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Lineage allocation and asymmetries in the early mouse embryo

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The mouse blastocyst, at the time of implantation, has three distinct cell lineages: epiblast (EPI), trophoblast and primitive endoderm (PE). Interactions between these three lineages and their directional growth and migration are critical for establishing the initial asymmetries that result in anterior–posterior patterning of the embryo proper. We have re-investigated the timing of specification of the three lineages in relation to the differential allocation of progeny of the first two blastomeres to the embryonic versus abembryonic axis of the blastocyst. We find that the majority of cells of the inner cell mass (ICM) are specified to be EPI or PE by the mid 3.5 day blastocyst and that this is associated with localized expression of GATA-6 in the ICM. We propose a model for molecular specification of the blastocyst lineages in which a combination of cell division order, signal transduction differences between inner and outer cells and segregation of key transcription factors can produce a blastocyst in which all three lineages are normally set up in an ordered, lineage-dependent manner, but which can also reconstruct a blastocyst when division order or cell interactions are disturbed.

Keywords: mouse embryo; blastocyst; transcriptional control; lineage analysis; primitive endoderm; GATA-6

1. INTRODUCTION

The E4.5 mouse blastocyst, at the time of implantation, contains three cell types: EPI, TE and PE. Lineage studies, mostly using chimeras and reconstituted blastocysts, have shown that the three cell types give rise to distinct tissues later in development (reviewed in Rossant 1987). TE and PE form only extraembryonic cell types, while the EPI gives rise to the entire foetus as well as extraembryonic mesoderm cells. The early differentiation of the extraembryonic cell lineages is important for their role in promoting the interchange of nutrients and other material with the maternal uterine environment. However, it has become clear in the last few years that these cell types also play key roles in signalling to the EPI to establish axial patterning in the embryo itself. In the early postimplantation embryo prior to gastrulation, extraembryonic ectoderm, a trophoblast derivative, is thought to provide general signals promoting expression of posterior mesoderm-specific genes, such as *Brachyury*, in the underlying EPI (Fujiwara *et al.* 2002). The PE gives rise to VE, overlying the extraembryonic ectoderm and the EPI, as well as parietal endoderm lining the inside of the external trophoblast giant cell layer. The VE, particularly the AVE, provides signals that restrict expression of genes like *Brachyury* to the posterior region, where the primitive streak arises (Beddington & Robertson 1999). Thus initiation of axial asymmetry can be attributed to an initial asymmetry in the VE. The AVE expresses a number of genes, such as *Hex* (Thomas *et al.* 1998) and *Cer1* (Belo *et al.* 1997), which define its limits. Monitoring the dynamic temporal and spatial expression of these genes, combined with lineage tracing, has shown that this asymmetry in the VE arises by directional movement of the VE from the distal tip of the egg cylinder to the anterior (Thomas & Beddington 1996). This asymmetry is preceded by non-random distribution of the progeny of different regions of the PE of the blastocyst to the surface of the EPI or extraembryonic ectoderm (Weber *et al.* 1999). Thus, understanding how the different lineages of the blastocyst are separately specified may give clues as to how asymmetries leading to axial specification are set up.

2. ALLOCATION OF CELLS TO BLASTOCYST LINEAGES

Early experiments, in which isolated ICMs from different stages of blastocyst development were studied, demonstrated that, by the fully expanded blastocyst stage, the ICM no longer contained cells capable of forming TE (Dyce *et al.* 1987; Gardner & Nichols 1991), but did contain EPI- and PE-forming cells (Rossant 1975). ICMs isolated from early blastocysts, however, could still form TE (Handyside 1978; Rossant & Lis 1979), as well as EPI and PE (Nichols & Gardner 1984). These results clearly defined the time of segregation of the TE and ICM, but did not address when PE is set aside. A series of experiments, using different genetic markers of increasing sensitivity to follow the fate of injected cells in blastocysts, has shown that the EPI and PE are clearly distinct by the E4.5 implanting blastocyst (Gardner & Rossant 1979; Gardner

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1982, 1984). At this stage the two cell types are also clearly distinguishable by morphology and gene expression (figure 1). What is less clear is when the two lineages become segregated. To address this question we have been re-investigating the fate of single cells in the E3.5 ICM, prior to morphological evidence of PE development.

