Disorganized epithelial polarity and excess trophectoderm cell fate in preimplantation embryos lacking E-cadherin

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SUMMARY
The first two cell lineages in the mouse, the surface trophectoderm (TE) and inner cell mass (ICM), are morphologically distinguishable by E3.5, with the outer TE forming a polarized epithelial layer enclosing the apolar ICM. We show here that in mouse embryos completely lacking both maternal and zygotic E-cadherin (cadherin 1), the normal epithelial morphology of outside cells is disrupted, but individual cells still initiate TE- and ICM-like fates. A larger proportion of cells than normal showed expression of TE markers such as Cdx2, suggesting that formation of an organized epithelium is not necessary for TE-specific gene expression. Individual cells in such embryos still generated an apical domain that correlated with elevated Cdx2 expression. We also show that repolarization can occur in isolated early ICMs from both wild-type and Cdx2 mutant embryos, indicating that Cdx2 is not required for initiating polarity. The results demonstrate that epithelial integrity mediated by E-cadherin is not required for Cdx2 expression, but is essential for the normal allocation of TE and ICM cells. They also show that Cdx2 expression is strongly linked to apical membrane polarization.

KEY WORDS: Trophectoderm, Inner cell mass, Blastocyst, Polarity, Polarization, Apical, Basolateral, E-cadherin, Cdh1, Cdx2, Yap, PKCzeta, Jam1, Lgl, Par6, P-cadherin, Mouse

INTRODUCTION
At E3.5, the mouse embryo consists of two cell types, the outside trophectoderm (TE), which generates the embryonic portion of the placenta, and the inner cell mass (ICM), which later forms the embryo itself along with other extraembryonic structures, including the yolk sac endoderm (Yamanaka et al., 2006). The mechanisms by which these two cell lineages emanate from what is likely to be a uniform and totipotent population of cells at the 8-cell stage is a continuous focus of research. In this paper, we focus upon the roles of cell adhesion and apical-basal polarity in the divergence of these two cell populations.

At the 8-cell stage a number of morphological changes occur that are believed to be important for the divergence of the first two cell lineages. The first of these is compaction, which involves a smoothing of the surface of the embryo, associated with an increase in intercellular adhesion mediated by cadherin 1 (Cdh1; hereafter referred to as E-cadherin) (Shirayoshi et al., 1983). Concomitant with compaction, individual blastomeres undergo apical-basal polarization (Ducibella et al., 1977; Johnson and Maro, 1986). This involves the formation of an apical microvillus-enriched polar region on the outer surface of the embryo associated with proteins including the atypical protein kinase (aPKC) isoforms PKCCε and PKCζ (Prkcz and Prkci), Par6 and ezrin (Ducibella et al., 1977; Louvet et al., 1996; Paulken and Capco, 2000; Reeve and Ziemek, 1981; Vinot et al., 2005). E-cadherin is involved in the formation of adherens junctions at points of cell-cell contact, which are regions enriched in proteins such as Lgl, Jam1 (F11r – Mouse Genome Informatics) and Par1 (Mark2 – Mouse Genome Informatics) (Thomas et al., 2004; Vinot et al., 2005; Yamanaka et al., 2003; Yamanaka et al., 2006).

Establishment of apical-basal polarity is thought to permit polarized divisions of blastomeres, whereby daughter cells inheriting the apical pole remain at the embryo surface and apolar progeny become enclosed within the embryo (Johnson and Ziemek, 1983; Johnson and Ziemek, 1981). By the 32-cell stage (E3.0), the outside polar cells strengthen their intercellular adhesion by formation of tight junctions so as to generate an epithelial monolayer, which will form the TE (Fleming et al., 2000). This epithelium ensures a seal that allows the formation of the blastocoele cavity and the generation of a blastocyst. The inside apolar cells, now the ICM, are compressed to one side of the cavity.

