vegetal pole as described in the legend for Fig. 3. (A) One such embryo that has been subjected to limited digestion with pronase in order to thin the zona pellucida (red arrow) so that it continues to maintain the tetrahedral morphology of the embryo and yet does not provide any resistance for micromanipulation of the individual blastomeres. (B) The embryo is held in the left-hand pipette by a m-blastomere while the e2 blastomere is withdrawn into the pipette on the right. (C,D) The procedure is repeated to remove an e1 blastomere into the right-hand pipette. (E,F) One of the m blastomeres, which is attached to the polar body, is then withdrawn into the right hand pipette. (G) The completed dissection showing all four four-cell stage blastomeres, the second polar body is still attached to one of the m cells. Scale bar: 60 μm. (H) An aggregate of e2 blastomeres from three such dissections. (I) The same aggregate as shown in H viewed under fluorescence optics to show the fluorescent beads used to label the vegetal poles. Scale bar: 18 μm in A,H,I; 33 μm in B-F.

abnormalities at subsequent stages, including apparent failure of development to the blastocyst (Fig. 5). We observed two types of abnormalities: apparently arrested morulae (14/33 of combined e1+ and e2+ chimaeras; 9/32 of e1– and e2– chimaeras); and formation of trophoblastic vesicles (3/33 of combined e1+ and e2+ chimaeras; 6/32 of e1– and e2– chimaeras). However, as many as 53% (17/32) of the combined groups of e1– and e2–, and 42% (14/33) of e1+ and e2+ chimaeras developed to apparently normal blastocysts, although often with reduced ICM. Such reduced size of ICM was especially apparent in e1+ and e2+ chimaeras (Fig. 5C,E,F). Optical sectioning indicated that such e1/e2– or e1/e2+ chimeric blastocysts had a reduced mean number of cells [mean of 33, range 23 to 43 (n=22); and mean of 29, range 20 to 37 (n=23), respectively] in comparison with control chimaeras derived from the earlier, meridionally dividing blastomere (mean of 49; range 30 to 56, n=20). A small series (n=10) of in situ experiments indicated that when blastocysts developed from either the e1 or e2 chimaeras, they expressed *Oct4* in the ICM (Fig. 5M-P).

With the knowledge that about half of the group of e1/e2– and some of the e1/e2+ chimaeras could develop into apparently normal, although small, blastocysts, a subset of embryos that had not been subjected to confocal miscroscopy or in situ hybridisation was transferred to foster mothers. Embryos were then allowed to develop until the early egg cylinder stage (E6.5) before being recovered. We found that the great majority (6/7) of the chimaeras constructed from m-blastomeres did not show any developmental defects (Fig. 5Q). However, when we recovered E6.5 e1+ or e2+ chimaeras, they were apparently retarded in comparison to control chimaeras (6/9 embryos) or showed in addition tissue disorganisation (1/9) (Fig. 5R-T), or appeared normal (1/9 embryos, not shown). Thus, the majority of e1/e2+ chimaeras could form ICM and the epiblast derived from it and therefore their demise cannot be solely explained by an inability of the e1+ or e2+ blastomere to form embryonic lineages of cells. Consistent with the reduced developmental survival of the chimaeras of the e1/e2– sister blastomeres from the equatorial division of ME embryos, we also found defects in their postimplantation development (5/7 were retarded in their development or showed abnormalities and 2/7 were normal) (Fig. 5U-W′). The e1– chimaera shown in Fig. 5U, for example, had asymmetric outgrowth of extra-embryonic tissue. Others did not show characteristic egg cylinder shape, and the extra-embryonic ectoderm particularly appeared to be poorly developed (Fig. 5V). Thus, the reduced survival of chimaeras built from a uniform type of blastomere arising from the later second cleavage of ME embryos correlates with abnormal development in both late preimplantation and early postimplantation stages.