Most of the work in the literature following the fate of single ICM cells injected into blastocysts has focused on the EPI contributions of such cells (Gardner 1985; Gardner & Cockroft 1998), so it is difficult to evaluate the range of contributions to PE and EPI. However, lineage tracing experiments of Zernicka-Goetz and colleagues, in which they injected GFP mRNA into single cells of the E3.5 ICM and followed their fate into early postimplantation stages, showed that cells injected on the surface of the ICM gave rise mostly to clones that were restricted to PE derivatives (Weber *et al.* 1999). EPI-only clones were also observed, but clones contributing to both lineages were not reported. There is, however, some concern that the marker system could make detection of EPI contributions difficult. Because there is considerable proliferation and mixing of the EPI postimplantation (Gardner & Cockroft 1998), the GFP mRNA could be diluted out and underestimate the EPI contributions. To avoid this problem, we have been carrying out lineage tracing experiments using a permanent GFP expression method (figure 2*a*). Instead of injecting GFP mRNA into single cells, we inject mRNA for the Cre recombinase (Sauer & Henderson 1989) into single cells of embryos derived from Z/EG reporter mice (Novak *et al.* 2000). GFP expression from a strong ubiquitous promoter is activated in cells expressing the Cre recombinase and hence in all progeny of the injected cell. Strong expression of GFP can be seen in all postimplantation tissues, allowing unequivocal identification of the progeny of single cells. When we collate data from a large number of experiments in which the fate of single injected ICM cells from early or expanded blastocysts is followed in early postimplantation stages, we find that clones segregate into either EPI or PE. So far no cell has contributed to both lineages (figure 2*b*,*c*).

These results suggest that, in the undisturbed blastocyst, single ICM cells are normally segregated to the EPI or PE lineages by E3.5, perhaps related to their position in the ICM. It does not, however, address whether the potential of these cells is restricted by this stage. To address this we have dissociated single GFP^+ cells from E3.5 ICMs (10–16 cells) isolated by immunosurgery from mid-blastocysts of the B5 ubiquitous GFP-expressing strain (Hadjantonakis *et al.* 1998). Dissociated single cells were then injected into unlabelled blastocysts. Chimeric conceptuses were examined for GFP expression in early postimplantation stages. Only one clone was found to contribute to both EPI and PE out of a fairly large series: all other clones were restricted to PE or EPI in roughly equal proportions. After injection of a single cell into the blastocyst, there may be too little time for the single cell to divide and contribute to more than one lineage before lineage separation. Thus, we also aggregated single ICM cells with eight-cell stage embryos so that the donor ICM cell has the opportunity to divide before formation of the host ICM lineages. When such aggregates were cultured to the blastocyst stage, one to four labelled progeny were observed. After transfer to the uterus, the majority of clones were again restricted to one or other lineage in early postimplantation conceptuses with only rare clones contributing to both EPI and PE. Marked cells were also occasionally seen in the TE confirming, at a single cell level, the plasticity of cells of the early ICM.

These three different experiments suggest that, although PE versus EPI fate is not absolutely fixed by the E3.5 blastocyst, there is a very strong bias towards separation of single cells to one or other lineage. This bias cannot be easily explained by the stochastic distribution of the progeny of each cell based on their position in the ICM.

3. TRANSCRIPTION FACTORS AND BLASTOCYST LINEAGE SPECIFICATION

As the different blastocyst lineages become set aside in early development, specific patterns of gene expression typifying each cell type become established. Lineage-specific transcription factors have been identified that are necessary for lineage specification. The POU domain transcription factor, Oct-4 (*Pou5f1*), has been shown to be important for development of the ICM of the blastocyst. Oct-4 is expressed in oogenesis and throughout early cleavage stages (Scholer *et al.* 1989) and becomes progressively restricted, first to the entire ICM and then to the EPI as blastocyst development proceeds (Palmieri *et al.* 1994). Knockout of Oct-4 leads to lethality at implantation and blastocysts fail to generate any ICM: all inside cells become trophoblast (Nichols *et al.* 1998). Oct-4 expression is thus a key in the first lineage decision. This has been emphasized by studies in ES cells, which normally express Oct-4. Conditional disruption of Oct-4 expression in ES cells led to differentiation of trophoblast-like cells, expressing markers typical of trophoblast cells *in vivo* (Niwa *et al.* 2000). When ES cells are forced to differentiate by removal of LIF from the culture medium, Oct-4 is also downregulated but no differentiation into the trophoblast pathway is observed (Niwa *et al.* 2000). Thus direct downregulation of Oct-4 seems to activate the TE pathway while indirect regulation is associated with later differentiation pathways. Oct-4 has been shown to be co-expressed with the SOX (SRY-box containing gene) factor, Sox2, in cleavage stages and in the ICM and EPI (Collignon *et al.* 1996), and to function with Sox2 in regulating FGF4, a key ICM-specific growth factor (Yuan *et al.* 1995). Knockout of *Sox2* also leads to early postimplantation lethality, with minimal development of the EPI (Avilion *et al.* 2003), suggesting that the Oct-4/Sox2 complex is critical for EPI formation. Sox2 is rapidly downregulated in PE, but, unlike Oct-4, it remains active in the TE, where it plays a later role in maintenance of trophoblast stem cells (Avilion *et al.* 2003), presumably in conjunction with other transcription factors.

