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Early Lineage Segregation between Epiblast and Primitive Endoderm in Mouse Blastocysts through the Grb2-MAPK Pathway

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Summary

It has been thought that early inner cell mass (ICM) is a homogeneous population and that cell position in the ICM leads to the formation of two lineages, epiblast (EPI) and primitive endoderm (PE), by E4.5. Here, however, we show that the ICM at E3.5 is already heterogeneous. The EPI- and PE-specific transcription factors, Nanog and Gata6, were expressed in the ICM in a random "salt and pepper" pattern, as early as E3.5, in a mutually exclusive manner. Lineage tracing showed predominant lineage restriction of single ICM cells at E3.5 to either lineage. In embryos lacking *Grb2* where no PE forms, *Gata6* expression was lost and all ICM cells were Nanog positive. We propose a model in which the ICM develops as a mosaic of EPI and PE progenitors at E3.5, dependent on Grb2-Ras-MAP kinase signaling, followed by later segregation of the progenitors into the appropriate cell layers.

Introduction

By the time of implantation, three distinct cell types are apparent in the mouse blastocyst which give rise to separate cell lineages in later development. Epiblast (EPI) gives rise to the entire fetus as well as extraembryonic mesoderm. Trophectoderm (TE) gives rise to all the trophoblast cell types that make up the majority of the fetal part of the placenta, and the primitive endoderm (PE) forms the extraembryonic endoderm layers of the visceral and parietal yolk sacs. The TE and PE extraembryonic lineages are required to support the growth of the mammalian fetus in the uterine environment and are sources of signals to the EPI to initiate axial patterning (Ang and Constam, 2004; Beddington and Robertson, 1998; Lu et al., 2001; Rossant and Tam, 2004).

By the late blastocyst, experimental chimera studies have indicated that the three lineages are restricted to their future fate (Gardner, 1985; Rossant et al., 2003) but the mechanisms leading to the initial segregation of the three lineages are still not well understood. At the eight-cell stage, all blastomeres retain the potential to form all cell lineages (Johnson and McConnell, 2004). Following compaction, the blastomeres become polarized and subsequent cell divisions generate outside polar cells and inside apolar cells, dependent upon the symmetric or asymmetric cell divisions of the polarized cells (Johnson et al., 1986). Polar cells remain outside and will form the TE, while apolar cells become enclosed by the polarized outer epithelium and form the inner cell mass (ICM). This segregation is accompanied by segregated expression of key transcription factors (TFs) required to establish cell fate, such as Oct4 (Nichols et al., 1998) and Cdx2 (Chawengsaksophak et al., 1997; Strumpf et al., 2005) for ICM and TE, respectively. Correct segregation of the two lineages is also dependent upon cell adhesion, involving E-cadherin (De Vries et al., 2004; Riethmacher et al., 1995), and the epithelial polarity pathway, involving genes such as Par3 and aPKC (Plusa et al., 2005).

Much less is known about the segregation of EPI and PE within the ICM. By E4.5, the PE layer is clearly morphologically distinct. When E4.5 EPI or PE cells were dissociated and injected into other blastocysts, their chimeric contributions were restricted to their own lineages (Gardner, 1982, 1984; Gardner and Rossant, 1979), indicating lineage restriction by this stage. Isolated E3.5 ICMs form PE over their exterior surface (Hogan and Tilly, 1978; Rossant, 1975), suggesting that EPI/PE segregation might also depend on some positional effects, with cells located in the inside of ICM becoming EPI and the cells lining the blastocoel becoming PE.

Recent genetic evidence has implicated Nanog and Gata family transcriptional factors in specifying EPI versus PE fate. Nanog, a homeodomain protein, was identified as a gene highly expressed in ES cells (Mitsui et al., 2003), which could substitute for LIF to promote self-renewal of ES cells (Chambers et al., 2003). Disruption of this gene leads to failure to maintain pluripotency in ES cells in vitro and peri-implantation lethality with failure of EPI formation in vivo (Mitsui et al., 2003). In both ES cells and ICMs from null mutants, loss of *Nanog* results in all cells taking up a parietal endoderm-like fate (Mitsui et al., 2003). This suggests that levels of *Nanog* expression are important for the EPI/PE fate decision. In a complementary manner, overexpression of *Gata6* or a close family member *Gata4* is sufficient to transform ES cells to PE lineages (Fujikura et al., 2002) and ES cells lacking *Gata6* or *Gata4* cannot form a functional visceral endoderm layer in in vitro embryoid body cultures (Koutsourakis et al., 1999; Morrisey et al., 1998; Soudais et al., 1995). In vivo, *Gata4* and *Gata6* mutants initiate PE lineage formation, but fail to form functional visceral endoderm in postimplantation embryos (Koutsourakis et al., 1999; Morrisey et al., 1998; Soudais et al., 1995). Gata4 and Gata6 may be redundantly required for PE formation in vivo.

In this study, we have carefully examined the expression of Nanog and Gata6 in early development and have

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shown that expression of the two factors is heterogeneous and complementary in the ICM as early as E3.5, although not in any obvious position-dependent manner. Cell lineage and chimera studies also support the concept that the E3.5 ICM is a mosaic of EPI and PE progenitors. We also show that signaling through Grb2 is required for the segregation of these progenitors at E3.5, because *Grb2* mutants, which fail to generate PE (Cheng et al., 1998), show conversion of all cells of the E3.5 ICM to Nanog-expressing EPI. These results suggest a reevaluation of the timing and mechanism of EPI/PE segregation in the mouse ICM is warranted.