Several lineage-specific transcription factors have been identified that play important roles in TE and ICM fate. Expression of the transcription factor Oct4, although ubiquitous early on, becomes restricted to the ICM after E3.5 (Dietrich and Hiragi, 2007; Palmieri et al., 1994). Embryos lacking Pou5f1, which encodes Oct4, generate an ICM that expresses TE markers (Nichols et al., 1998; Ralston et al., 2010). Cdx2 expression, by contrast, becomes restricted exclusively to outside TE progenitors prior to Oct4 restriction at ~E3.5 (Dietrich and Hiragi, 2007; Niwa et al., 2005; Ralston and Rossant, 2008). Loss of Cdx2 leads to the ectopic expression of ICM markers in the TE and an inability to sustain TE development (Strumpf et al., 2005). The early restriction of Cdx2 expression, along with its role in inhibiting the expression of ICM-specific transcription factors in the TE, indicate that Cdx2 is an essential factor for the divergence of TE and ICM lineages. Cdx2 expression itself is regulated by the transcription factor Tead4 and its co-activator partner Yap (Yap1 – Mouse Genome Informatics) (Nishioka et al., 2009; Nishioka et al., 2008; Yagi et al., 2007). Although Tead4 is expressed in all cells of the preimplantation embryo, nuclear Yap is restricted to the developing TE, thus restricting Cdx2 expression to these outside cells. Yap...
nuclear localization has been shown to be regulated by phosphorylation and cytoplasmic sequestration mediated by the Hippo pathway member Lats2 (Nishioka et al., 2009).

The close association between restriction of Cdx2 expression and formation of the outer polarized epithelium raises the question of whether there is a causal link between cell polarity, epithelium formation and TE cell fate. Previous work has demonstrated that E-cadherin is the primary mediator of cell adhesion in the preimplantation embryo and that embryos lacking zygotic E-cadherin are preimplantation lethal (De Vries et al., 2004; Larue et al., 1994; Ohsugi et al., 1997) with failure to form an organized blastocyst. However, some cells in mutants lacking zygotic E-cadherin still express Krt8, which is usually a marker of TE fate (Ohsugi et al., 1997). Other research has demonstrated a link between apical-basal polarization and cell allocation. Notably, disruption of the activity of Par3 and PKCa proteins localized to the apical pole can direct the progeny of early blastomeres preferentially to the inside of the embryo (Pluza et al., 2005). Although fate markers were not examined in these embryos, these observations suggest that polarization is likely to be upstream of lineage-specific transcription factor expression. In Cdx2 mutants, initial polarization occurs but the outer polarized epithelium fails to be maintained, suggesting that Cdx2 is not required to initiate polarity (Strumpf et al., 2005). Although this study found no evidence for a maternal contribution of Cdx2, a recent study suggests that maternal Cdx2 is present in the early embryo. Furthermore, knockdown of maternal Cdx2 suggested that it regulates the expression level of polarity proteins in early blastomeres (Jedrusik et al., 2010). Also, when isolated inside apolar cells repolarize (Eckert et al., 2004; Johnson and Ziomek, 1983) they re-express Cdx2 (Suwinska et al., 2008), again supporting a close correlation between polarity and lineage specification. A recent study suggested that increased expression of Cdx2 could polarize cells and bias them towards TE fate, whereas reduced expression of Cdx2 led to blastomeres being preferentially allocated to the ICM (Jedrusik et al., 2008; Ralston and Rossant, 2008; Strumpf et al., 2005). However, no similar bias was observed when Cdx2 mutant blastomeres were mixed with wild-type cells in chimaeric aggregates (Ralston and Rossant, 2008). It is therefore still unclear whether polarization drives the TE differentiation program or Cdx2 expression drives morphogenetic changes associated with TE fate.

The focus of the current study was to further clarify the relationship between formation of an organized polar epithelium, apical-basal polarity and lineage segregation. We generated mouse embryos by flushing dissected oviducts or uteri with M2 medium (Speciality Media, Chemicon). Embryos and ICMs were cultured in 15 μl drops of KSOM under mineral oil at 37°C in a CO2 incubator for 4 days, if not examined immediately (Nagy et al., 2003).

