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Developmental fate and lineage commitment of singled mouse blastomeres

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SUMMARY

The inside-outside model has been invoked to explain cell-fate specification of the pre-implantation mammalian embryo. Here, we investigate whether cell-cell interaction can influence the fate specification of embryonic blastomeres by sequentially separating the blastomeres in two-cell stage mouse embryos and continuing separation after each cell division throughout pre-implantation development. This procedure eliminates information provided by cell-cell interaction and cell positioning. Gene expression profiles, polarity protein localization and functional tests of these separated blastomeres reveal that cell interactions, through cell position, influence the fate of the blastomere. Blastomeres, in the absence of cell contact and inner-outer positional information, have a unique pattern of gene expression that is characteristic of neither inner cell mass nor trophectoderm, but overall they have a tendency towards a 'trophectoderm-like' gene expression pattern and preferentially contribute to the trophectoderm lineage.

KEY WORDS: Cell fate, Mouse embryo, Blastomere

INTRODUCTION

It has been suggested that the inside cells of the 16-cell stage mouse embryo adopt an inner cell mass (ICM) fate and those from the outside adopt a trophectoderm (TE) fate (Tarkowski and Wróblewska, 1967). By the 32-cell stage, there is a clear delineation between the inside cells and the outside epithelial monolayer, and these two components herald the formation of the first two cell lineages: TE and ICM (Cockburn and Rossant, 2010). Differential gene expression proceeds in parallel, though seemingly lagging behind the morphological changes by a cell division. Indeed, the clear positional differences present at the 16-cell stage are only reflected in differential gene expression at the 32-cell stage (Guo et al., 2010).

The discovery of blastomere polarization, and the ability of blastomeres to divide asymmetrically to produce both polar (outer cell) and non-polar (inner cells) daughter cells, led to the establishment of the cell polarity model of lineage specification (Johnson and Ziomek, 1981). Polarized blastomeres adopt a TE fate, whereas the non-polarized cells contribute to the ICM. In the two proposed models (inner-outer and polar/non-polar, which are most probably complementary) cell-cell contact seems to be the key factor in determining whether a cell adopts a TE or ICM fate.

In addition to the inner-outer and cell polarity models, another hypothesis has been proposed for lineage specification through pre-patterning in the zygote (Piotrowska et al., 2001; Piotrowska and Zernicka-Goetz, 2001). This model predicts that the two-cell stage embryo can be superimposed on the embryonic-abembryonic axis of the blastocyst so that the progeny of one blastomere gives rise to the polar TE, while the other contributes mainly to mural TE and

ICM (Zernicka-Goetz, 2004). However, the idea that lineage decisions are pre-determined in the egg or the early cleavage stage mammalian embryo is still controversial and has not been universally accepted (Gardner, 2001; Piotrowska et al., 2001; Hiragi and Solter, 2004).

E-cadherin mutant embryos have been used to study the impact of cell-cell interaction on embryo development and cell fate determination. The absence of E-cadherin results in disorganized epithelial polarity and an increase in the number of blastomeres expressing CDX2 (De Vries et al., 2004; Stephenson et al., 2010). However, in these studies, E-cadherin mutant embryos are kept in an intact zona pellucida. Therefore, the inner-outer cell environment is still partially present and other cadherin proteins are expressed, which may allow for cell-cell communication. In addition, E-cadherin is linked with several major developmental signaling pathways that are crucial for normal development (Stepniak et al., 2009; Kim et al., 2011); thus, elimination of E-cadherin could have additional effects beyond simply affecting cell-cell adhesion.

Here, we aimed to investigate how cell-cell interactions influence fate specification of the blastomeres by completely inhibiting cell-cell contact and, therefore, cell positioning by mechanically separating the blastomeres at the two-cell through to the 32-cell stage. This approach can also determine whether blastomeres of the two-cell stage embryo are pre-patterned, as analysis of individual blastomeres throughout the pre-implantation development should give predictable ratios of cells expressing ICM or TE lineage markers. We used a high-throughput single-cell analysis platform to study the impact of cell-cell interaction loss on mouse embryo development, by analyzing the gene expression patterns of individual blastomeres separated from embryos at the two-cell through to the 32-cell stage. Our results show no lineage pre-patterning in the separated blastomeres. Rather, we find the extensive cell-cell interaction of inner-outer positioning is required for ICM-lineage commitment. In the absence of this contact, blastomeres tend to develop a unique pattern of gene expression that leans toward that of the trophectoderm. Furthermore, embryos reconstructed from long-term separated blastomeres are incapable of gastrulation.

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MATERIALS AND METHODS

Nomenclature

Individual blastomeres after separation were named using a '1/original embryonic stage' system; i.e. 1/2 refers to a single separated blastomere of a two-cell stage embryo. Freshly separated (FS) blastomeres from intact embryos are referred to as 1/2FS, 1/4FS, 1/8 FS or 1/16FS. In the analysis of long-term separated (LS) blastomeres, two-cell stage blastomeres were mechanically separated and individually cultured. Mechanical separation was then repeated at every division until the desired stage (supplementary material Fig. S1). Blastomeres derived from the long-term separation procedure were named according to the stage of blastomere at the point of analysis, i.e. 1/2LS, 1/4LS, 1/8LS, 1/16LS and 1/32LS. Blastomeres separated at the eight-cell stage (each separate blastomere is a 1/8 blastomere) and then re-aggregated with their sister blastomeres right after separation, are referred to as 8/8FS. Blastomeres separated at the two-cell stage and then continuously separated at each cell division until the eight-cell stage, then left as single blastomere for ~6 hours before re-aggregation, are called 8/8LS. Similar criteria are used for blastomeres of 16-cell stage embryos. Two types of recipient embryos were used: zona-free intact eight-cell stage embryo (i8C) and separated eight-cell stage embryo (s8C).

Embryo collection and blastomere separation

Embryos were collected from superovulated B6D2F1 females as previously described (Lorthongpanich et al., 2008). Zonae pellucidae were removed by 0.5% pronase (Sigma, St Louis, MO, USA) at the two-cell stage and sister blastomeres were separated by gentle pulling using two open pulled-bore pipettes controlled by a micromanipulator (Narishige). The blastomeres were separated 2-3 hours after completion of cytokinesis. Pipette size varied according to the embryonic stage (supplementary material Fig. S1A,B). The isolated blastomeres were cultured individually in 72-well plates (Nunc) filled with 10 μ l/well KSOMCAA media (Chemicon), under mineral oil, in a 5%O₂, 5% CO₂, 90% N₂ atmosphere, at 37°C, for 6-8 hours before mRNA isolation. For 1/2 to 1/16 blastomeres, only full sets of sister blastomeres were used for analysis. Obtaining individual blastomeres from the 32-cell stage proved to be difficult because of the fragility of the membrane at this stage. Accordingly, the 1/32 blastomeres used in this experiment were not from full sets, but rather from a pool of 1/32 blastomeres from six different embryos. Embryos were randomly selected from each experimental replication, and then blastomeres were randomly chosen from these embryos. We selected several blastomeres/embryo rather than 1-2 blastomeres from all surviving embryos because we wanted to determine the developmental tendency of the blastomeres that originated from the same embryo. All mouse work was approved by the BRC IACUC (A*STAR, Biopolis).