To examine the molecular patterning of some of the above chimaeras at E6.5 we performed a small series in situ hybridisation to detect Fgf8, Cer1 or Bmp4. *Fgf8* is first expressed in the posterior epiblast and in the anterior visceral endoderm (AVE) at pre-streak stages and is maintained in the primitive streak after the onset of gastrulation (Crossley et al., 1995). *Cer1* is expressed in the distal and then AVE cells from E5.5 to E6.5 (Belo et al., 1997; Stanley et al., 2000). BMP4 is expressed in a ring of extra-embryonic ectoderm abutting the proximal epiblast from pre-streak stages onwards (Winnier et al., 1995; Lawson et al., 1999). In agreement with their normal morphology control m chimaeras show normal expression of *Fgf8* and *Cer1* in the posterior epiblast and the AVE respectively (Fig. 5X). An example of an e2+ chimaeric embryo with delayed development is shown in Fig. 5X′. This embryo showed a thick *Cer1*-positive AVE that is reminiscent of wild-type E5.75 embryos, when distal visceral endoderm cells have just reached an anterior position. However, although the embryo is small, the expression of *Bmp4* appears normal. This is also the case in another larger e2+ chimaera (Fig. 5Y). Other small e2+ chimaeras (Fig. 5Y′) showed no expression of *Fgf8* and *Cer1*, indicating their patterning was severely affected. Nevertheless, three germ layers could be distinguished morphologically.

An example of an e1– chimaera with a visceral endoderm layer as well as an inner epithelial layer, but with an abnormally placed ectoplacental cone (on one side of the embryo) is shown in Fig. 5Z. *Bmp4* expression is relatively normal in a ring of cells in the inner epithelium, presumably marking the boundary