Specification of the trophoblast lineage is not simply the default pathway of development in the absence of Oct-4. The caudal-related homeodomain protein, Cdx2, shows a reciprocal pattern of expression to Oct-4 in the early embryo, becoming restricted to the TE by the blastocyst stage (Beck *et al.* 1995). Knock-out of *Cdx2* results in homozygous embryos that fail to implant, indicative of a major trophoblast defect (Chawengsaksophak *et al.* 1997). We have examined these embryos in more detail (D. Strumpf and J. Rossant, unpublished data) and show that

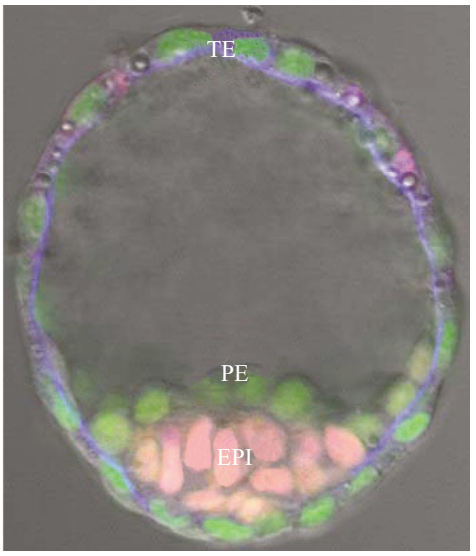

Figure 1. Composite confocal image of E4.5 mouse blastocyst. Green nuclei, YOYO nuclear stain; pink nuclei, Oct-4 antibody staining merged with YOYO stain; blue basement membrane staining, anti-α7 integrin antibody stain.

they develop to the early blastocyst stage, but rarely form an expanded blastocoele. All cells in the mutant blastocysts continue to express Oct-4. Mutant blastocysts fail to attach and outgrow in culture and no differentiated trophoblast is detected. Further, no trophoblast stem cell lines can be obtained from the *Cdx2*−/− embryos, although homozygous ES cells can be obtained. TE formation thus requires loss of Oct-4 and gain of Cdx2 expression. It is not yet known whether ectopic expression of Cdx2 is sufficient to drive ES cells to the TE pathway, or whether ectopic expression of Oct-4 will drive TS cells to the EPI/ES pathway. It is also not clear whether there is a direct regulatory network between Oct-4, Sox2 and Cdx2 in the early embryo. However, to date, these three transcription factors appear to be the earliest regulators of cell fate in the embryo. The upstream mechanisms that lead to their restricted expression by the blastocyst stage are not known.

When the PE develops in the E4.5 blastocyst, it activates a number of transcription factors, including HNF4 (Duncan *et al.* 1994), GATA-4 (Arceci *et al.* 1993; Heikinheimo *et al.* 1994) and GATA-6. Mutations in all three genes lead to defective development of the later VE derived from the PE (Chen *et al.* 1994; Molkentin *et al.* 1997; Morrisey *et al.* 1998; Koutsourakis *et al.* 1999), and both GATA-6 and GATA-4 deficient ES cells fail to form PE-type cells in embryoid bodies (Soudais *et al.* 1995; Morrisey *et al.* 1998). GATA-6 has been shown to be upstream of HNF4 (Morrisey *et al.* 1998), which, in turn, is upstream of a number of other VE-specific regulators that activate differentiated products of the VE (Duncan *et al.* 1998). Although GATA mutations do not lead to complete absence of PE formation, GATA factors are at the top of the genetic hierarchy of PE development as defined to date. This is consistent with the conserved role for GATA factors in specifying endoderm cell through evolution (Patient & McGhee 2002). Some redundancy of activity between GATA-6 and GATA-4, and perhaps some persistence of maternal protein, may explain the failure to completely block PE development *in vivo*. Recent work has shown that ectopic expression in ES cells of GATA-4 or GATA-6, but not downstream genes like HNF4, is sufficient to drive PE differentiation even in the presence of LIF (Fujikura *et al.* 2002), consistent with a major role for these factors in specifying PE. GATA-6 was also shown to activate PE-specific genes, like COUP-TF I and II (Fujikura *et al.* 2002), which in turn may repress expression of Oct-4 (Ben-Shushan *et al.* 1995).

We have re-examined the early expression pattern of GATA-6, by confocal imaging of both fluorescent *in situ* hybridization and antibody localization. By E4.5 there is clear segregation of Oct-4 and GATA-6 to the EPI and the overlying PE, respectively (figure 3*c*). However, at E3.5 there are cells co-expressing GATA-6 and Oct-4 within the ICM (figure 3*a*). As blastocyst development proceeds, there appears to be a segregation of the GATA-6 expression to the surface of the ICM (figure 3*b*), followed by a downregulation of Oct-4 in these cells. If GATA-6 is an initiator of PE development, this might suggest that individual cells of the ICM vary in their potential to form PE or EPI depending on whether or not they express GATA-6. This would help explain the data showing that single ICM cells are usually restricted to one or other lineage by E3.5. Direct proof of this requires separate evaluation of the potential of the GATA-6-expressing and non-expressing ICM cells, which may be possible with GFP-reporter lines.