Immunocytochemistry

Embryo or blastomere immunofluorescence was performed as previously described (Dietrich and Hiragi, 2007). Briefly, primary antibodies include these to OCT4 (1:250; Santa Cruz, Biotechnology, Santa Cruz, CA, USA), CDX2 (1:250; BioGenex), ZO1 (1:200; Invitrogen, CA, USA), aPKC (1:200; Santa Cruz, CA, USA), p-YAP (1:200; Cell Signaling Technology, MA, USA), YAP (1:200; Cell Signaling Technology, MA, USA), FLOPED, MATER and TLE6 (gifts from Dr Jurrien Dean, National Institutes of Health, Bethesda, MD, USA) (Li et al., 2008). After an overnight incubation with first antibody, the samples were then incubated with secondary antibodies conjugated with the appropriate fluorescent tag, followed by staining with 5 mg/ml Hoechst 33342 (Sigma, St Louis, MO, USA). Filamentous-actin (F-actin) was detected with Alexa Fluor 488 phalloidin (1:50; Invitrogen, CA, USA). Embryos were imaged on a Zeiss LSM510 confocal microscopy using a 40 \times /1.3 oil immersion objective lens. Alexa 594 excitation was performed with a HeNe laser set to 543 nm, and emission was detected by a 560BP nM filter. Excitation for Hoechst 33342 was performed using a UV laser at 364 nm and emission was detected by a 450BP filter. The embryos were stained with Hoechst 33342, for total cell counts, and CDX2 antibody, for TE cells counts. The counts were obtained from z-stacks of confocal images, taken at 2 μ m intervals.

RNA preparation and cDNA synthesis

Total RNA of each blastomere was purified using a PicoPure RNA Isolation Kit (Arcturus Bioscience). All real-time PCR primers were first tested on the Prism 7900HT Sequence Detection System 2.2 (ABI), and Ct values were calculated by the system software. High expression is defined by Ct values of 31 or lower, and undetectable by Ct values of 35 or greater.

High-throughput single-cell qPCR

Sequence-specific assays (Roche) were pooled to a final concentration of 20 μ M for each assay (supplementary material Table S2). The cDNA of each individual blastomere was subjected to sequence-specific amplification prior to analysis with the Universal Probe Library (UPL) for gene expression assays (Roche) in 48 \times 48 Dynamic Arrays on a BioMark System (Fluidigm) as previously described (Guo et al., 2010).

Single-cell data processing

All Ct values obtained from the BioMark System were converted into relative expression levels by subtracting the values from the assumed baseline value of 28 (Guo et al., 2010). Cells with low or absent endogenous control gene expression levels were removed from analysis (<5%). The resulting values were at times normalized to the endogenous control by subtracting, for each cell, the average of its Act β gene expression levels. As the Ct scale is logarithmic (a difference of one Ct corresponds to a doubling of measured transcript), a subtraction of the average of Act β genes on this scale corresponds to taking the geometric mean on a linear scale. In Fig. 1A-F, *Oct4* and *Cdx2* average transcript abundance of separated blastomeres, TE and ICM were plotted by Prism, whereas the transcript abundance heat map hierarchy, shown in Fig. 2A, was made using Mev software (the Artistic License v2.0). Principal component analysis, using a covariance matrix to classify cells into the hypothetical cell types shown in Fig. 2B, was performed using the princomp command in R version 2.12.2 on cell expression data from 67 cells (48 1/32 blastomeres, nine ICM and 10 TE cells). These data were standardized by mean subtraction.

Blastomere aggregation and embryo transfer

A 1/8 or 1/16 blastomere of a GFP⁺ embryo, from B6.129S1Tg(CAG-EGFP)S1C^{Tpo/KnwPoa} mice, was co-cultured with a zona-free eight-cell or 16-cell stage carrier embryo in microdrops (28 drops/dish). The aggregated embryos were cultured to the blastocyst stage (E4.5) before fixation for immunofluorescent analysis or transfer into surrogate mothers. E2.5 pseudopregnant ICR female mice were used as recipients for blastocyst-derived aggregated embryos.

RESULTS

Effect of blastomere separation

To determine the effect of cell-cell contact on cell lineage decision, we established long-term separated (LS) blastomeres by mechanically separating blastomeres at each of the five cell divisions up to the 32-cell stage and culturing them individually (supplementary material Fig. S1). We found that physical separation did not affect early development from the two- to the eight-cell stage as blastomeres developed normally at these stages. Out of 336 embryos manipulated, 300 embryos proceeded normally to the third division, giving rise to 2400 1/8 LS blastomeres (300/336; 89.3%). However, the number of blastomeres reaching the 16-cell and 32-cell stage was reduced. Only 25% (85/336) and 7% (25/336) of manipulated embryos gave rise to a complete set of daughter blastomeres at the 16-cell and 32-cell stage, respectively. This decrease in developmental potential could have resulted from both technical and biological effects. Blastomeres that do not divide or die may increase for various technical reasons; for example, the extensive time in vitro, repeated handling at room temperature or mechanical damage. Additionally, we observed some flattening and increased membrane fragility of the 1/16LS blastomeres, slight 'compaction' between 2/16LS and

2/32LS blastomeres that were not promptly separated and also the previously reported phenomenon of larger blastomeres engulfing smaller ones (Dietrich and Hiiragi, 2007). However, overall, long-term separation did not significantly affect the morphological properties of the separated blastomeres.

Cell-cell interaction is required to maintain *Oct4* and *Cdx2* expression levels in embryonic blastomeres

To address the impact of cell-cell interaction on regulating cell-fate decisions, we analyzed the expression of two genes, *Oct4* and *Cdx2*, which mark the ICM and TE lineages in the LS blastomeres. LS blastomeres were collected 6-8 hours after separation for analysis, and FS blastomeres, from intact embryos of the corresponding stage, were collected concurrently as controls. At the 2- and 4-cell stages, we analyzed 1/2 and 1/4FS and LS blastomeres, and found that *Oct4* expression is comparable between 1/2FS and LS, and 1/4FS and LS, whereas expression of *Cdx2* in these blastomeres was essentially undetectable (data not shown). At the eight-cell stage equivalent, most 1/8LS blastomeres express *Oct4* at a similar level to that of 1/8FS blastomeres, although there are a few exceptions that display higher levels of *Oct4* transcripts (Fig. 1A). Whereas the *Cdx2* levels are low in all blastomeres, we detected relatively higher levels of *Cdx2* transcripts in significantly more 1/8LS blastomeres than 1/8FS blastomeres (Fig. 1A; $P < 0.05$, χ^2 analysis). Indeed, comparison of the average expression levels of *Oct4* and *Cdx2* in 1/8LS and 1/8FS blastomeres (Fig. 1B) revealed that 1/8LS blastomeres contained higher levels of both *Oct4* and *Cdx2* transcripts than the 1/8FS blastomeres.