Results

"Salt and Pepper" Expression of *Nanog* and *Gata6* in the ICM of E3.5 Blastocysts

We examined protein and mRNA expression of the EPI and PE lineage-related transcription factors Gata6, Nanog, and Oct4 (Chambers et al., 2003; Koutsourakis et al., 1999; Mitsui et al., 2003; Nichols et al., 1998) at E3.5–E4.5 using laser confocal microscopy. Expression of *Gata6* and *Oct4* are clearly segregated to the PE and EPI, respectively, by E4.5 (Figure 1A). Using a heterozygous *lacZ* knock-in *Gata6* mutant mouse, Koutsourakis et al. reported *Gata6* expression at the E3.5 blastocyst stage in a portion of ICM cells as well as in some trophoblast (Koutsourakis et al., 1999). With fluorescent in situ hybridization and immunohistochemistry, we found *Gata6* and *Nanog* mRNA and protein expression at high levels in subsets of cells within the E3.5 ICM (Figures 1B–1E). *Oct4* was expressed at equivalent levels in all ICM cells at this stage (Figure 1G). No other PE markers such as *Pem*, *Hex*, or *Dab2* could be detected at this stage (data not shown). Double in situ hybridization revealed that domains of *Gata6* and *Nanog* expression were mainly exclusive (Figure 1F), suggesting that the ICM is a mosaic of Nanog+ and GATA6+ cells. There was no apparent spatial restriction of Nanog+ or GATA6+ cells to particular regions of the ICM; rather, the ICM showed a random "salt and pepper" pattern of expression of the two genes. This mosaic expression of two functionally important lineage-specific transcription factors suggested the possibility that there might be lineage segregation of EPI and PE progenitors as early as the E3.5 ICM.

Labeled Single ICM Cells in Early Blastocysts Give Rise to Either EPI or PE at E5.5

To address the question of when EPI and PE progenitors are set aside in development, we reexamined the fate of single ICM cells in E3.5 blastocysts prior to morphological evidence of PE formation. Initially, we microinjected GFP mRNA into single ICM cells (Weber et al., 1999) and followed the development of labeled clones postimplantation. Although this technique has been used successfully to study PE clonal distributions (Weber et al., 1999), we found that the high proliferation rate of EPI cells led to dilution of the GFP mRNA and difficulties in unambiguously identifying contributions of labeled cells to EPI (data not shown). To avoid the problem of dilution, we injected mRNA for the Cre recombinase along with a small amount of tracer GFP mRNA into single ICM cells of embryos derived from ICR females crossed with Z/EG reporter males (Novak et al., 2000). In the Z/EG line, GFP expression from a strong ubiquitous promoter is activated only after Cre excision is induced. Hence the progeny of the Cre mRNA-injected cell is labeled by GFP permanently. Three hundred fifty-eight embryos were injected and 186 embryos, selected because they had single GFP-positive cells in ICM at 1 hr after injection, were transferred into recipient mothers. At E5.5, 146 embryos were recovered and 45 embryos out of 146 were GFP positive (Table 1). Examination by confocal microscopy revealed that progeny of labeled cells contributed to either EPI (Figure 2A) or PE (Figure 2B) lineage but never both: 18 embryos had GFP cells only in EPI and 24 embryos only in PE (n = 45; Figure 2C).

This result suggested that in the E3.5 blastocyst, the fate of single ICM cells is to become either EPI or PE. This lineage tracing experiment does not address, however, whether single cells are restricted in their potential when placed in different environments. To test this, we isolated single GFP-expressing cells dissociated from immunosurgically isolated early E3.5 ICMs (the mean number of cells in ICM is 13.8 cells) and injected them into ICR blastocysts. Injected cells were from embryos heterozygous for a ubiquitously expressed GFP transgene (Hadjantonakis et al., 1998). Of the 30 embryos containing GFP-positive cells recovered at E5.5 (Table 2), 14 embryos showed GFP-positive cells only in the EPI and 15 embryos showed GFP-positive cells only in the PE lineage (Figure 2D). Only 1 embryo had GFP contributions in both lineages. This was consistent with the previous cell-tracing data.

This suggested restriction of cell potential in individual E3.5 ICM cells. However, it is also possible that injected cells did not have enough time to divide and contribute to more than one lineage before lineage restriction at E4.5. 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 GFP-positive cells were observed in the embryos. Thirteen out of 167 GFP-positive blastocysts had GFP cells in TE and were not used. One hundred forty-seven embryos were transferred and 75 embryos were recovered at E5.5. Sixty were GFP positive (Table 3). Nineteen embryos had GFP cells restricted to EPI and 36 embryos showed restriction to the PE lineage. Only 5 embryos had GFP cells in both lineages (Figure 2E).

Thus, both lineage tracing and chimera analysis suggest that EPI/PE cell fate restriction is largely initiated by the early blastocyst stage—1 full day before these tissues are recognizable morphologically.

Lineage Segregation Is Not Based on Either Blastomere History or Position in the E3.5 ICM

It is important to ask whether the apparent lineage bias seen at E3.5 is related to the position of cells in the ICM, as cells lining the surface of the ICM have been thought to become PE. However, attempts to reproducibly inject surface versus enclosed cells of the E3.5 ICM were not successful. Moreover, later examination of aggregated blastocysts in culture showed that cells did not necessarily maintain their position. Time-lapse movies revealed considerable movement and displacement of