Although no mutation in a single transcription factor has produced embryos completely lacking PE, mutation of the signal adaptor protein, Grb2, has been reported to result in absence of PE in both embryos and ES cells (Cheng *et al.* 1998). We examined the expression of GATA-6 in *Grb2* mutant embryos and confirmed absence of both morphological PE and expression of GATA-6 by E4.5 (not shown). We then examined E3.5 *Grb2* mutant blastocysts, which appear morphologically normal and showed complete absence of GATA-6 expression (figure 3*d*). This suggests that activation of GATA-6 in PE progenitors is dependent on a signal transduction pathway involving Grb2. Ras/Map kinase activation has been shown to activate endoderm differentiation in F9 terato-carcinoma and ES cells (Burdon *et al.* 1999; Verheijen *et al.* 1999), consistent with this model. The FGF signalling pathway has been shown to be involved in PE proliferation in blastocyst explants (Rappolee *et al.* 1994; Feldman *et al.* 1995). FGF signalling, acting via Grb2, may thus be involved in GATA-6 activation and induction of PE differentiation. FGF signalling has also been shown to be involved in promoting TE stem cell development and differentiation (Tanaka *et al.* 1998), and transient GATA-6-lacZ expression has been reported in the TE (Koutsourakis *et al.* 1999). Endogenous GATA-6 mRNA expression was not seen in the TE of the E3.5 blastocyst but protein expression persisted, suggesting that the lacZ expression represented perdurance of lacZ from earlier GATA-6 transcription. We examined late morulae/early blastocysts and found low levels of GATA-6 mRNA and protein specifically in the outer cells of the morula (not shown). This is an intriguing result because it represents a clear example of spatial segregation of important cell fate