At the 16-cell stage, we observed a wide range of *Oct4* transcript abundance in individual 1/16FS blastomeres (Fig. 1C). By contrast, the 1/16LS blastomeres showed less variation in *Oct4* expression levels and no blastomere expressed high levels of *Oct4* (Fig. 1C). The overall levels of *Cdx2* transcripts were more variable and higher in the 1/16LS blastomeres than in the 1/16FS blastomeres (Fig. 1C,D).

At the 32-cell stage, we used TE and ICM cells isolated from intact embryos as the 1/32FS controls. We observed that *Oct4* transcript abundance was significantly lower in 1/32LS blastomeres than in the ICM cells but higher than that in TE cells (Fig. 1E,F). ICM cells and 1/32LS blastomeres had, respectively, 300- and 100-fold higher *Oct4* expression than did TE cells. *Cdx2* expression was detected in 1/32LS blastomeres at a higher level than that in TE cells whereas it was absent in ICM cells. These data suggest cell-cell interaction is important for the regulation of *Oct4* and *Cdx2* expression levels in 1/8, 1/16 and 1/32 blastomeres. In the absence of cell-cell interactions, blastomeres do not maintain the high *Oct4* levels typical of ICM cells and *Cdx2* levels are upregulated. In particular, the expression of *Cdx2* appears to be strongly influenced by cell-cell contact.

We observed that *Cdx2* transcription was first detectable at the eight-cell stage and more *Cdx2*-positive blastomeres with higher *Cdx2* transcript levels were found after long-term separation. Ten percent of the 1/8FS and 25% of 1/8LS blastomeres, respectively, expressed *Cdx2* ($P < 0.05$; Chi-square test). A similar trend was seen at the next stage, where 77% of the 1/16LS blastomeres were *Cdx2* positive compared with 70% of the 1/16FS group. Not only did all the analyzed 1/32LS blastomeres express *Cdx2*, but they expressed it at a higher level than did TE cells (Fig. 1G; supplementary material Table S1; $n=48$). These observed changes in *Cdx2* and *Oct4* expression suggest a shift in fate specification upon the loss of cell-cell contact.

Lack of cell-cell interaction throughout pre-implantation development results in blastomeres of undetermined lineage

To determine the lineage identity of these separated blastomeres, individual 1/32LS blastomeres were analyzed for expression of gene markers of the ICM, primitive endoderm (PE) and TE lineages (Fig. 2). Analysis of their transcription profiles showed that the 1/32LS blastomeres have a unique signature, different from that of either TE or ICM cells. *Oct4* was the only ICM lineage marker highly expressed in most of the 1/32LS; none of the 1/32LS blastomeres expressed *Sox2* and most expressed *Nanog* and *Sall4* at low levels. The 1/32LS blastomeres also expressed the PE-initiation marker *Grb2* (Fig. 2A). An intriguing heatmap-signature was found for the TE-lineage markers *Cdx2*, *Eomes*, *Borg5*, *Elf5* and *Dppa1*, which were expressed in a different pattern from that in isolated TE cells (Fig. 2A). In normal development, high *Cdx2* expression corresponds to reduced *Oct4* expression (Guo et al., 2010). However, we found that isolated blastomeres maintained high *Oct4* expression, despite high *Cdx2* expression. This finding is consistent with a previous report on *Cdx2-Oct4* co-expressing cells found in E-cadherin mutant embryos (Stephenson et al., 2010). Taken together, these observations suggest these cells have an indeterminate gene expression profile characteristic of neither ICM nor TE blastomere.

Expression analysis of additional genes in the 1/32LS blastomeres may contribute to a more precise lineage identity. With this in mind, we analyzed expression of genes in the Hippo signaling pathway, the Wnt canonical pathway, DNA methyltransferases and genes involved in post-translational control (supplementary material Table S2). Hierarchical clustering of the gene expression profiles of the 67 analyzed cells showed two main clusters with the 'ICM cluster' standing alone and the 1/32LS blastomeres clustering with, but distinct from, the TE cells (Fig. 2A).

To visualize these data, we employed principal component analysis (PCA) based on the expression of these genes in the TE, ICM and 1/32LS blastomeres. Projection of the blastomere expression patterns onto component 1 and 2 clearly separated the individual cells into three distinct clusters (Fig. 2B). The ICM cluster is distinguished from the TE cells along the PC1 axis. The gene expression pattern of freshly isolated TE cells were all similar, with all 10 cells plotting closely to one another, whereas ICM cells were more spread out in a pattern similar to that previously reported (Guo et al., 2010). The 1/32LS blastomeres plotted to the middle, suggesting they represent an uncertain cell type leaning more towards the TE lineage rather than the ICM lineage. Moreover, each of the 1/32LS blastomeres derived from different embryos (represented in different colors) showed slightly different gene expression profiles. Some plotted closer to the TE cluster, whereas their sister blastomeres plotted closer to the ICM cluster. This suggests a certain plasticity of the 1/32LS blastomeres, with each blastomere, albeit derived from the same embryo, trying to capture a different fate, thus resulting in a heterogeneous population of blastomeres. Even though the sample number was 5-11 cells/embryo, the results showed a similar tendency among all the singled blastomeres and across embryos. This consistency lends confidence the interpretation that data are representative and reproducible.

Localization of polarity proteins is altered in singled blastomeres

To determine whether the localization of polarity proteins is dependent on cell-cell interaction, we examined cell polarity by