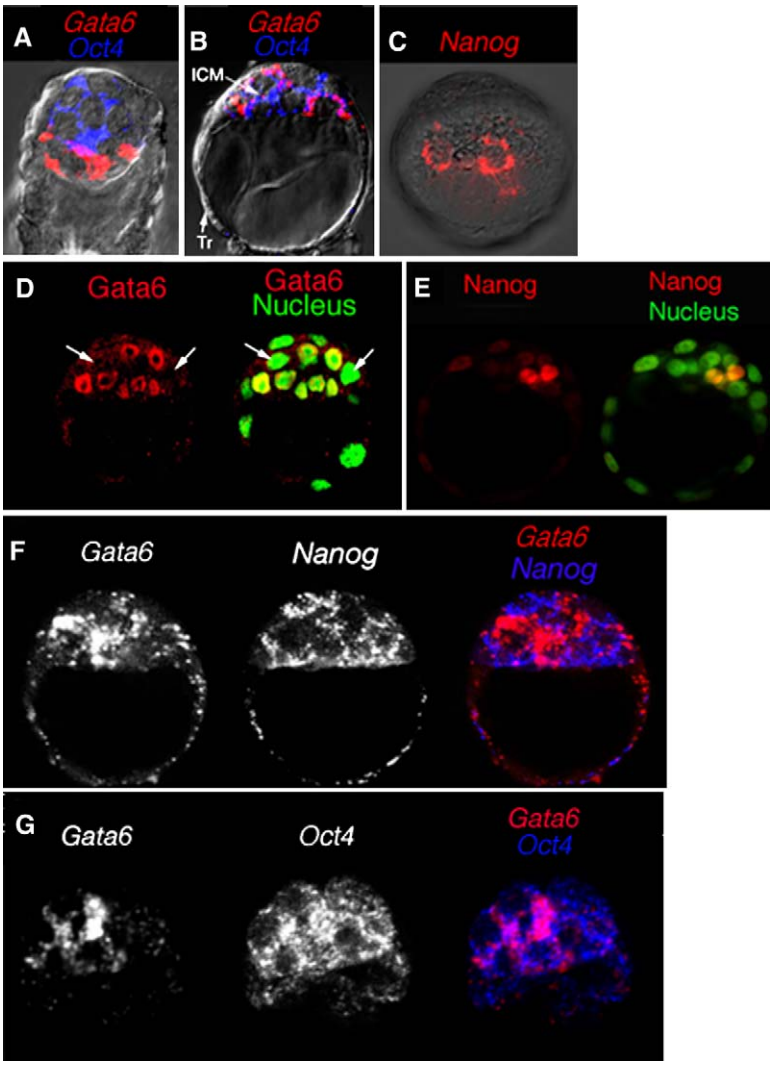

Figure 1. "Salt and Pepper" Expression of *Nanog* and *Gata6* in the ICM of E3.5 Blastocysts

Fluorescent whole-mount in situ hybridization (A–C, F, and G) and fluorescent whole-mount immunostaining (D and E).

(A and B) *Gata6* (red) and *Oct4* (blue) expression in the E4.5 implanting embryo (A) and the E3.5 blastocyst (B).

(C) *Nanog* expression in the E3.5 blastocyst.

(D) Gata6 immunostaining (red) in the E3.5 blastocyst. Gata6 was detected in some ICM cells. Nuclei (green) were stained by YOYO1 in the right panel. White arrows indicate Gata6-negative ICM cells.

(E) Nanog immunostaining (red) in the E3.5 blastocyst. Nanog was detected in some ICM cells. Neither Nanog nor Gata6 staining was localized to a specific area within ICM.

(F) Double in situ hybridization with *Gata6* (red) and *Nanog* (blue) in the E3.5 blastocyst. Most of the cells were expressing either *Nanog* or *Gata6*. A few cells were weakly co-expressing both markers.

(G) Double in situ hybridization with *Gata6* (red) and *Oct4* (blue) in the E3.5 blastocyst.

cells over time (see Supplemental Data available with this article online). As another way to label the blastocoel-facing surface of the ICM, we labeled single blastomeres at the two-cell stage, then selected those in which the progeny of one of the two-cell blastomeres has contributed to the polar TE and inner cells of the ICM, while the other cell contributed progeny to the mural TE and the surface of the ICM (Gardner, 2001; Piotrowska et al., 2001). In this way, surface cells can be labeled and their potential assessed in aggregation experiments such as those described above.

One of the blastomeres of two-cell stage embryos expressing GFP ubiquitously was labeled with DiI noninvasively (Piotrowska et al., 2001). Blastocysts were recovered 2 days after transfer to the oviduct and those embryos that showed a clear boundary between progeny of the individual two-cell blastomeres at the embryonic-abembryonic border were selected for further use (Figure 3A). ICMs were isolated from these embryos and DiI-labeled and -nonlabeled cells were counted. In all embryos, both two-cell blastomeres contributed to the ICM and there was no significant difference in the contribution derived from the embryonic or abembryonic blastomere (abembryonic ICM cells/embryonic ICM cells = 0.91 ± 0.07; n = 12). Single isolated ICM cells from either the embryonic or abembryonic side were separately aggregated with eight-cell embryos (Table 4). No statistically significant bias was observed in the

Table 1. Single ICM Cell Labeling with Cre mRNA Injection into Z/EG Blastocysts

Number of Injected Embryos	Number of Blastocysts with Single GFP-Positive Cells	Number of Transferred Embryos	Number of Embryos Recovered at E5.5	Number of Embryos Containing GFP-Positive Progeny at E5.5
358	186	186	146	45

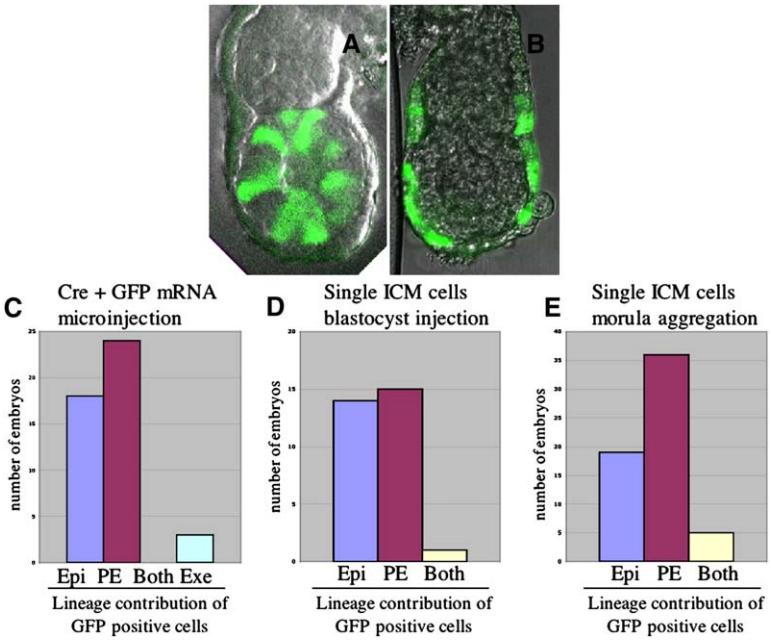

Figure 2. Single ICM Cells in Early Blastocysts Give Rise to Either EPI or PE Lineage at E5.5

(A and B) Representative embryos with GFP-positive cells in EPI (A) or visceral endoderm (B).