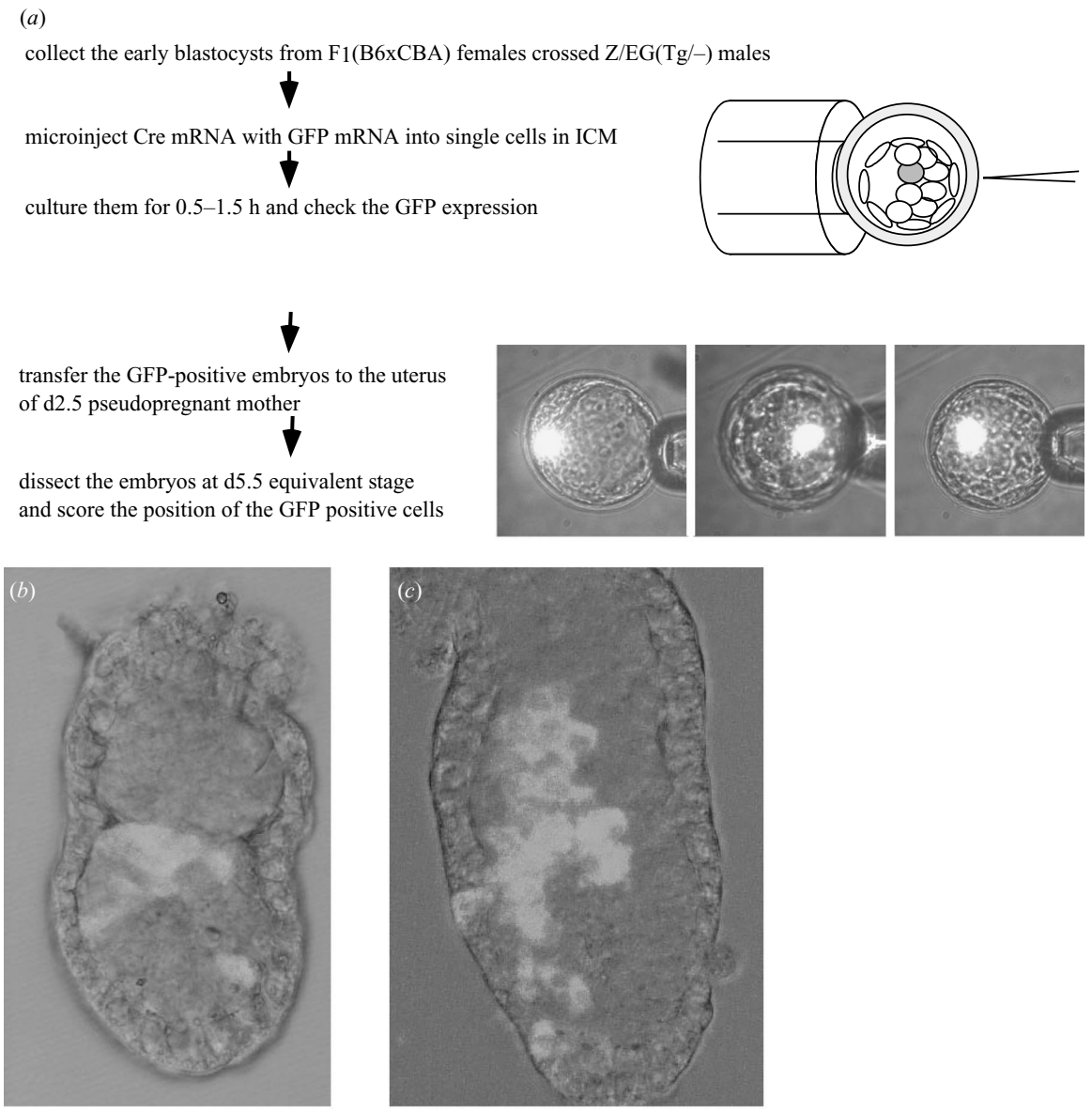

Figure 2. Lineage tracing by single cell injection of Cre mRNA. (*a*) Experimental design. Z/EG mice contain a transgene in which a floxed *lacZ* gene is expressed under the control of the strong, ubiquitous PCAGGS promoter. When the Cre recombinase is expressed, the *lacZ* gene is excised and a *GFP* gene comes under the control of the promoter instead. Thus Cre excision leads to loss of lacZ expression and gain of GFP expression in all progeny of the initial cell in which Cre is expressed. Injection of Cre into one cell of the ICM can thus mark all the progeny of that cell with GFP expression at later stages. (*b*) EPI only clone derived from a single Cre-injected ICM cell. (*c*) PE only clone derived from a single Cre-injected ICM cell.

determinants prior to formation of separate lineages at the blastocyst stage.

4. A MODEL FOR MOLECULAR SPECIFICATION OF THE BLASTOCYST

From these combined lineage and molecular experiments, it becomes apparent that the establishment of the early cell lineages involves a progressive segregation of key lineage-specific transcription factors that act positively to specify cell fate, and induction of other factors that repress opposing cell fate. It is also apparent that, although the three lineages are not entirely restricted by the E3.5 blastocyst, there is already a clear predisposition of cells to one of the three lineages. What is still unclear is how this molecular and cellular segregation is initiated.

Here, we propose a possible model for this process, which incorporates information on how cell division planes drive the formation of inside or outside cells during cleavage, the knowledge of how the order of cell division affects the contribution of cells to different parts of the embryo and some of the molecular knowledge recently accumulated. Extensive work from Martin Johnson's group has shown that the enclosed cells of the cleavage stage embryo are generated by differentiative divisions of the polarized outside blastomeres at the 8–16 and 16–32 cell stage (Fleming & Johnson 1988). There is also extensive evidence, including the recent analysis of the distinct fates of the blastomeres of the two-cell stage (Gardner 2001; Piotrowska *et al.* 2001), to suggest that earlier dividing blastomeres generate more inside progeny than late-dividing blastomeres (Graham & Deussen 1978). Lin-

eage tracing with fluorescent beads suggested that 75% of the ICM derived from inside cells generated at the 8–16 cell transition and that these were predominantly derived from the earlier dividing blastomeres (Fleming 1987). The advantage of the early dividing cells relates to the changes in cell behaviour that occur at this cell division, including intercellular flattening. When groups of early dividing 2/8 blastomeres were aggregated with later-dividing cells, the early dividing cells flattened first and contributed disproportionately to the inside cells. However, treatment of the cells with low calcium medium blocked the cellular changes and removed the bias for the early dividing cell to contribute to the inside group (Garbutt *et al.* 1987). Thus division order is important but more for its effect on cell behaviour than as a true determinant of cell fate.

In normal development, if one of the first two blastomeres divides ahead of its neighbour, its progeny will retain this early dividing advantage throughout normal development. This will result in the early dividing blastomere contributing disproportionately to the ICM, because of the cell behaviour differences noted above. Indeed, the progeny of the earlier dividing blastomere preferentially contribute to the polar TE and inner cells of the ICM, while the other blastomere contributes to mural TE and the surface cells of the ICM (Gardner 2001; Piotrowska *et al.* 2001). The position of sperm entry has been reported to be associated with the earlier-dividing two-cell blastomere (Piotrowska & Zernicka-Goetz 2001), and parthenogenetic embryos tend not to show the same predominance of the early dividing blastomere to the embryonic region (Piotrowska & Zernicka-Goetz 2002). This might suggest that the sperm entry point somehow drives the contribution of the early dividing cell to the embryonic region. However, the role of the sperm entry point in determining the plane of first cleavage is still controversial (Davies & Gardner 2002). Here, we build our model around the fact that division order is set at the two- to four-cell stage and that there is usually relatively coherent growth of the progeny of the first two blastomeres. We show that it is possible to generate a blastocyst with all three cell lineages more or less specified by E3.5, without recourse to segregation of determinants from the egg or segregation of determinants by asymmetric cell division (figure 4).