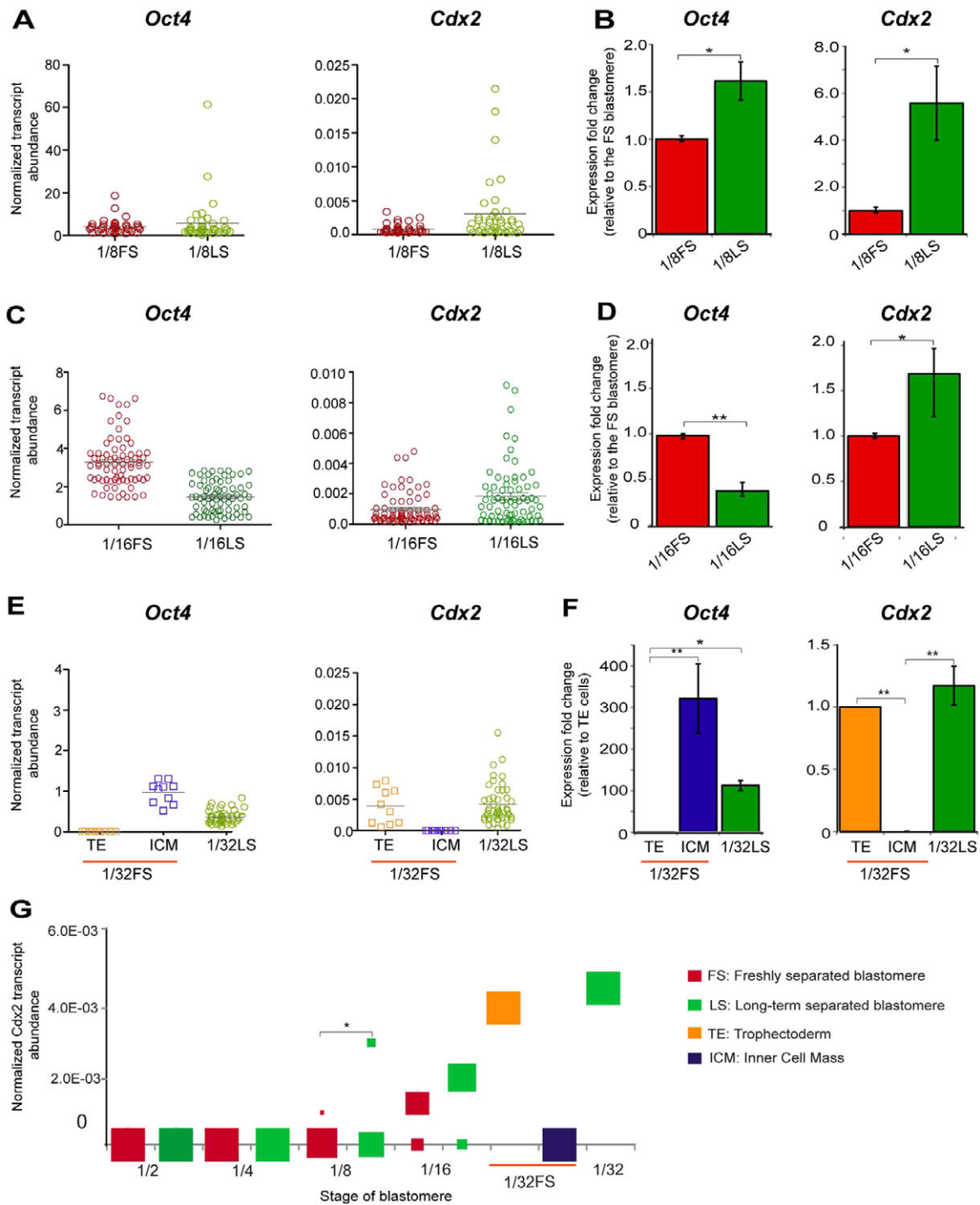


Fig. 1. Transcriptional profile of single blastomeres. (A,C,E) Single cells of (A) 1/8, (C) 1/16 and (E) 1/32 blastomeres derived from FS and LS blastomeres were analyzed for *Oct4* and *Cdx2* expression (embryo-isolated TE and ICM cells represent 1/32FS cells). Levels of *Oct4* and *Cdx2* transcript abundance from individual blastomeres (circle), each group of blastomeres is shown in a different color, horizontal bar in each data group represents the median. These data, normalized against those for *Actb* expression (which increases as development proceeds), are shown as a scatter plot. (B,D,F) Relative expression of *Oct4* and *Cdx2* from LS blastomeres were analyzed against FS blastomeres of the equivalent stages: (B) 1/8, (D) 1/16 and (F) 1/32 blastomeres. $n=40$ blastomeres each of 1/8FS and LS; $n=80$ blastomeres each of 1/16FS and LS; $n=48$ blastomeres of 1/32LS; 10 TE cells; and nine ICM cells. Each histogram has a different scale. Transcript abundance of less than 2.0×10^{-4} was considered as background (Ct value=28). (G) Comparison of expression level and number of *Cdx2*-positive blastomeres between FS and LS blastomeres across the same developmental stages. Data plotted on three-dimensional axes. The value of the *Cdx2* transcript abundance is shown on the y-axis, the embryonic stage on the x-axis and number of blastomeres with detectable *Cdx2* transcription is indicated by the size of the square box. Transcript abundance of less than 2.0×10^{-4} was considered background (Ct value=28; $P < 0.05$, Student's *t*-test). Comparison between 1/8FS and 1/8LS blastomeres ($n=40$ per group, $P < 0.05$; χ^2 analysis) and 1/16FS and 1/16LS blastomeres ($n=80$ per group; $P > 0.05$; χ^2 analysis).