(C–E) Number of embryos with GFP-positive cells in EPI or PE lineage in Cre + GFP mRNA microinjection (C), single ICM cells blastocyst injection (D), and single ICM morula aggregation (E).

contribution of the embryonic- versus abembryonic-derived cells to EPI or PE and, as before, the majority of embryos showed restricted contribution from the single cell to one lineage or the other (Figure 3B). Thus, there is no evidence that lineage segregation is based on either blastomere history or position of cells in the E3.5 ICM.

Receptor Tyrosine Kinase Signaling Is Required for Segregation of Lineages in the ICM

Grb2 is an adaptor molecule that plays an important role in signal transduction downstream of several different receptor tyrosine kinases (Pawson and Scott, 1997). Mutation of *Grb2* leads to loss of the ability to form the PE lineages in embryos in vivo and in embryoid bodies in vitro (Cheng et al., 1998). We reexamined the early phenotype of $Grb2^{-/-}$ embryos in detail using markers of EPI and PE lineages. No morphologically distinct PE was apparent in mutants at E4.5 but a homogeneous, compact ICM was found (Figures 4A–4C). Moreover, none of the PE markers such as *Pem* (n = 7), *Hex* (n = 2), and *Dab2* (n = 3) were expressed at E4.5 in $Grb2^{-/-}$ embryos (Figures 4A–4C) but all the remaining ICM cells expressed *Oct4* (Figure 4A; n = 5). As *Gata6* is expressed at E3.5 before the PE becomes morphologically apparent (Figures 1 and 4D), we examined whether *Gata6* expression was initiated at all in $Grb2^{-/-}$ mutants. At E3.5, $Grb2^{-/-}$ blastocysts were morphologically completely normal but *Gata6* expression was absent by in situ hybridization (n = 5) and immunohistology (n = 5) (Figure 4D and data not shown). In contrast, *Nanog* mRNA (Figure 4E; n = 5) and Nanog protein (Figure 5A) were expressed at uniformly high levels in all ICM cells of E3.5 $Grb2^{-/-}$ embryos (Figures 4E and 5A), rather than patchily as in wild-type embryos.

Cell counts were performed on stacks of confocal z-series images to determine whether there was a selective loss of Nanog-negative cells in E3.5 *Grb2* mutants by counting the number of ICM cells (Figure 5B). Because it was difficult to collect absolutely identical stage-matched litters, embryos were compared within litters. Litter 1 contained mostly expanding blastocysts, while litter 2 contained mostly fully expanded blastocysts. In the first litter, ICM cell numbers were 16.3 ± 2.8 in mutants (n = 7) and 16.9 ± 1.9 in wild-type embryos (n = 6). In the second litter, ICM numbers were 28.5 in mutants (n = 2) and 24.6 ± 1.5 in wild-type (n = 5). In both cases, there was no significant statistical difference in the number of ICM cells between mutant and wild-type.

Next, we examined the percentage of cells expressing high levels of Nanog in ICMs (Figure 5C). In litter 1, the percentage of Nanog-positive cells was 44.7 ± 7.5 in wild-type and 74.3 ± 7.0 in mutants. In litter 2, the slightly later stage embryos, the percentage of positive cells was 49.7 ± 3.7 in wild-type (n = 5) and 100 in mutants (n = 2).

These results suggest that all E3.5 ICM cells take up a Nanog+ EPI fate in $Grb2^{-/-}$ mutants and indicate that establishing lineage segregation requires signaling through Grb2.

Table 2. Single Early ICM Cell Injection into Blastocysts

Number of Injected Embryos	Number of Transferred Embryos	Number of Embryos Recovered at E5.5	Number of Embryos Containing GFP-Positive Progeny at E5.5
210	210	123	30

Table 3. Single Early ICM Cells Aggregated with Morula Embryos

Number of Embryos Aggregated	Number of GFP-Positive Blastocysts	Number of Transferred Embryos	Number of Embryos Recovered at E5.5	Number of Embryos Containing GFP-Positive Progeny at E5.5
366	167: (154 in ICM, 13 inTE)	147	75	60

Discussion

The idea of position driving cell fate segregation in the ICM of the mouse blastocyst has long held sway, as the primitive endoderm is recognized as a monolayer on the surface of the ICM directly facing the blastocoel. In addition, in isolated ICMs (Hogan and Tilly, 1978; Rossant, 1975) and in ES cell-derived embryoid bodies, an in vitro model of PE formation (Murray and Edgar, 2004), the PE lineage forms on the outer surface of the embryoid bodies (EBs). However, there are few experimental data to suggest how position might drive cell fate determination in the ICM. In this study, we carefully analyzed the onset of expression of the PE- and EPI-specific TFs, Gata6 and Nanog, before these two lineages are morphologically distinct. Expression of each TF was detected in a subset of cells in E3.5 blastocysts. No consistent spatial pattern was observed—the two genes were expressed in a complementary "salt and pepper" manner. In addition, lineage tracing and chimera data showed that the majority of single early E3.5 ICM cells were already restricted to be either EPI or PE. Although some early ICM cells were capable of generating both EPI and PE when placed back in the eight-cell stage embryo, the predominant pattern was of restricted fate, suggesting that there is heterogeneity in the E3.5 ICM. These results suggest that there is a need to consider an alternative model for segregation of the two ICM lineages, perhaps not by position in the ICM.