At the 8–16 cell transition, a group of inside cells is generated, probably predominantly derived from the first dividing two-cell blastomere. Once this has occurred, the embryo has two distinct populations of cells, allowing possible signalling between inside and outside cells. We propose that this results in preferential activation of transcription factors, such as GATA-6, in the outside cells (as we have observed). Whether GATA-6 is the key factor, whether it is activated at the protein level from maternal transcripts or whether it is newly transcribed in the outside cells of the embryo, remains to be proved. The identity of the signalling pathway that leads to GATA-6 expression is also not clear. However, it is likely to be a receptor tyrosine kinase driven pathway, as GATA-6 expression is absent in *Grb2* mutant embryos (figure 3*d*). Grb2 is a key adaptor molecule in receptor tyrosine kinase signal transduction. FGF signalling has been shown to be required for cell divisions leading to the blastocyst (Chai *et al.* 1998) and for proliferation of both TE and PE at the blastocyst stage (Rappolee *et al.* 1994), and could be a candidate also for this early signalling event. This will require further investigation.

At the 16–32 cell transition, another round of inside, apolar cells is generated by differentiative divisions of the outer cells. Because the outer cells now express GATA-6, the protein can be inherited by both the polar and apolar cells generated at this division, but will be absent from the cells already inside the embryo. This allows the generation of two populations of ICM cells—GATA-6^{+} and GATA-6^{-}. This is consistent with our observations on GATA-6 expression in cells in the E3.5 ICM. GATA-6 expression in the outside TE progenitors is transient but may be involved in initiating the downregulation of Oct-4 that is required for TE development. In ES transfections, at any rate, GATA-6 can turn on expression of COUP-TF (Fujikura *et al.* 2002), which can repress expression of Oct-4. GATA-6 probably plays this role in the PE progenitors too. It is known that COUP-TF I and II are expressed in the PE of the blastocyst (Murray & Edgar 2001), as Oct-4 expression declines. In our experience, there is no transient upregulation of Oct-4 in the developing PE, as reported previously (Palmieri *et al.* 1994), but Oct-4 does stay on longer than in the TE, consistent with an earlier onset of expression of the possible repressive pathway initiated by GATA-6 in the TE. We have not yet been able to confirm expression of COUP-TF in the outside cells of the morula, as predicted by this model. Further specification of the TE lineage is associated with the induction of expression of Cdx2 in the outer TE cells and the maintenance of expression of Sox2.

Because GATA-6 is capable of inducing PE in undifferentiated ES cells and is totally absent from mutant embryos that fail to make any PE, we propose that the GATA-6-expressing ICM cells are PE progenitors while the negative cells are EPI progenitors. The coherent growth and differential contributions of the first two blastomeres will lead to a normal situation in which the later dividing blastomere from the two-cell stage is likely to contribute more inside cells at the 16–32 rather than the 8–16 stage. Thus, proportionately more inside progeny of this cell will inherit factors like GATA-6, and become PE. Because the later-dividing cell also contributes predominantly to cells on the surface of the ICM (and the mural trophoblast), this will put GATA-6-expressing cells in the right position to become PE. There will be other GATA-6-expressing cells in other positions in the ICM, derived by differentiative divisions of other 16-cell blastomeres. GATA-6 is known to turn on molecules like Dab2 (Fujikura *et al.* 2002), which are involved in correct epithelial organization (Sheng *et al.* 2000). Mutants in Dab2 (Yang *et al.* 2002) and Laminin C1 (Smyth *et al.* 1998), an important extracellular matrix component of the PE, produce PE, but the cells are mixed throughout the ICM. This suggests that GATA-6 can promote cellular properties that will allow correct sorting of PE progenitors that are in the wrong position in the ICM. We have monitored the progression of GATA-6 expression in the ICM from E3.5 to 4.5 and find a progressive accumulation of GATA-6 expression at the ICM surface, as predicted.

Thus, in the normal embryo, the likelihood of the first two blastomeres producing PE is not equal and division order will lead to appropriate distribution of most PE pro-