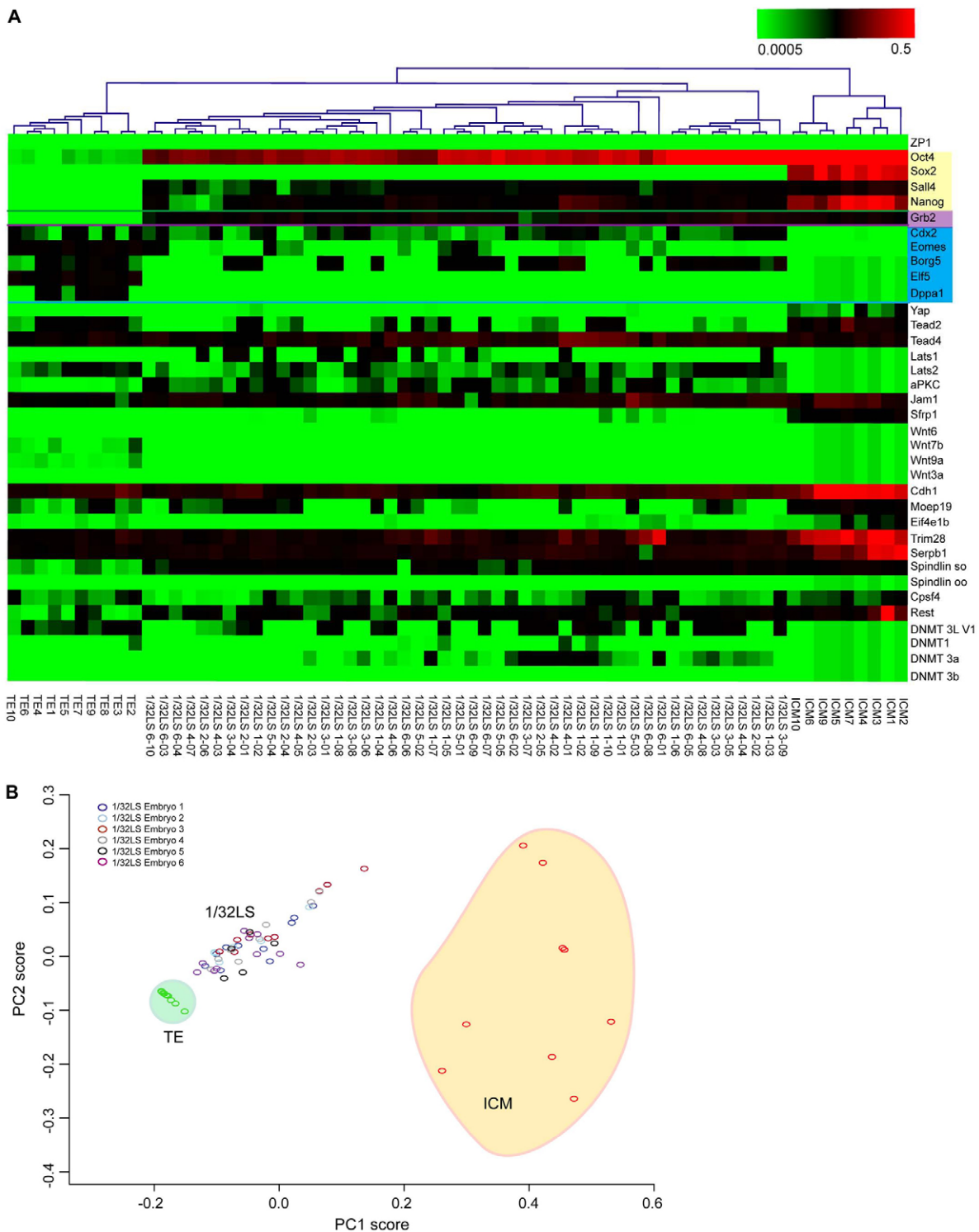


Fig. 2. Gene expression and lineage analysis of 1/32 LS blastomeres. Individual blastomeres analyzed for ICM, PE and TE lineage markers using microfluidigm single cell q-PCR. **(A)** Heat maps represent transcriptional abundance of 36 genes, including ICM, PE and TE lineage markers, and developmentally relevant genes, comparing 1/32LS with freshly isolated TE and ICM cells. The ICM, PE and TE lineage-specific markers are highlighted in yellow, purple and blue, respectively. Analysis conducted on 48 1/32LS blastomeres, nine ICM cells and 10 TE cells. **(B)** Principal component analysis (PCA) of 1/32LS blastomeres with cells from TE and ICM. The PCA bi-plot is based on expression of the 36 genes shown in Fig. 2A. Values from blastomeres are plotted in two dimensions using their projections onto the first two principal components. The first two principal components are shown, which account for over 89% of the variants. Principal component (PC) projections of 48 1/32 blastomeres (each circle represents a single cell, colored according to its embryo of origin) compared with nine ICM and 10 TE cells. Cells with a PC1 score greater than 0.2 are classified as ICM, cells with a PC1 score lower or equal to -0.15 are classified as TE, the remainder were considered to be uncertain. Graphical comparison of these data with those previously published (Guo et al., 2010) from 32-cell stage embryos are shown in green and orange highlights, which represent TE and ICM cells, respectively.

observing the apical distribution of F-actin, localization of the apical membrane markers (aPKC), a tight junction protein (ZO1) and members of the subcortical maternal protein complex (FLOPED, MATER and TLE6) (Li et al., 2008) in the long-term separated blastomeres (Fig. 3A). In 1/8LS blastomeres, only F-actin maintained a polarized distribution in the absence of cell-cell contact (Fig. 3B, arrow). Interestingly, its distribution corresponded to that found in the outer cells of the intact morula, suggesting that the separated blastomeres are more likely to adopt an outer cell fate. We found that aPKC failed to establish polarity and ZO1 showed cytoplasmic localization, whereas the subcortical maternal protein complex, TLE6, FLOPED and MATER, showed subcortical localization with a loss of polarity (Fig. 3B).

Earlier works demonstrated that with reduced cell-cell contact (through the inhibition of E-cadherin mediated cell-cell interaction), embryonic blastomeres can still become polarized (Shirayoshi et al., 1983; Stephenson et al., 2010). It is, however, important to note that there were residual cell adhesion molecules, such as cadherin 3, present at cell-cell junctions in the E-cadherin mutant embryos, which weakly link the blastomeres together (Stephenson et al., 2010). This residual contact could permit communication among blastomeres, resulting in activation of signaling pathways and establishment of polarity. We hypothesized

that correct polarity cannot be established in the absence of any cell-cell contact, but that if cell-cell contact is restored, polarity could be re-established. To test this hypothesis, daughters of 1/8LS blastomeres were allowed to remain together (2/16 blastomeres) before fixation and staining. We found that F-actin, aPKC and ZO1 protein markers were indeed able to re-establish their positions, albeit slightly misplaced ($n=16$ /protein marker; Fig. 3C, arrow) in the 2/16 blastomeres. We demonstrate that in the complete absence of cell-cell contact, cell polarity can not be set up, but partial contact between blastomeres is sufficient for the re-establishment of polarity. Together, these results strongly suggest that cell interactions are required for blastomeres in pre-implantation embryos to establish apical-basolateral polarity.

Activation of Hippo signaling pathway in singled blastomeres is independent of cell interactions

It has previously been reported that the Hippo pathway is activated in the inner cells of the embryo, which form extensive cell-cell contact with their neighboring cells on all surfaces. The result is phosphorylation of Yes-associated-protein (YAP) in their cytoplasm. On the contrary, the Hippo signaling pathway is not active in the outer cells that have contact-free surfaces. YAP is therefore not phosphorylated, and can shuttle into the nucleus and