The best way to test whether the position in the E3.5 ICM is important for PE formation would be to directly label the surface versus enclosed cells in the ICM. We attempted that, but it was hard to judge the precise location of injected cells in living embryos. Instead, we used embryos in which one of the two-cell blastomeres had been labeled by lipophilic dye and showed a clear two-cell progeny boundary across the embryonic-abembryonic axis of the blastocyst. In these embryos, a surface layer of ICM derived from one of the two-cell blastomeres could be distinguished from the enclosed cells of ICM derived from the other blastomere. This allowed us to analyze the developmental potential of surface or enclosed ICM cells. There was no evidence from these experiments that position of cells in ICM related to their later fate.

Furthermore, the results of the single ICM cell blastocyst injection chimeras also supported the lack of influence of cell position. If position in the ICM were important, one would expect a bias toward PE contribution, because the injected cells were always positioned on the surface of ICM after injection. However, no such bias was observed: the results showed almost equivalent contributions to either EPI or PE. Therefore, both gene expression and experimental analysis provide evidence for a new model of generation of the E3.5 ICM as a mosaic of EPI and PE progenitors, with later

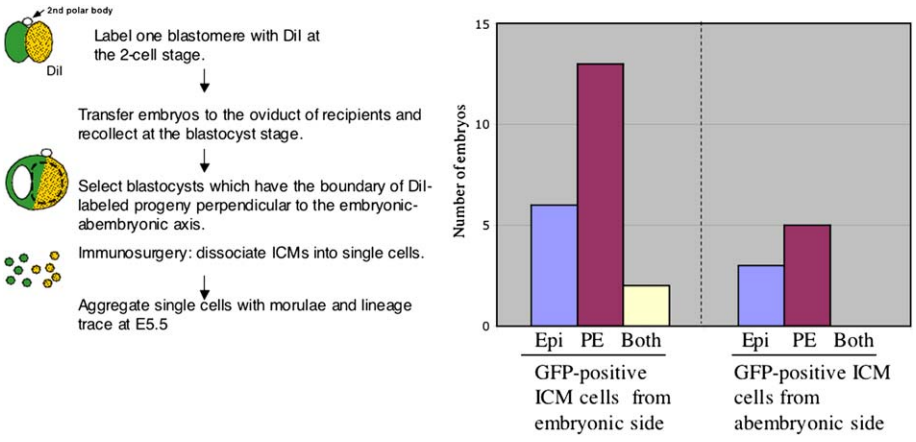

Figure 3. Potential between ICM Cells from the Embryonic Side and Abembryonic Side to Contribute to EPI or PE

(A) Method used for labeling surface ICM cells.

(B) Comparison of the number of embryos with GFP-positive cells contributing to EPI or PE between ICM cells derived from the embryonic or the abembryonic side. There was no significant statistical difference between these cells ($P < 0.05$, Chi-squared test) in their ability to contribute to PE or EPI.

Table 4. Single ICM Cells Aggregated with Morula Embryos

	Number of Embryos Aggregated	Number of GFP-Positive Blastocysts Transferred	Number of Embryos Recovered at E5.5	Number of Embryos Containing GFP-Positive Progeny at E5.5
Abembryonic	76	35	11	8
Embryonic	83	41	22	21

segregation of these EPI and PE progenitors into appropriate positions. Proof of a direct relationship between TF expression profile and lineage restriction, however, will await the development of new tools, such as GFP fusion knock-in alleles of *Nanog* and *Gata6*, that will allow us to trace directly the fate and potential of cells expressing the specific TFs in the future.

What mechanism could regulate establishment of the two lineage progenitors in early blastocysts? Two groups have suggested the possibility that the progeny from the two different blastomeres at the two-cell stage may have different fates or potencies, as their progeny tend to colonize either the embryonic or abembryonic side of the blastocyst (Gardner, 2001; Piotrowska et al., 2001). However, our results showed no difference in potential to form EPI and PE between the progeny of the different two-cell blastomeres (Figure 3B). Another possibility is that the cleavage pattern during the 8- to 32-cell stage may create this difference. Inner cells, future ICM cells, are produced from two successive rounds of asymmetric division, from the 8- to 16- and 16- to 32-cell stage (Johnson and McConnell, 2004). An attractive model would suggest that EPI and PE derive separately from these two successive rounds of asymmetric division. This idea was originally suggested by Chisholm and Houliston (1987). They analyzed the localization of the extraembryonic lineage-specific cytokeratin (ENDO-A) in early embryos using the TROMA-1 antibody. They showed that those cells in the ICM of early blastocysts, which did possess filaments, were almost exclusively the apolar progeny of polar 16-cell blastomeres rather than progeny of apolar blastomeres generated at the 8- to 16-cell stage. However, this model has never been rigorously tested.