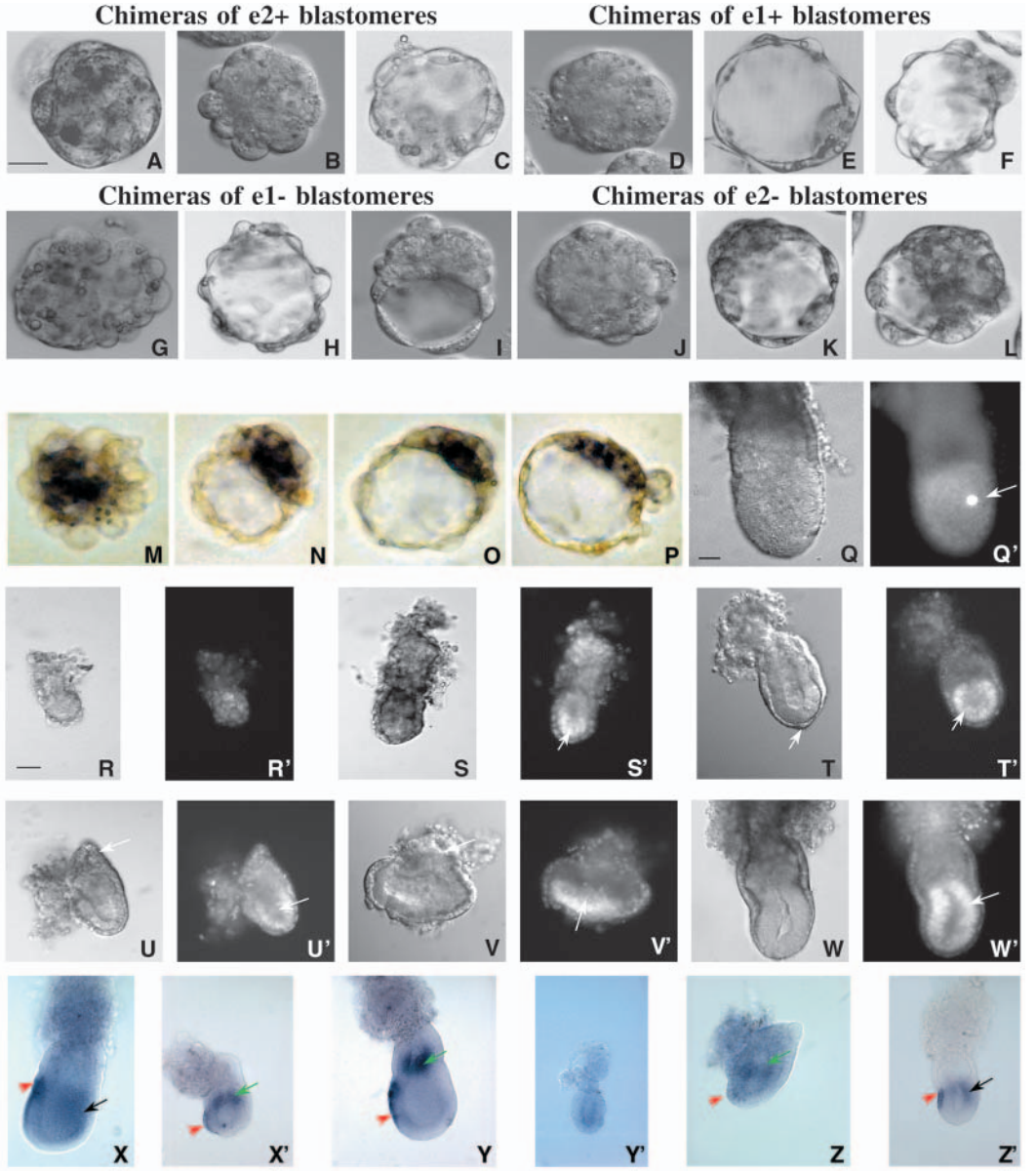


Fig. 5. Defects in preimplantation and postimplantation development of chimaeras derived from e1–, e1+, e2– and e2+ blastomeres. (A-L) The rows of vegetally labelled e1 and e2 (A-F) or non-vegetally labelled (e1 and e2) (G-L) chimaeras display embryos in which the marked vegetal membrane had either not undergone displacement or had been displaced during their generation in the second cleavage. A similar range of defects were seen in all groups. Many chimaeras developed to a late morula-like stage (A,B,D,G,J). Some embryos appeared to have begun to cavitate (D). Other embryos developed to form trophoblastic vesicles (F). When chimaeras developed to the blastocyst stage, they could contain very little ICM (E,H) or have a normal appearance (C,I,K,L). Scale bar: 20 μm. (M-P) In situ hybridisation showing *Oct4* expression in ICM cells or their precursors in such chimaeras. Blastocysts derived from e1– or e2– (M,N) and e2+ or e1+ (O,P) chimaeras. (Q-W′) Chimaeras constructed as described in Fig. 4 were allowed to develop in culture to the equivalent of the late blastocyst stage (see Materials and methods) and transferred to foster mothers together with carrier embryos. The chimaeras, which are recognised by their fluorescent nuclei, were recovered at E6.5, equivalent to the onset of gastrulation. (Q-Q′) m chimaera: a gastrulating embryo of normal appearance. The embryo is also shown under fluorescent optics (Q′) to reveal GFP-H2B expression and a fluorescent bead (arrow in Q′) applied in subset of experiments also in m chimaeras. This shows that the bead did not interfere with embryo development. The GFP marker is more strongly fluorescent in epiblast cells. Scale bar: 100 μm. (R-T) e2+ chimaeras: embryos either appear almost normal morphologically, although delayed in their development in comparison with m chimaeras (S,T) or are abnormal (R). The characteristic thickening of the visceral endoderm (presumably anterior visceral endoderm) is still at the distal tip of the egg cylinder (arrow in T) as found in E5.5, but not in E6.5 wild-type embryos. (R′-T′) Fluorescent images of embryos in R-T. Arrows indicate epiblast. None of the e2 cells used to make these chimaeras had undergone displacement of the vegetal membrane at the time of transition to the four-cell stage. Scale bar: 80 μm. (U-W) e1– and e2– chimaeras: two embryos that show significant levels of tissue disorganisation (U,V). (U) Asymmetric outgrowth of extra-embryonic tissue occurs (arrow in U). This is a e1– chimaera in which there was no displacement of the vegetally marked membrane. (V) e2– chimaera lacking or with reduced extra-embryonic structures (arrow in V). (W) Embryo (e1–) of apparently normal morphology. (U′-W′) Fluorescent images of embryos in U-W. Arrows indicate epiblast. (X-Z′) In situ hybridisation showing *Fgf8, Cer1* or *Bmp4* expression in m chimaera (X), e1+ chimaeras (X′,Y), e2+ (Y′) e1– chimaeras (Z,Z′). (X′) This embryo shows a thick *Cer1*-positive AVE (red arrow) that is reminiscent of wild-type E5.75 embryos. However, although the embryo is small, the expression of *Bmp4* appears normally localised (green arrow). (Y) An embryo that shows normal expression of Cer1 and Bmp4 (red and green arrows, respectively). (Y′) Small e2+ chimaera showing no expression of *Fgf8* and *Cer1*, indicating a delay or a complete arrest in development. Nevertheless, all three germ layers could be distinguished morphologically. (Z) An example of an e1– chimaera with a visceral endoderm layer as well as an inner epithelial layer, but with an abnormally placed ectoplacental cone (on one side of the embryo rather than proximally). The same embryo is shown in U. *Bmp4* expression is relatively normal in a ring of cells in the inner epithelium (green arrow), presumably marking the boundary between the epiblast and the extra-embryonic ectoderm. *Cer1* (red arrow) is expressed in a group of visceral endoderm cells presumably marking the distal tip of the embryo. (Z′) An e1– chimaera showing expression of *Cer1* (red arrow) and *Fgf8* (black arrow) as observed in control m chimaeras (X).