ICM isolation
Zona pellucidae were removed with acid-Tyrode's solution. Embryos were allowed to recover for 2 hours and were then treated with non-adsorbed rabbit anti-mouse lymphocyte serum (Cedarlane) diluted 1/8 in KSOM (50 μl) for 20 minutes in a CO2 incubator at 37°C. Embryos were washed six times with KSOM and then added to Alexa 488 anti-rabbit antibody for 10 minutes in a CO2 incubator at 37°C. Embryos were washed six times with KSOM and then added to standard guinea pig complement (Cedarlane) diluted 1/4 in KSOM (70 μl) for 8 minutes in a CO2 incubator at 37°C. Finally, embryos were washed six times in M2 medium and dead cells were removed from ICMs by pipetting through a fine pulled glass needle. Efficiently isolated ICMs, i.e. those lacking fluorescence upon examination under a microscope, were then cultured in KSOM to the time points indicated.

Whole-mount immunofluorescence
Embryos were fixed in 4% formaldehyde at room temperature for 30 minutes, washed three times in PBS containing 0.1% Triton X-100, permeabilized for 30 minutes in 0.25% Triton X-100 and then blocked in PBS with 10% foetal calf serum overnight at 4°C. After washing in Na/K ATPase β1 immunohistochemistry, embryos were fixed for 15 minutes in 10% trichloroacetic acid at 4°C. Primary antibodies were diluted in blocking solution and embryos were incubated in this solution overnight at 4°C (19-20 hours). Embryos were then washed three times in PBS and incubated in secondary antibodies diluted 1/400. Primary antibodies were: mouse anti-Cdx2 (1/400, CDX-88, Biogenex), rabbit anti-Cdx2 (1/400) (Chawengsaksophak et al., 1997), rabbit anti-Nanog (1/200, Cosmobio), mouse anti-Oct4 (1/1000, Upstate), goat anti-human GATA6 (1/200, R&D Systems), goat anti-claudin 6 (1/200, Santa Cruz), rat anti-Jam1 (1/400, HyCult), rabbit anti-Lgll (Plant et al., 2003), mouse anti-Na/K ATPase β1 (1/1000, Upstate), goat anti-P-cadherin (1/200, R&D Systems), rabbit anti-PKCa (1/400, Santa Cruz), rat anti-Uvomorulin (E-cadherin) (1/100), rabbit anti-dcx2 was only used for experiments involving co-staining with mouse anti-Oct4, otherwise mouse anti-dcx2 was used. Secondary antibodies included: Alexa 488 goat anti-mouse and anti-rabbit, Alexa 488 donkey anti-goat, Alexa 488 goat anti-mouse and anti-rabbit, and Alexa 647 donkey anti-mouse (all Molecular Probes), Dylight 549 donkey anti-rabbit (Jackson ImmunoResearch) was only used for reactions when embryos were co-stained for Gata6 and Nanog. Nuclei were labeled using either Hoechst 33342 (2.5 μg/ml, Molecular Probes) or Draq5 (1/400, Alexis Biochemicals).

MATERIALS AND METHODS
Mouse lines and genotyping
Cdh1tm2Kem mice (with a floxed allele of Cdh1) (Boussadia et al., 2002) were backcrossed to ICR mice for four generations and intercrossed to generate mice homozygous for the floxed allele. Mice lacking zygotic, maternal or both maternal and zygotic E-cadherin were generated as shown in Fig. S1 in the supplementary material. Additional mice used in this study include wild-type ICR, Tg(Zp3-cre)3Mrt mice carrying the Zp3-cre transgene (Lewandoski et al., 1997) and Cdx2tm1Fbe mice, which carry a null allele of Cdx2 (Chawengsaksophak et al., 1997). Genotyping of mice was performed on DNA extracted from ear punches or blastocysts using the RedExtract-N-Amp Kit (Sigma). DNA preparation for ears was performed as per manufacturer’s specifications. Blastocysts were lysed in 4.4 μl Extraction and 1.1 μl of Tissue Preparation solutions from the RedExtract-N-Amp Kit for 30 minutes at 25°C then 30 minutes at 56°C; then, 4.4 μl Neutralization Buffer was added after incubation at 95°C. To determine the presence of the Zp3-cre transgene, a primer pair specific for cre was used: Zp3-cre F5’-TGGTAGG-GTTCGCAAGAAC-3’ and Zp3-creR 5’-CCATGAGTGAAAGCA-ACTTG-3’. PCRs for the presence of the Cdh1tm2Kem and Cdx2tm1Fbe alleles were performed as described previously (Boussadia et al., 2002; Chawengsaksophak et al., 1997).