Our evidence strongly suggests that the lineage segregation requires receptor tyrosine kinase (RTK)-Ras-MAP kinase signaling. In *Grb2* mutants, lack of activation of this signaling pathway caused all early ICM cells to become Nanog positive and no PE was formed. Interestingly, homozygous mutants of *Fgf4* and *Fgfr2* cannot form PE and have very similar phenotypes to *Grb2* mutants (Arman et al., 1998; Feldman et al., 1995; Wilder et al., 1997). Although lineage markers have not been examined in those mutants, the similar phenotypes suggest that FGF signaling might be the major RTK pathway involved upstream of Grb2. Furthermore, EBs from ES cells overexpressing a dominant-negative Fgf receptor construct cannot differentiate into PE but this phenotype can be rescued by overexpression of Gata factors (Li et al., 2004). This result suggests that activation of Gata factor expression is the key PE-promoting element downstream of RTK-Grb2 signaling. Because Nanog protein is initially found throughout the early embryo (Strumpf et al., 2005; data not shown) and then becomes restricted to a subset of ICM cells as *Gata6* expression begins, Fgf signaling through Grb2 may promote PE formation by activating *Gata6* expression, which leads secondarily to loss of *Nanog* expression. *Fgf4*, *Fgfr2*, and *Grb2* are expressed throughout the ICM (Arman et al., 1998; Niswander and Martin, 1992; Rappolee et al., 1994; data not shown), so restricted localization does not explain how *Gata6* expression becomes heterogeneous in the ICM. Individual ICM cells may acquire differential sensitivity to RTK-Ras-MAPK activation

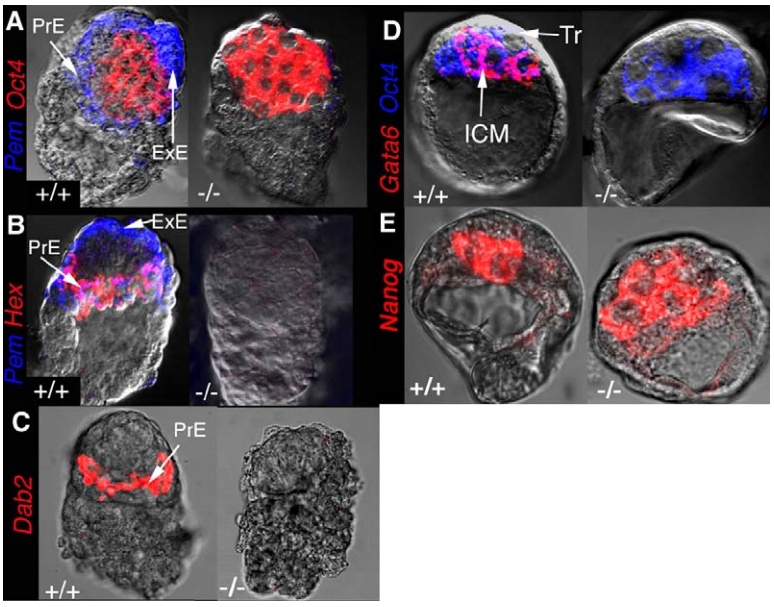

Figure 4. Absence of PE in *Grb2*$^{-/-}$ Embryos

Embryos on the left are *Grb2*$^{+/+}$ or *Grb2*$^{+/-}$ and on the right are *Grb2*$^{-/-}$. There was no morphologically apparent PE layer in mutant E4.75 embryos (A–C).

(A) Expression of *Oct4* (red) and *Pem* (blue).

(B) Expression of *Hex* (red) and *Pem* (blue).

(C) Expression of *Dab2* (red). Neither PE marker was expressed in *Grb2*$^{-/-}$ embryos at E4.75.

(D) Expression of *Gata6* (red) and *Oct4* (blue) in the E3.5 blastocyst.

(E) Expression of *Nanog* (red) at E3.5. *Gata6* expression was not detected but *Nanog* expression was detected in all ICM cells in *Grb2*$^{-/-}$ embryos. Expression of *Oct4* was not affected in *Grb2*$^{-/-}$ embryos (D).

ExE, extraembryonic ectoderm; ICM, inner cell mass; PrE, primitive endoderm; Tr, trophoblast.

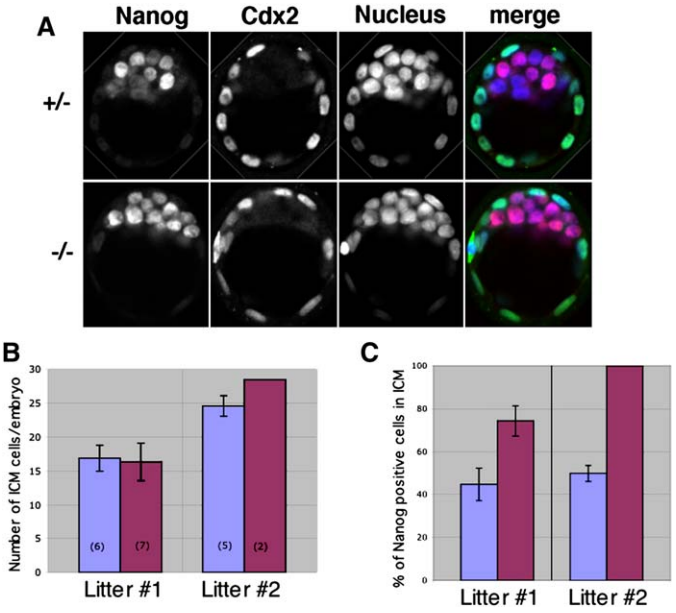

Figure 5. All ICM Cells Express Nanog in *Grb2*$^{-/-}$ Embryos

(A) Immunostaining of Nanog and Cdx2 in *Grb2*$^{+/-}$ (upper panels) and *Grb2*$^{-/-}$ (lower panels). All ICM cells in *Grb2*$^{-/-}$ expressed Nanog (red in merged image).

(B) Number of ICM cells in wild-type and mutant blastocysts. Nuclei (YOYO3-blue) were counted as ICM if they did not express Cdx2 (green). There was no significant difference in total number of ICM cells between *Grb2*$^{+/+}$ and *Grb2*$^{+/-}$ (blue bar) and *Grb2*$^{-/-}$ embryos (purple bar). Numbers of embryos in individual samples are shown inside of bars.

(C) The percentage of strong Nanog-positive cells in the ICM. In early blastocysts (litter 1), there are strong and weak Nanog-positive cells in the ICM. However, in expanding blastocysts (litter 2), we did not detect weak positive cells anymore.

(Figure 6), perhaps based on a combination of their lineage derivation during cleavage and their interactions with each other and with the TE.