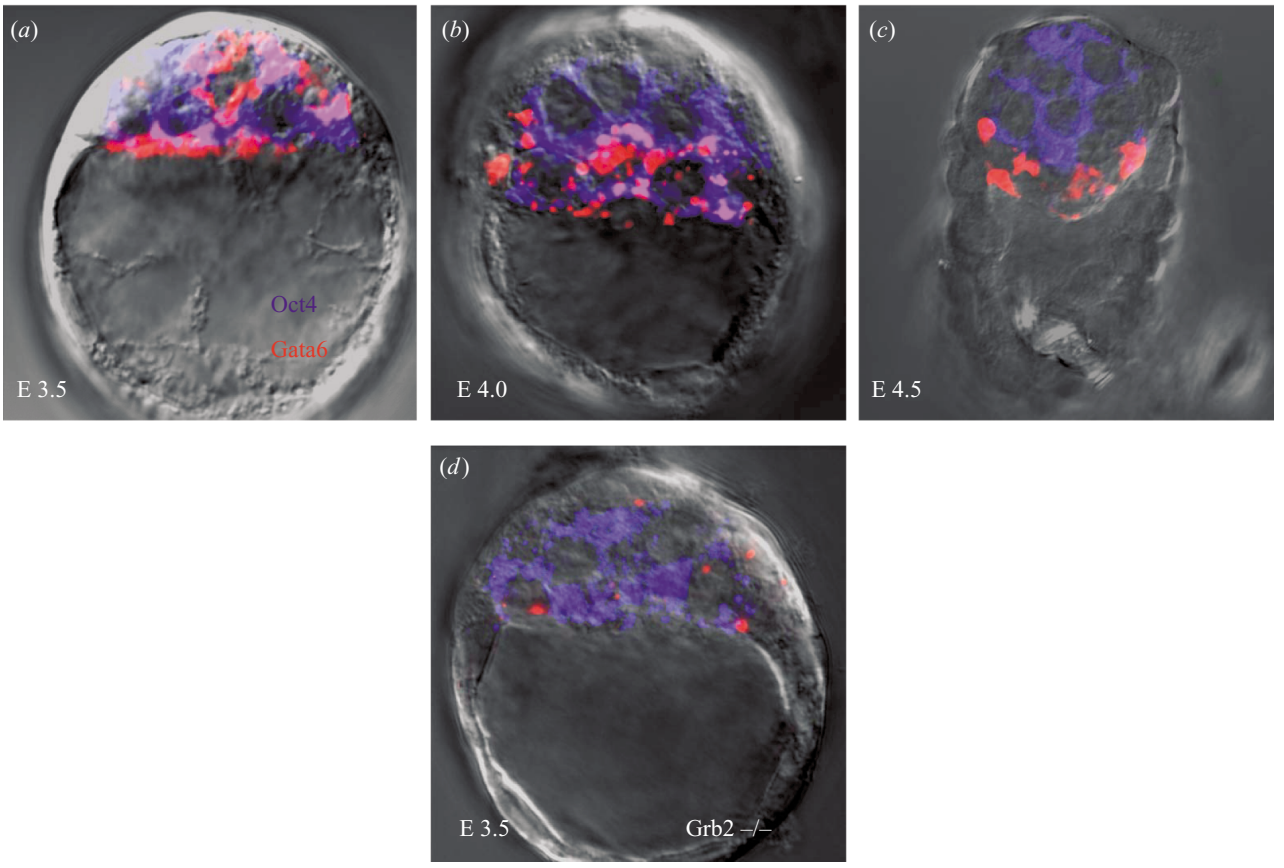

Figure 3. GATA-6 and Oct-4 expression in wild-type and Grb2−/− mutant developing blastocysts. Double *in situ* hybridization using fluorescent-labelled probes to Oct-4 (blue) and GATA-6 (red). Confocal images of the two fluorophores were merged so co-expression appears as pink. (*a*,*b*,*c*) Progressive restriction of GATA-6 expression to the surface cells of the ICM and then the PE. (*d*) Absence of GATA-6 expression in morphologically normal Grb2 mutant blastocyst.