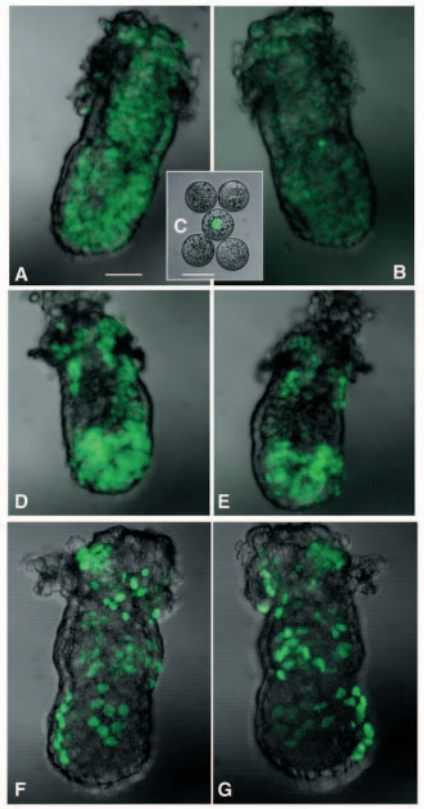


Fig. 6. Developmental potential of vegetally marked four-cell blastomeres when surrounded in chimaeras by four-cell blastomeres from random positions. Three chimaeras recovered at E5.5 to observe the distribution of H2B-GFP marked cells in comparison to progeny of not expressing GFP. (A,B) Two sides of the embryo in which the e2+-derived progeny contributed mainly to epiblast and extra-embryonic ectoderm. The contribution of labelled cells appears to be stronger on one side of the embryo (A). (C) Chimaera of e2+ four-cell stage blastomere (visible bead) and four wild-type blastomeres of F1 (wild-type) strain. (D,E) Two sides of embryo in which e2+ blastomere contributed to epiblast, extra-embryonic ectoderm and some visceral endoderm. (F,G) Two sides of chimaera in which e2+ contributed predominantly to visceral endoderm. Scale bar: in A, 50 μm for A,B,D-G; in C, 30 μm for C.

between the epiblast and the extra-embryonic ectoderm. *Cer1* is expressed in a group of visceral endoderm cells, presumably marking the distal tip of the embryo. Another example of an e1–chimaera shows normal expression of *Cer1* and *Fgf8*, as observed in control m chimaeras (Fig. 5Z′ compare with 5X). Thus, it appears that chimaeras of like-blastomeres arising from the later E-division show variety of defects during their pre- and postimplantation development.