How could EPI and PE progenitors be sorted out from a "salt and pepper" pattern to a separate EPI and PE layer? XEN cells, newly identified PE-derived stem cell lines (Kunath et al., 2005), form derivatives of the PE lineage only when they are injected into blastocysts. When XEN cells are aggregated with ES cells in suspension culture, they form EB-like structures in which XEN cells and ES cells sort out such that XEN cells always surround the outside of ES cells (M. Ryczko and J.R., unpublished data), the same arrangement as occurs in the developing ICM. Thus, EPI and PE cells must have different adhesive characteristics that allow them to sort out from each other. In ES cells, *Gata* overexpression leads to a strong upregulation of LamininB1, which changes the adhesion properties of *Gata*-expressing cells (Fujikura et al., 2002). Interestingly, there are several targeted mouse mutants in genes involved in cell adhesion and cell migration that fail to generate a cohesive PE layer. These include Dab2 (Morris et al., 2002; Yang et al., 2002), a PTB domain-containing adaptor protein, Maspin (Gao et al., 2004), a noninhibitory Serpin family protein and a putative tumor suppressor, LamC1 (Smyth et al., 1999), an ECM protein, and integrin β1 (Stephens et al., 1995), a cell adhesion molecule. In all cases, scattered cells labeled with PE markers can be detected in the middle of ICM of these mutants. These results suggest that PE lineage progenitors were formed in these mutants but that there was a failure of the mechanism necessary to ensure that the PE cells are segregated to the surface of the ICM. Interestingly, *Dab2* has been shown to be a direct target of Gata6 (Morrisey et al., 2000). Dab2 modulates the cellular adhesiveness during megakaryocytic differentiation of the human chronic myeloid leukemic cell line K562 (Tseng et al., 2003) and transiently interacts with integrin β1 to regulate its activation during transforming growth factor β-induced epithelial to mesenchymal transition in normal murine mammary gland cells (Prunier and Howe, 2005). These suggest that activation of pathways leading to appropriate cell sorting is one of the key roles for early expression of *Gata6* in PE progenitors in the E3.5 ICM (Figure 6).

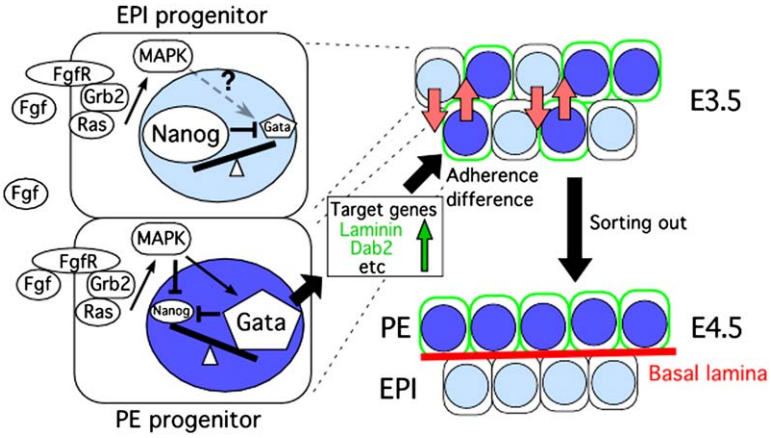

Figure 6. Model of EPI/PE Formation

The ICM of E3.5 blastocysts is a mosaic of EPI progenitors and PE progenitors. Grb2-dependent signals (likely Fgf signal) induce Gata and repress Nanog in PE progenitors, although the mechanism of selective activation of this pathway in PE progenitors is unknown. The absence of Grb2-dependent signals allows Nanog expression in EPI progenitors. In PE progenitors, Gata induces target genes such as Laminin and Dab2, which modulate cellular adhesive function to initiate the sorting of the two lineages. By E4.5, the basal lamina (consisting mainly of Laminin) forms and separates the two lineages.

It is well known that ES cells cannot contribute to the PE lineage in chimeras, even though they can produce PE-like cells in EBs (Beddington and Robertson, 1998). ES cells thus behave like EPI progenitors in chimeras but behave as bipotential progenitors in vitro. Unlike the embryo itself, where PE formation is an early event and limited in its duration, PE formation from ES cells takes several days and requires changes in either growth factor conditions (Murray and Edgar, 2004) or direct alteration of levels of expression of *Nanog* (Mitsui et al., 2003) or *Gata6* (Fujikura et al., 2002). It seems likely that the same downstream TF pathways used in the early embryo are reactivated in ES cells to drive PE formation but the upstream mechanisms activating expression are different. When placed in the blastocyst environment, ES cells do not respond by making PE but behave as restricted EPI cells, consistent with this proposal. Future experiments combining live cell imaging with cell fate analysis and genetic interventions will help determine what mechanisms are used to drive early lineage segregation in the embryo and will lead to better understanding of lineage development and cell behavior.

Experimental Procedures

Microinjection of mRNA into Single Cells of Preimplantation Embryos

Capped mRNAs were synthesized according to the manufacturer's protocol (MEGAscript; Ambion), using pCS2 EGFP and pCS2 CreNLS as templates. Both plasmids were linearized by NotI and transcribed mRNA using SP6 RNA polymerase.

For microinjection, we used a Leica microscope (Leica Microsystems) and manipulators with FemtoJet (Eppendorf) for pressure injection and a Cyto721 intracellular amplifier (WPI) was used to create the negative capacitance. The injection was performed in an open glass chamber in M2 solution. ICM cells were injected through either the polar or mural TE. Injected embryos were cultured for 1 hr in KSOM solution in a 5% CO_2 incubator and GFP expression was checked under a fluorescent UV microscope. Only embryos that had single GFP cells were transferred to pseudopregnant recipient mothers.