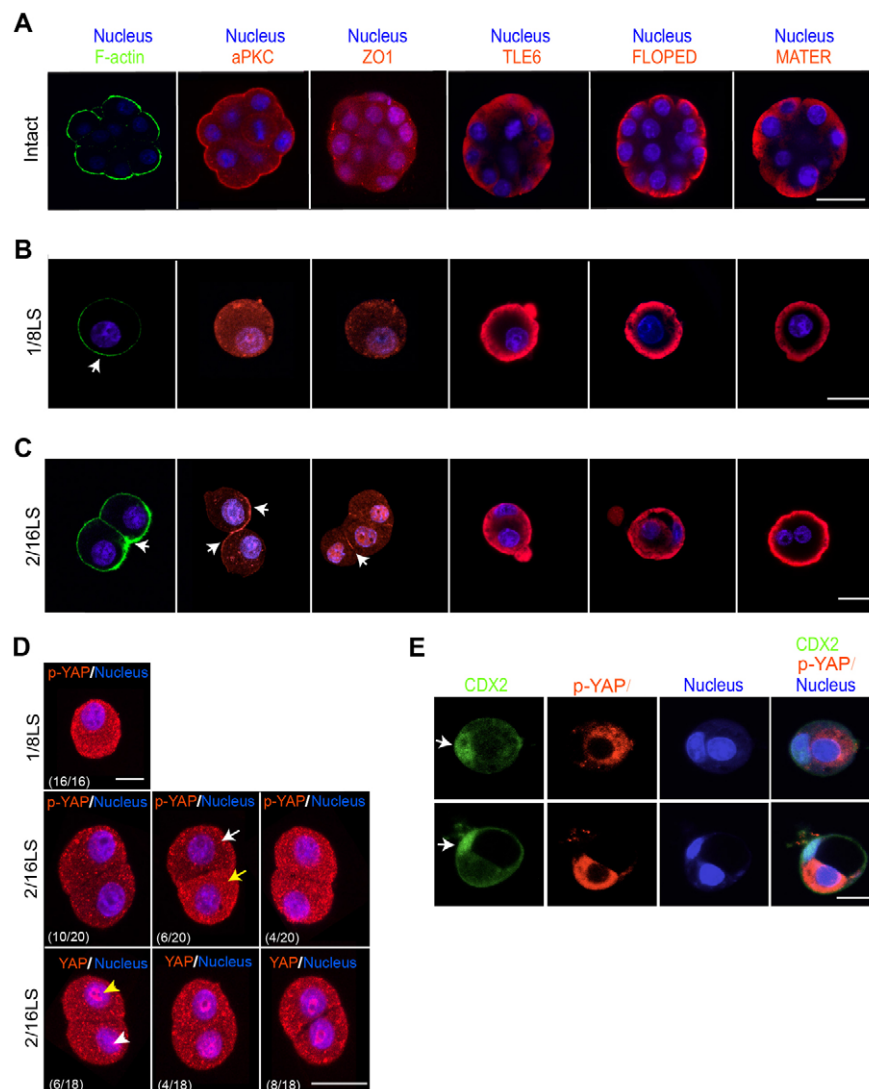


Fig. 3. Polarity-protein localization and Hippo signaling pathway activity in singled blastomeres.

(A-C) Localization of F-actin, membrane protein marker aPKC, ZO1 tight junction protein and the subcortical maternal complex TLE6, FLOPED and MATER in intact control embryos (A), in 1/8LS blastomeres (B, arrow indicates asymmetric expression of F-actin) and in 2/16LS blastomere (C, arrow indicates irregular expression of F-actin, aPKC and ZO1 proteins). Note that blastomeres stained for TLE6, FLOPED and MATER are fully compacted. Scale bars: 50 μ m. (D) Hippo signaling pathway activity in 1/8LS and 2/16LS blastomeres, white and yellow arrows/arrowheads show negative and positive blastomeres, respectively. Numbers in brackets indicate sample numbers. Scale bars: 20 μ m. (E) CDX2 and p-YAP expression in 2/16 blastomeres. Nuclear staining for CDX2 (arrow) is present only in blastomeres in which cytoplasmic p-YAP is absent. Scale bar: 20 μ m.

bind to its partner protein, TEAD4, to activate *Cdx2* expression (Nishioka et al., 2009). To study the activity of Hippo pathway in singled blastomeres, the 1/8LS blastomeres were stained with antibodies recognizing total or phosphorylated Yap (p-YAP). We found that the cytoplasm of most 1/8LS blastomeres was strongly stained with p-YAP antibodies (Fig. 3D), suggesting the Hippo pathway is activated in singled cells. However, we did not observe any nuclear staining for YAP, a result consistent with the report that there is no accumulation of nuclear YAP in 1/8 blastomeres (Nishioka et al., 2009), and that Hippo signaling is regulated by cell morphology and F-actin (Sansores-Garcia et al., 2011; Wada et al., 2011; Zhao et al., 2012). Our results indicate that Hippo signaling does not depend on cell contact because singled blastomeres, which become depolarized after separation, exhibit activated Hippo signaling resulting in p-YAP in the cytoplasm. Thus, cell contact is not the only factor activating the Hippo signaling pathway; however, the polarized state does appear to be inhibitory to the Hippo signaling cascade.

Coupling this result with the finding that *Cdx2* expression was increased in 1/8LS blastomeres (Fig. 1A,B), our data suggest the ability of the Hippo pathway to inhibit *Cdx2* expression is altered in singled blastomeres. Thus, we hypothesized that the activity of the Hippo pathway, in terms of controlling *Cdx2* expression, would be restored when the blastomeres re-establish cell-cell contact. To test this hypothesis, 1/8LS blastomeres were allowed to develop to 2/16 blastomeres and then stained for p-YAP, total YAP and CDX2. Heterogeneous patterns of p-YAP and total YAP localization was observed in 2/16LS blastomeres, even though each cell had an equal area of cell-cell contact (Fig. 3D). There were some 2/16

blastomeres that exhibited low p-YAP and total YAP signals in both cells, some of which exhibited high p-YAP and total YAP in both cells, and some that exhibited a high signals in only one of the two blastomeres. When examined CDX2 expression in the 2/16LS blastomeres, we found CDX2 expression restricted to p-YAP negative cells ($n=15/15$; Fig. 3E, arrow). These results demonstrate that, upon establishment of cell-cell contact, the Hippo pathway again functions in a canonical manner in the regulation of *Cdx2* expression.

Long-term separated blastomeres preferentially contribute to the trophectoderm of the blastocyst

To determine whether the observed gene expression patterns observed correlate with the developmental behavior of LS blastomeres, we aggregated separated blastomeres, from a transgenic mouse that abundantly expresses green fluorescent protein (GFP) in each cell (Frank et al., 2003), with zona-free intact eight-cell stage embryos (i8C) (Fig. 4A). Visualization of GFP-positive (GFP⁺) cells in blastocysts derived from 1/8FS blastomeres showed they can contribute to both the ICM and TE lineages (Fig. 4B), whereas 1/8LS blastomeres in the majority of blastocysts contributed to the TE lineage alone (Fig. 4C).

We also determined whether disaggregation of the eight-cell stage recipient embryos into single cells (s8C) before they were re-aggregated with 1/8FS or 1/8LS blastomeres facilitates integration and ICM lineage allocation of singled blastomeres. The average cell number (~68-82 cells/embryo) in the blastocysts of all four aggregate groups was comparable, with each embryo containing 6-10 GFP⁺ cells. All blastocysts derived from 1/8FS (6/6) had GFP⁺