Embryo collection and culture
Embryos were collected from timed natural matings. Preimplantation embryos were collected by flushing dissected oviducts or uteri with M2 medium (Speciality Media, Chemicon). Embryos and ICMs were cultured in 15 μl drops of KSOM under mineral oil at 37°C in a CO2 incubator for specified times, if not examined immediately (Nagy et al., 2003).

Whole-mount immunofluorescence
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Confocal microscopy and image analysis
Embryos were mounted in PBS containing 0.1% Triton X-100 in wells of Secure Seal spacers (Molecular Probes) and placed between two cover glasses for imaging. Images were captured using a Zeiss Axiosvert 200 inverted microscope equipped with either a Hamamatsu Orca AG CCD camera and Quorum spinning disk confocal scan head or a Zeiss LSM510 META emission scan head. The spinning disk confocal was driven by Velocity Acquisition software (Perkin Elmer), and the laser-scanning microscope was driven with the LSM510 software package (Zeiss). Images were taken using a 25× or 30× water-immersion objective.

Settings were unchanged within a given session to permit direct comparison of expression levels among embryos stained in the same batch. Image series were taken with 0.5 μm between z-sections. Expression levels were estimated from Cdx2, Oct4 and Yap immunohistochemistry by measuring average pixel intensity and dividing by corresponding values for Hoechst using ImageJ software. Cells were binned as expressing strong, weak or no Cdx2 by eye for half of control embryos (wild type or unmanipulated) and expression levels were recorded for each cell to determine bin ranges. Cells in remaining controls were binned based exclusively on expression levels and bins were validated by visual inspection. Scoring of PKCC and Par6 localization was carried out blind from Cdx2 expression data.

Time-lapse imaging was performed on a Zeiss Axiovert inverted microscope equipped with an environment controller as previously described (Yamanaka et al., 2010). Embryos were placed in a glass-bottom dish (MatTek) in KSOM covered with mineral oil. A 20× dry (NA=0.75) objective lens and Axiocam MRm (1388×1044 pixels) camera were used. Images were taken every 15 minutes for 48 hours.

RESULTS
E-cadherin is not required for TE differentiation
To investigate the roles of compaction and cell adhesion in the generation of the ICM and TE, we used mouse embryos totally deficient in E-cadherin. Crossing a floxed allele of Cdh1 in the Klemier laboratory (Bousadina et al., 2002) with mice expressing oocyte-specific Zp3-cre (Lewandoski et al., 1997) we generated mutants lacking maternal E-cadherin, zygotic E-cadherin or both maternal and zygotic E-cadherin (see Fig. S1 in the supplementary material for crosses and images of embryos). As previously reported, embryos lacking maternal E-cadherin compacted late, but otherwise formed normal blastocysts (De Vries et al., 2004). Embryos lacking zygotic E-cadherin compacted normally, but consisted of loosely associated cells by E3.5, as shown previously (Larue et al., 1994). In the absence of both maternal and zygotic E-cadherin (mz mutants), embryos never compacted but developed as a loose aggregate of cells (see below). Preliminary E-cadherin mutant crosses demonstrated that although most embryos divided normally to E.5 (12/14 wild-type embryos, 30/37 heterozygous embryos, 18/23 maternal mutants, 20/28 zygotic E-cadherin mutants and 21/32 mz E-cadherin mutants showed little cell death and normal cell numbers), some never developed past the 8- to 16-cell stage, before the decision to form TE or ICM (see Table S1 in the supplementary material).