Development of vegetally marked four-cell stage blastomeres when surrounded in chimaeras by blastomeres from random positions

The preceding experiments indicate that when four-cell blastomeres of equivalent type are aggregated into chimaeras their development can be compromised to an extent that depends upon their spatial location and history. As the greatest defects were seen in chimaeras derived from either e1+ or e2+ blastomeres, we wished to see the extent to which a single such cell placed into a chimaera surrounded by other four-cell blastomeres from random positions would contribute to the development of different lineages. We thus selected individual e1+ or e2+ cells from a line expressing GFP-H2B and aggregated these with four randomly selected non-labelled four-cell blastomeres of a wild-type strain (Fig. 6C). The chimaeras were cultured in vitro to the advanced morula/blastocyst stage, transferred to foster mothers and then recovered for examination at E5.5. The experiments showed that, for nine such embryos recovered, the labelled e1+ or e2+ derived cells could contribute to all tissue types (Fig. 6). Similar results were found for m-blastomere controls (n=20). Thus, when surrounded by randomly selected blastomeres, it would appear that the e1+ and e2+ blastomeres have full developmental potential.

Discussion

Recent lineage tracing studies have allowed us to identify a relationship between the distinct patterns of cleavage divisions that generate the four-cell mouse embryos and the contribution of progeny of four-cell blastomeres to specific regions of the blastocyst (Piotrowska-Nitsche and Zernicka-Goetz, 2005). One of the major patterns of cleavage, in which a meridional second division (an M-division) precedes an oblique/equatorial one (the E-division in ME embryos), is associated with the development of defined polarity to the future embryonic-abembryonic axis. Thus, in this group of embryos, the earlier dividing two-cell blastomere shows a tendency to contribute to the embryonic part of the blastocyst. In such embryos, the later-dividing two-cell blastomere appears to undergo a division that, were it truly equatorial and if cell components were distributed without mixing, would generate one four-cell blastomere with ‘vegetal’ and another with ‘animal’ components of the egg (Gardner, 2002). Both of these four-cell blastomeres are found to contribute most of their progeny to extra-embryonic rather than embryonic tissues, i.e. either to mural trophectoderm or to the boundary zone between the embryonic and abembryonic parts (Piotrowska-Nitsche and Zernicka-Goetz, 2005). Here, we show that chimeric embryos derived from such four-cell blastomeres resulting from the later equatorial/oblique division have a reduced chance of developing to term. However, ‘animal’ and ‘vegetal’ egg components do not have to retain the spatial relationships of their maternal origins to the four-cell stage. Indeed the four-cell blastomere that is furthest away from the polar body, the e2 cell, had ‘vegetally’ marked membrane in only 52% (68/132) of embryos studied in our experiments. In the remaining cases, the marked ‘vegetal’ membrane was found in the e1 cell, the cell more proximal to the polar body. Irrespective of its disposition, the four-cell blastomere with this ‘vegetal’ membrane marker tends to contribute most of its progeny to the mural trophectoderm and its sister to the boundary zone between the embryonic and abembryonic parts. Furthermore, none of the chimaeras built from either e1 or e2 cells with the ‘vegetal’ membrane marker survived to term. This was in contrast to chimaeras of their ‘animal’ sisters that did develop, although with significantly reduced frequency. This indicates that the developmental success of blastomeres arising from a later equatorial/oblique second cleavage division is reduced when combined with cells of the same type, but this is most severe for the cells inheriting this part of ‘vegetal’ membrane.