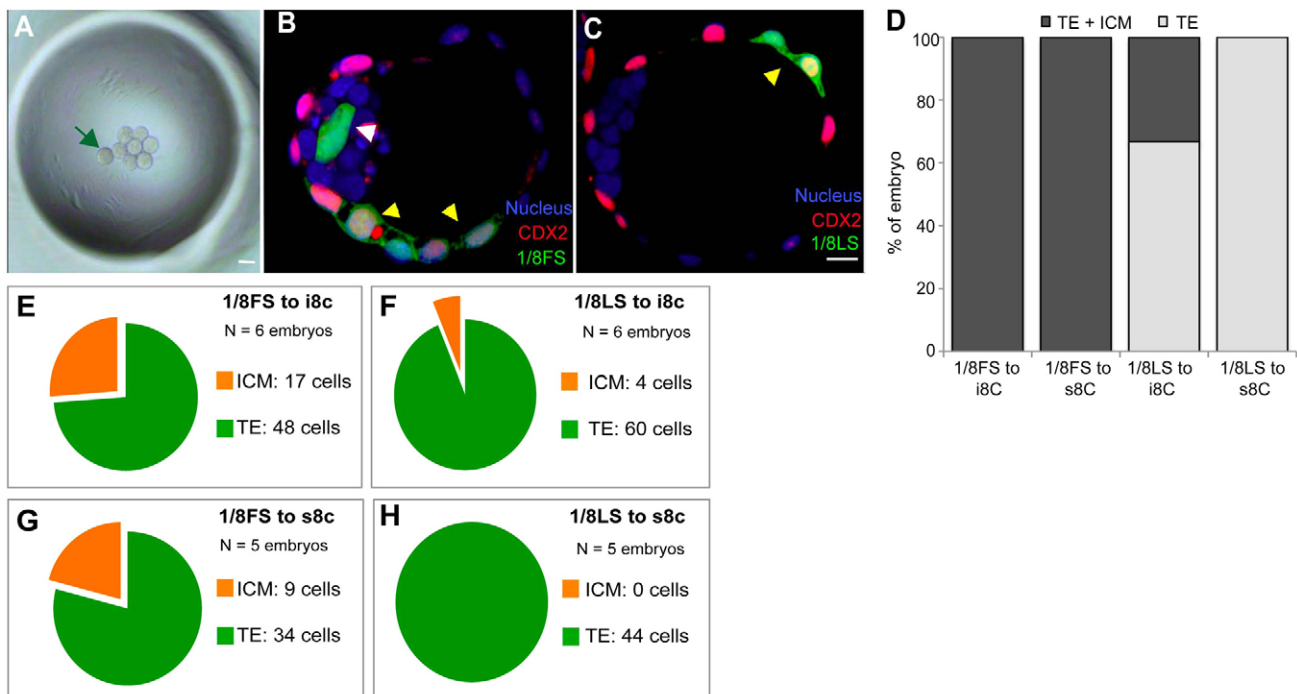


Fig. 4. Aggregation of 1/8 LS and 1/8FS GFP⁺ blastomeres with eight-cell stage embryos. (A) An 1/8 FS blastomeres (arrow) was co-cultured with a zona-free intact eight-cell stage (i8C) recipient embryos. Scale bar: 20 μ m. (B) Progeny of 1/8FS blastomere contribute to TE and ICM ($n=38$), yellow and white arrowheads, respectively. (C) 1/8LS blastomere generated daughter cells (arrowhead) contributing to the TE ($n=40$). Trophectoderm cells stained with a CDX2 (red). Scale bar: 10 μ m in B,C. (D) Ratio of re-aggregated embryos derived from aggregation assays, with i8C as above or with eight-cell stage embryos separated into individual blastomeres to facilitate aggregation (s8C), that have either GFP⁺ cells in their ICM and TE or in which GFP⁺ cells are restricted to TE. (E,F) Total progeny cell number from 1/8FS (E) and 1/8LS (F) blastomeres that contributed to TE and ICM after re-aggregation to zona-free intact eight-cell stage embryos. (G,H) The same experiment using separated eight-cell stage recipient embryos (s8C) as recipients of 1/8FS (G) and 1/8LS (H) blastomeres.

cells in both TE and ICM (Fig. 4D), whereas fewer blastocysts derived from the 1/8LS-i8C embryos had GFP⁺ cells in their ICM. None of the 1/8LS-s8C embryos (0/5) gave rise to blastocysts containing GFP⁺ cells in their ICM.

When GFP⁺ cells, from blastocysts derived from 1/8FS and 1/8LS aggregation to i8C recipient embryos, were counted and lineage classified, one quarter of the cells derived from 1/8FS were found to contribute to the ICM (Fig. 4E), whereas fewer than 10% of cells derived from 1/8LS blastomeres contributed to the ICM (Fig. 4F). GFP⁺ cells derived from aggregating 1/8FS or 1/8LS with s8C recipient embryos showed a similar trend, with 20% of the progeny from the 1/8FS cells contributing to the ICM, whereas none from the 1/8LS aggregated with s8C contributed to the ICM (44 cells were found in the TE; Fig. 4G,H). Thus, even at the eight-cell stage, LS blastomeres preferentially acquire the TE cell fate and, in the majority of these aggregated embryos, they contribute exclusively to the trophoctoderm. However, this tendency was not absolute, LS blastomeres did contribute to the ICM in some embryos.

Long-term separated blastomeres display reduced developmental plasticity

To further test the developmental plasticity of LS blastomeres, we tested their capacity to contribute to the ICM in a non-competitive environment, i.e. in the absence of cells from a normal embryo. LS blastomeres were re-aggregated to their sister blastomeres at the 8- (8/8LS) or 16-cell (16/16LS) stage and allowed to develop to blastocysts. Freshly separated blastomeres, which were immediately re-aggregated to their sister blastomeres, served as controls. The re-aggregated blastomeres were able to develop to blastocysts (Fig. 5), although by morphology, the 16/16LS-derived embryos exhibited a less defined clump of inner cells compared with the other experimental groups. Blastocysts derived from 8/8FS and 16/16FS contained similar cell numbers compared with non-manipulated embryos (Table 1), and they contained the same number of TE cells as the controls ($P > 0.05$; ANOVA). However, 16/16FS-derived blastocysts contained significantly fewer ICM cells than those from 8/8FS and controls ($P < 0.05$; ANOVA). Embryos derived from 8/8LS cell stage (8/8LS) reaggregations contained significantly fewer cells than did 8/8FS-derived blastocysts ($P < 0.05$; ANOVA), although the number of ICM cells was not significantly different. Significantly, the 16-cell stage reaggregates (16/16LS) contained fewer total cells than 16/16FS ($P < 0.05$; ANOVA), and most embryos do not have a clearly defined clump of cells in the blastocoel cavity. These results indicate ICM lineage commitment is clearly affected by the absence of cell-cell interaction at the 16-cell stage.

To determine whether cell-cell interaction at the 16-cell stage is crucial for embryo implantation and the ability to make the embryo proper, we transferred blastocysts derived from 8/8LS, 8/8FS, 16/16LS and 16/16FS reaggregations into pseudopregnant foster mothers (five to eight embryos/group). There was no significant differences in the capacity of the 8/8FS- and 8/8LS-derived

blastocyst to implant, whereas the 16/16FS reaggregates had a higher implantation rate than blastocyst derived from 16/16LS. At E7.5, developing embryos were found in all categories but embryos of the 16/16LS group were developmentally delayed (Table 2). We also examined E10.5 embryos and found that all the 16/16LS embryos were resorbed as all decidua were empty, whereas every deciduum in the 8/8FS, 8/8LS and 16/16FS groups contained phenotypically normal embryos. The high rate of 16/16LS embryo resorption at E10.5 suggests cell-cell contact at the 16-cell stage may be important for the ICM lineage fate decision, which in turn has a direct effect on post-implantation development.