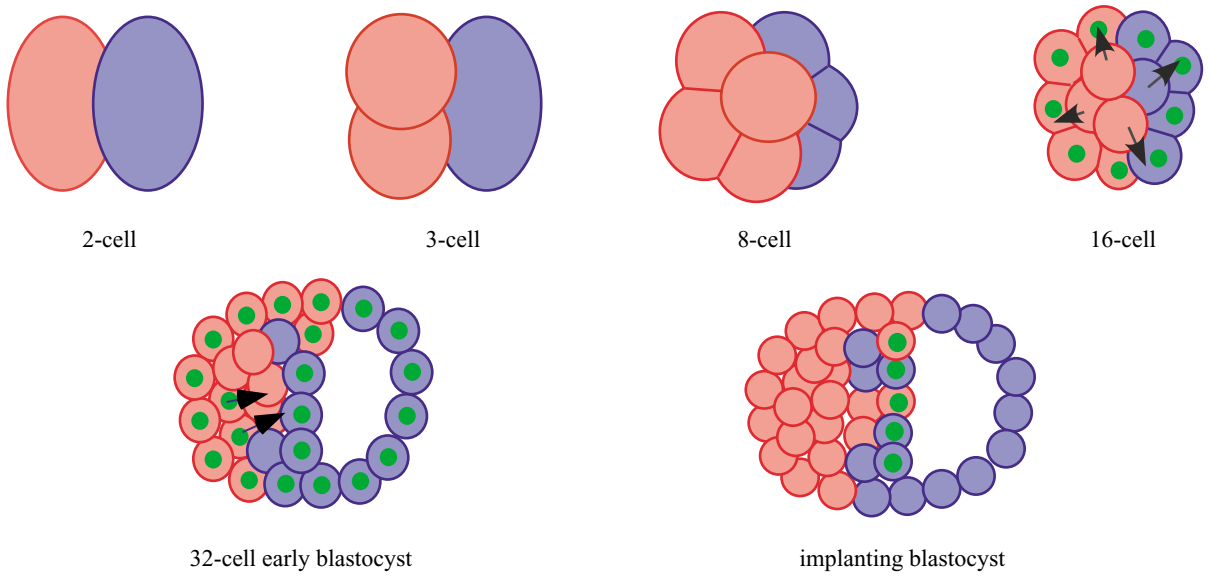

Figure 4. Speculative model for establishment of the early lineages of the mouse embryo. One of the first two blastomeres (red) divides ahead of the other at the three-cell stage and retains this division advantage, generating more inside cells at the 16-cell stage. The inside cells at the 16-cell stage signal to the outer cells (arrows) inducing expression of GATA-6 (green). More inside cells are generated from outer GATA-6-expressing cells at the 16–32 cell transition, generating GATA-6-expressing cells in the ICM, predominantly on the cell surface. GATA-6-expressing cells in the interior of the ICM sort to the ICM surface (arrows). By implantation, GATA-6 is repressed in TE and all GATA-6-expressing cells are in the PE.

genitors on the surface of the ICM. However, if division order is disturbed or cells are removed, added or altered in position during cleavage, the model will still allow normal development. PE progenitors will still be generated by inheritance of GATA-6 from outside cells at the 16–32 cell transition, but the progenitors will be more randomly distributed, and will have to sort out in order to generate an intact PE. This model allows for the resistance of the mammalian embryo to experimental perturbation and the observed variation in the order of division and the extent of cell mixing in normal development. We are currently testing some of the cellular and molecular predictions of the model.

The complex interactions of cell lineage, cell division order and segregation of molecular determinants involved in setting aside the lineages of the blastocyst also provide opportunities to develop asymmetries within lineages that could lead to axial patterning in the postimplantation embryo. Cell division order, for example, could lead to some PE cells developing ahead of others, or being in the correct position for later expansion ahead of others. This could drive the asymmetric growth and expansion of the VE seen postimplantation that presages the formation of the AVE. Continued careful lineage tracing experiments, combined with molecular markers, will help elucidate the significance of the events in the preimplantation embryo for later development.

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Discussion

M. Jones (*Institute of Cancer Research, London, UK*). Do you think that cell division is critical, particularly in the Grb2 mutant, to establish the PE versus EPI lineages? Are there the same number of cells in Grb2 mutants compared to wild-type?

J. Rossant. We have not examined cell number in great detail in the Grb2 mutant embryos, but at the E3.5 blastocyst, morphology and cell number appear normal, and yet GATA-6 expression is absent. There certainly are deficiencies in cell proliferation at later stages, but we think the initial effect is not on cell division.

E. J. Robertson (*Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, USA*). Why do cultured ES cells reintroduced into blastocysts fail to contribute to extraembryonic lineages?

J. Rossant. ES cells never form trophoblast *in vitro*, except when Oct-4 is conditionally disrupted (Niwa *et al.* 2000), so it is not surprising that they do not contribute to trophoblast in chimeras. ES cells do make PE *in vitro*, but undifferentiated ES cells do not initially express PE-inducing genes like GATA-6. When undifferentiated ES cells are put back in a blastocyst, there are already GATA-6 expressing cells in the ICM, which we think are poised to be PE. Presumably ES cells are therefore shunted into the EPI lineage and fail to contribute to PE, despite their ability to do so after longer periods of differentiation *in vitro*.

A. Smith (*Centre for Genome Research, University of Edinburgh, Edinburgh, UK*). What is the distribution of GATA-6 protein during cleavage and in the ICM?

J. Rossant. We are currently examining GATA-6 expression at earlier cleavage stages. GATA-6 protein in the ICM is distributed similarly to the mRNA.

R. G. Edwards (*Reproductive BioMedicine Online, Dry Drayton, Cambridge, UK*). There are recent reports that one blastomere in a four-cell human embryo secretes either HCG-β or LH-β (Hansis *et al.* 2002; Hansis & Edwards 2003). We do not know why a blastomere in the four-cell stage secretes LH-β: that is a very interesting point for later studies. But to secrete HCG-β mRNA, that blastomere must surely be on its way to forming TE. It means by the four-cell stage one cell appears to be allocated or committed in a specific way. How does this fit into your current model on early differentiation? I think you believe germline develops much later.

J. Rossant. That is an interesting observation. Our model, or indeed any other current model, would not predict very early segregation of the TE lineage, but it could be that some blastomeres turn on markers of TE earlier than others.

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GLOSSARY

AVE: anterior visceral endoderm
COUP-TF: chicken ovalbumin upstream promoter transcription factor
E: embryonic day
EPI: epiblast
ES: embryonic stem
FGF: fibroblast growth factor
GATA: GATA-binding protein
GFP: green fluorescent protein
HNF: hepatocyte nuclear factor
ICM: inner cell mass
LIF: leukaemia inhibitory factor
PE: primitive endoderm
TE: trophectoderm
VE: visceral endoderm