These results demonstrate that when surrounded by like cells, not all the four-cell blastomeres of the mouse embryo are equally able to achieve their full developmental capability. How does this outcome relate to previous findings about developmental capacities of individual mouse blastomeres? Earlier studies testing whether individual four-cell blastomeres were able to develop into mice, selected cells without referring to their site of origin or fate before they were aggregated into chimaeras with 'carrier' blastomeres (Kelly, 1977; Tarkowski et al., 2001). To our knowledge, the only previous attempt to generate chimaeras from blastomeres with spatially defined origins was from our own laboratory, in which we selected and isolated one four-cell blastomere with an attached polar body and let it divide to give one one-eighth of a blastomere with an attached polar body (by definition an 'animal blastomere') and another that we presumed to be a 'vegetal blastomere' (Ciemerych et al., 2000). When we made chimaeras of five of such one-eighth 'animal blastomeres' or five presumptive 'vegetal blastomeres', we found that 26-31% of them developed to term. However, our lack of understanding of the exact early cleavage pattern at that time meant that we could not have excluded with certainty 'animal blastomeres' or others with greater developmental potency from the 'vegetal blastomere' chimaeras.

The present findings focus on the development of particular four-cell blastomeres from one specific pattern of cleavage that allow us to predict not only the origin of individual blastomeres but also their developmental fate up to the blastocyst stage. However, we cannot exclude the possibility that blastomeres that inherit 'vegetal' egg components in some other cleavage patterns may also differ in their developmental success. Indeed, in experiments in which we monitored the developmental success of the two minority groups of embryos in which the second cleavage divisions appear to be either successively meridional (MM) or successively equatorial/oblique (EE) using a polar body as a marker (Piotrowska-Nitsche and Zernicka-Goetz, 2005), the EE embryos showed significantly poorer development. Whether this reflects 'animal-vegetal' partitioning in all of the cells of such four-cell embryos would require further study.

However, the reduced ability of the products of the later equatorial cleavage in ME embryos to develop as single-cell type chimaeras could also in part reflect their parentage from the later dividing blastomere. We note that, in general, chimaeras generated from later dividing blastomeres survived less well than those made from the earlier dividing cells. This can be seen by the better survival of chimaeras made from four-cell blastomeres descended from the earlier dividing blastomere of EM embryos (Table 1). In chimaeras generated from three cells from different regions of the four-cell ME embryo this may be accentuated (Table 2). Thus, chimaeras of the earlier dividing m-cells attained a mean size of 49 cells at the blastocyst stage and achieved 88% developmental success to term. This developmental success correlates with the ability of specific cells to proliferate when compared with chimaeras generated from the e– progeny of the later second cleavage. These e– chimaeras together achieved 23% developmental success. The mean cell number of e– chimaeras at the blastocyst stage was 33. However, although blastocysts from e+ cell chimaeras (with marked vegetal membrane) attained a similar mean cell number (29), none of these developed to term. Thus, a combination of at least two 'factors' could affect blastomeres achieving their full developmental ability. First of all, it would appear that cells arising in the later division have a reduced proliferative ability. Second, the products of the equatorial division appear to have inherent differences. Further study will be required to resolve the relative contribution of these factors. Thus far, there is no evidence that would allow us to conclude that there are any specific components in mouse egg that are spatially distributed along the oocyte animal-vegetal axis. We therefore wish to stress that in interpreting these results it is important to bear in mind that differences might arise between blastomeres, at least in part, owing to their specific arrangements and interactions with each other.