Immunosurgery and Dissociation of ICM

Early blastocysts were flushed from the uteri of the ICR females crossed with B5 males expressing GFP ubiquitously (Hadjantonakis et al., 1998). Immunosurgery was performed according to Nagy et al. (2003). Briefly, the zona was removed with acid-tyrode solution, embryos were treated with nonabsorbed rabbit anti-mouse lymphocyte cells antibody (Cedarlane), and diluted 1 in 6 to 1 in 10 in KSOM, for 10 min in a CO_2 incubator at 37ºC. After five washes with M2, they were treated with complement (Cedarlane) diluted 1 in 6 to 1 in 10 in KSOM. Embryos were observed periodically under the dissecting microscope to check lysis of outer cells. Usually, we observed clear membrane blebbing of TE within 10–15 min. ICMs were then washed in M2 and treated with 1% trypsin in PBS-EDTA to dissociate them to single cells. Collected single cells were used for morula aggregation or blastocyst injection.

Lipophilic Dye Labeling

DiI or DiA (Molecular Probes) was dissolved in corn oil. A small droplet of dye solution was deposited on one of the two-cell blastomeres using the microinjection system. Embryos were transferred to the oviducts of recipients and flushed from uteri 2 days later at the blastocyst stage. We checked the distribution of labeled progeny in blastocysts using a UV fluorescent microscope and selected embryos exhibiting a boundary of two-cell progenies at the embryonic-abembryonic border.

Whole-Mount Immunostaining

Embryos collected from the oviduct or the uterus by flushing were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4ºC. Embryos were permeabilized with 0.5% Triton X100 in PBS for 15 min. Embryos were blocked in 10% fetal bovine serum in PBS-0.1% Triton for 1 hr. We used mouse anti-Cdx2 monoclonal Ab, 1/100 dilution (BioGenex, Cdx288), rabbit anti-Nanog polyclonal Ab, 1/200 dilution (a gift from Dr. S. Yamanaka, Japan and Cosmo Bio, Japan), and rabbit anti-Gata6 Abs, 1/200 dilution (a gift from Dr. M. Nemer, Montreal). Fluorophore-conjugated secondary Abs were purchased from Jackson Immuno Research and Molecular Probes.

To counterstain nuclei, embryos were treated with 10 μM YOYO1 or YOYO3 (Molecular Probes) in PBS with 200 μg/ml RNaseA.

Fluorescent Whole-Mount In Situ Hybridization

A detailed protocol can be found on the Rossant lab home page (http://www.sickkids.ca/rossant/protocols/doubleFluor.asp). Briefly, after flushing or dissection, embryos were fixed with 4% PFA in PBS overnight at 4ºC and stored in methanol at −20ºC. Embryos were rehydrated with 0.1% Tween 20 in PBS (PBT) and permeabilized with RIPA solution (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 1 mM EDTA, 50 mM Tris [pH 8.0]) for 10–20 min at room temperature (RT). Embryos were postfixed with 4% PFA + 0.2% glutaraldehyde for 10 min at RT, and prehybridized in hybridization buffer (50% formamide, 5× SSC, 1% SDS, 100 mg/ml yeast tRNA, 50 mg/ml heparin) for 1 hr at 65ºC. Hybridization with probes was done overnight at 65ºC. Embryos were washed twice for 30 min with 50% formamide, 5× SSC, 1% SDS, once for 20 min with 50% formamide, 2× SSC, 1% SDS for 20 min at 65ºC, and then transferred to PBT. Embryos were blocked with manufacturer's blocking solution for 1–2 hr at RT. Embryos were treated with anti-DIG or -FITC antibody conjugated to peroxidase (1/200; Roche) overnight at 4ºC. Fluorescent staining with TSA-Cy3, -Cy5 kits was carried out according to the manufacturer's instructions (Perkin Elmer). Embryos were treated with tyramide-Cy3 or -Cy5 (1/50 in amplification diluent) for 30–60 min in the dark at RT. For the second color reaction, the first antibody was inactivated in 0.1 M glycine-HCl (pH 2.2), 0.1% Tween 20 for 10 min and the procedures were repeated from the blocking step. To amplify the staining, a TSA-biotin amplification kit (Perkin Elmer) was used.

Confocal Microscopy

Zeiss LSM 510 and Leica TCS2 confocal microscopes fitted with Zeiss Fluar 20× (NA = 0.75) or Leica HC PL APO 20× (NA = 0.70) were used for capturing images. The pinhole was set to 1–1.2 Airy unit and a series of optical sections was taken for all samples. Embryos were mounted in wells of Secure Seal (Molecular Probes) between two cover glasses for observation.

Genotyping of Blastocysts

Genotyping of blastocysts was performed after confocal microscopy as follows. Embryos were lysed in 10 μl of DNA solution with proteinase K for 15 min at 65ºC, then treated at 100ºC for 5 min to inactivate proteinase K. Three microliters of DNA solution was used in a 20 μl PCR reaction. Primer pairs used for Grb2 genotyping were 5′-TT GGGTCCAGGTGAACACGAGGA-3′ with 5′-CCTTCTATCGCCTTCTT GACGAG-3′ for the mutant allele and 5′-TTGGGTCCAGGTGAACACG AGGA-3′ with 5′- CAGAGCCAGGTAAGAGCCCCAG- 3′ for the wild-type allele. An annealing temperature of 60ºC and Advantage Taq (Clontech) were used for all PCR reactions.

Supplemental Data

Supplemental Data including one figure and a time-lapse movie are available at http://www.developmentalcell.com/cgi/content/full/10/5/615/DC1/.

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Note Added in Proof

A paper was recently published online supporting the molecular heterogeneity in the E3.5 ICM (Kurimoto, K., Yabuta, Y., Ohinata, Y., Ono, Y., Uno, K.D., Yamada, R.G., Ueda, H.R., and Saitou, M. 2006. An improved single-cell cDNA amplification method for efficient high-density oligonucleotide microarray analysis. Nucl. Acids Res. *34*, e42).