DISCUSSION

In this study, we aimed to investigate whether cell-cell interaction can influence the fate specification of the embryonic blastomeres by mechanically separating blastomeres at the two-cell stage embryos and continuing separation after each cell division throughout pre-implantation development. We examined gene expression profiles, polarity establishment and Hippo signaling pathway activity, and performed functional tests of the individual separated blastomeres. Our results demonstrate that in the absence of cell-cell contact, each blastomere develops a unique pattern of gene expression, though there is tendency to partially mimic the gene expression pattern of trophoctodermal cells. Our results also show that, in the complete absence of cell-cell contact, the singled blastomeres fail to establish polarity. We further show, in contrast to the current hypothesis, that activation of the Hippo/Yap signaling pathway in single blastomeres and their daughter cells is achieved independently of cell contacts. Additionally, we show that singled blastomeres fail to make the embryo proper, and preferentially contribute to the TE lineage. Our overall conclusion is that cell-cell interaction plays a significant role in the fate specification of the embryonic blastomeres and post-implantation development.

Analysis of gene expression in separated mouse blastomeres could hypothetically result in several different patterns, depending on alternative views of how lineage commitment is achieved in pre-implantation mouse embryo. One possibility is that in the absence of positional and cell-cell contact clues, the gene expression pattern of the two-cell stage embryo will continue without change through subsequent cleavage divisions. Alternatively, based on the pre-patterning model of development (Gardner, 1997; Gardner, 2001; Piotrowska et al., 2001) one might anticipate that singled progeny of one of the two-cell blastomere will display a trophoctoderm gene expression pattern whereas the other will give rise to blastomeres with either an ICM or TE gene expression pattern. The third option, based on the stochastic model of development (Motosugi et al., 2005) would predict random distribution of blastomeres expressing an ICM or TE gene expression pattern among the progeny of both 'singled' two-cell stage embryo blastomeres. Finally, the inside-outside hypothesis (Tarkowski and Wróblewska, 1967) would predict that essentially all 'singled' 32 blastomeres (32LS) would end up with a TE gene expression pattern.

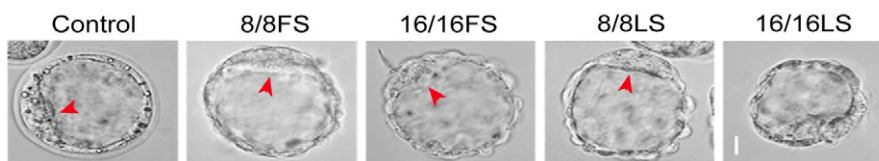


Fig. 5. Developmental fates of eight- and 16-cell blastomere aggregates on embryo implantation. Morphology of blastocysts derived from intact embryos and re-aggregated blastomeres of 8/8FS, 16/16FS, 8/8LS and 16/16LS. Red arrowheads indicate the ICM cell cluster. Scale bar: 20 μ m.

Table 1. Embryonic cell number

| Embryo | Total | TE | ICM |
|---------|---------------------|---------------------|--------------------|
| Control | 78±2.2* | 59±1.7* | 19±0.8* |
| 8/8FS | 75±1.8* | 57±1.5* | 18±0.6* |
| 16/16FS | 70±1.4* | 61±1.4* | 9±0.5 [‡] |
| 8/8LS | 57±1.8 [‡] | 43±1.5 [‡] | 14±0.5* |
| 16/16LS | 44±2.8 [§] | 42±0.3 [‡] | 2±2.7 [§] |

Total cell, TE and ICM cell number in blastocysts derived from intact and re-aggregated blastomeres.

*,[‡],[§]Significant differences at P<0.05; ANOVA.

In reality, none of these predictions exactly matches the observed results. Each of the singled blastomeres expresses its own unique gene pattern that represents a random mixture of ICM- and TE-specific genes. The simplest explanation of this result is that, during normal development, each blastomere gradually activates essentially all ICM and TE genes. As cleavage progresses and positional clues start to play a role, expression of some genes is downregulated and that of others is upregulated, depending on the position and cell-cell interaction of each blastomere. This process continues during subsequent cleavage divisions, resulting in an ever increasingly specific gene expression pattern. Finally, in expanded blastocysts, three committed cell lineages, each with a unique gene expression pattern, are established. In the absence of any outside information, each singled blastomere will randomly express a haphazard mixture of activated ICM-specific and TE-specific genes. Results describing progression and gradual specification of gene expression patterns during cleavage (Dietrich and Hiiragi, 2007; Guo et al., 2010) are in agreement with this interpretation. Finally, when we examine the gene expression patterns of 32LS blastomeres, we note that, though each pattern is unique, there is also a tendency towards a ‘trophectoderm-like’ pattern. This is to be expected as all singled blastomeres are receiving ‘outside’ signals but these are obviously not sufficient, in the absence of cell-cell contact, to result in a true trophoctoderm pattern of gene expression.

A crucial role of cell-cell contact in establishing and maintaining the developmental potential of the pre-implantation embryo was suggested previously by experiments in which four-cell stage mouse embryos were cultured in the absence of a zona pellucida. In zona-free conditions, four-cell stage embryos can assume different configurations, among them linear (minimal cell-cell contact) and tetrahedral (maximal cell-cell contact). Linear four-cell stage embryos develop into blastocysts with significantly fewer ICM cells (Graham and Lehtonen, 1979). When blastocysts derived from these different configurations were re-implanted, those from the linear-configured four-cell stage the embryos developed less well than those from the tetrahedral embryos (Suzuki et al., 1995). If such relatively modest interference with cell-cell communication is detrimental for subsequent development, it is not at all surprising that prolonged absence of cellular contact and positional signaling can, as we have observed, completely eliminate developmental potential.

In our experiments, the signals present in normal development: cell-cell contact and positional information are clearly lacking, yet the developmental clock, in terms of gene expression, is obviously still running. However, the differential gene expression underlying cell-fate commitment is absent, with the pattern of novel gene expression in each separated blastomere subtly different, but tending towards a trophoctodermal gene expression pattern. Thus,

Table 2. Embryo development after implantation

| Type | Embryo/decidua | |
|---------|----------------|-------|
| | E7.5 | E10.5 |
| 8/8FS | 10/10 | 10/10 |
| 8/8LS | 8/8 | 7/8 |
| 16/16FS | 5/5 | 5/5 |
| 16/16LS | 3/3* | 0/6 |

Development of re-aggregated embryo after transplantation to pseudo-pregnant females.

*Delayed embryo development.

activation of the embryonic genome and random expression of lineage-specific genes is driven by a developmental clock, but correct patterning of lineage-specific gene expression requires positional signals and cell-cell interaction.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.086454/-/DC1>

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