We cannot fully account for the mechanism whereby marked 'vegetal' membrane becomes displaced so that it lies more proximal to the polar body in some equatorially dividing cells at the four-cell stage. It has been reported that in rabbit embryos the cross-wise arrangement of blastomeres at the four-cell stage was the consequence of two meridional divisions in which one group of cells underwent a 90° rotation (Gulyas, 1975). It has also been suggested that this might be a possibility in the mouse embryo. However, a cell labelling study with beads by Gardner (Gardner, 2002) concluded that the most common tetrahedral form of the mouse embryo arose from the meridional division of one two-cell blastomere and the approximately equatorial division of the other. Thus, although the possibility of predominantly sequential meridional divisions associated with 90° rotation of cells as suggested by Gulyas (Gulyas, 1975) was dismissed, our observations of the displacement of the marked 'vegetal' membrane make it of interest to re-examine this question once again in future. Indeed it is not clear why the study of Gardner (Gardner, 2002) did not apparently detect the movement of 'vegetally' marked membrane. One possibility is that by labelling a larger area of 'vegetal' membrane with more diffusely distributed microspheres, the movement of a smaller restricted region was not detectable. An alternative possibility is that because Gardner's study used a smaller number of embryos, this membrane behaviour was not seen as we detected it only in about half of the ME embryos. In this light, we also note that beads placed at the 'vegetal' pole of meridionally dividing cells showed little movement in relation to the polar body position. Our study indicates that some of the equatorial/oblique divisions may be difficult to classify as parts of the membrane may behave independently of cytoplasmic components, let alone the spindle itself. Thus, it seems that these divisions may be equatorial in some aspects and not others. Consequently, it appears that at least some potential 'animal' and 'vegetal' egg components do not retain their initial spatial positioning in the second equatorial division.

Importantly, irrespective of whether the 'vegetal' membrane marked in our experiments is displaced, the four-cell blastomere that carries it tends to have specific blastocyst fate and developmental properties. We stress that this does not mean the fate of this cell is absolutely fixed. Indeed, when surrounded by cells of random origins it can contribute to a variety of embryonic lineages. However, when surrounded by cells of similar origins, as in the e1+ and e2+ chimaeras, it seems less able to do so. Thus, the e1– and e2– blastomeres are pluripotent in their ability to respond to developmental signals. However, they may be in the process of loosing their own ability to generate such signals as a result of their placement in the embryo. Alternatively, they may have other deficiencies in

comparison with their neighbouring blastomeres, a component of which can relate to slower rate of division as discussed above. Although we observed that a significant number of e1+and e2+ chimaeras already had defects at the preimplantation stages, the ICM of some was able to develop into epiblast, indicating that they were able to make not only extra-embryonic but also embryonic tissues. Interestingly, the extra-embryonic tissues in these chimeric embryos were not always properly organised. This was also evident in e1– and e2– chimaeras of their sister cell type. Although understanding the precise reasons behind the demise of such chimaeras will require much further study, our results indicate that developmental abnormalities appear to be not only due to the effects of a delay in the formation of the ICM and subsequently the epiblast, even though this could be a contributing factor.

In summary, a better understanding of the spatial and temporal cleavage patterns of the mouse embryos has allowed us to undertake studies of the developmental properties of individual four-cell stage blastomeres. This indicates that blastomeres differ in realising their developmental abilities from as early as the four-cell stage and that blastomeres inheriting at least some 'vegetal' component(s), partitioned in the later second cleavage, are significantly compromised in their development when aggregated with cells of the same origin. Alternatively, such four-cell blastomeres could be lacking critical components from the animal part of the egg. It has long been known that it is difficult to obtain identical quadruplets in mice from individual four-cell stage blastomeres. Even when combined together to increase total cell numbers at the time of blastocyst formation, not all combinations of individual four-cell blastomeres have similar chances for successful development to term. Our findings that not all four-cell stage blastomeres have equal ability to achieve their full development when surrounded by like cells could account in significant part for this difficulty.

The work was supported by the Wellcome Trust Senior Fellowship to M.Z.G. K.P. is a Marie Curie Fellow. We are grateful to David Glover, Anne McLaren, Jonathon Pines and John Gurdon for their invaluable support and comments on the manuscript. We thank I. Mason for the Fgf8 probe.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/3/479/DC1

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