Similarly, a delay of cell division is observed in embryos cultured in the presence of E-cadherin-blocking antibody and in calcium-free medium (Dietrich and Hiiragi, 2007; Ducibella et al., 1975). This indicates that E-cadherin might have a role in early cell division. However, mutant embryos that survived past this stage showed little effect with regard to total cell number (Fig. 1G). Since the focus of our current study is on the role of E-cadherin in cell fate, we excluded developmentally compromised embryos from the experiments described below.

When the zona pellucida was removed from mz mutants, there was still some residual cell adhesion (not shown) even though the absence of E-cadherin was confirmed by immunohistochemistry in confocal sections (see Fig. S2 in the supplementary material). Indeed, we found that cadherin 3 (also known as P-cadherin), which is normally present at cell-cell junctions in wild-type embryos at E3.5, was also present at residual cell-cell junctions in E-cadherin mutants (see Fig. S3A,B,C in the supplementary material). We further found claudin 6, a component of tight junctions, at a few of these residual cell-cell junctions in mz mutants, suggesting that residual cell adhesion in E-cadherin mutants could permit the formation of tight junctions (see Fig. S3D-F in the supplementary material).

To determine the effects of loss of E-cadherin on cell fate, we examined the expression of the TE- and ICM-specific transcription factors Cdx2 and Oct4 in E-cadherin mutant embryos. Expression of Oct4 in E.5 was not fully restricted to the ICM in wild-type embryos by E3.5, we flushed embryos from the uterus at E3.5 and cultured them for one additional day before fixation (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008; Strumpf et al., 2005). Expression of Oct4 and Cdx2 in heterozygous and maternal mutant embryos was virtually indistinguishable from that in wild-type embryos. There was a clear restriction of expression of Oct4 and Cdx2 to the ICM and TE, respectively (Fig. 1A-C). Examination of zygotic and mz mutant embryos revealed that Cdx2 and Oct4 expression was mutually exclusive in most cases, although cells expressing these markers were not necessarily arranged in the usual spatial manner (Fig. 1D,E, arrowheads and arrows, respectively; see also Fig. S4 in the supplementary material). This indicates that cells in E-cadherin mutants can adopt a TE-like fate despite the fact that these embryos formed neither a blastocoel cavity nor organized TE epithelia. Some Cdx2 and Oct4 co-expressing cells persisted in zygotic and mz mutants (Fig. 1D,E, asterisks; see Fig. S4 in the supplementary material). These could represent cells that were developmentally delayed and had not yet resolved expression of these fate markers. When we counted the number of Cdx2-positive cells in embryos of all genotypes, we found a significant increase in the proportion of Cdx2-positive cells in both zygotic and mz mutant embryos in comparison to wild type and heterozygotes (Fig. 1F). In the most extreme cases, almost all of the cells in these embryos were Cdx2 positive (4 of 8 zygotic mutant embryos had greater than 95% Cdx2-positive cells and 5 of 7 mz mutant embryos had over 95% Cdx2-positive cells). Importantly, there was no difference in total cell numbers between embryos of all genotypes (among embryos that developed past the 16-cell stage), indicating that there was unlikely to be a difference in cell proliferation or death (Fig. 1G). Categorization of Cdx2-expressing cells as strongly or weakly positive by quantitation of fluorescence levels revealed that the increased proportion of Cdx2-expressing cells in zygotic and mz mutants was primarily due to an increase in the number of weakly positive cells (Fig. 1F). This suggested that the absence of cell adhesion prevented the downregulation of the early, widespread, low-level expression of Cdx2 observed in normal development (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008).

To confirm that Cdx2-positive cells in these embryos constituted cells initiating a TE fate, we examined the expression of the TE-specific intermediate filament protein Krt8 with respect to Cdx2 (Fig. 1H-J). We scored cells in mutant and wild-type embryos as Krt8- or Cdx2-positive and then overlaid the datasets, counting the number of co-expressing cells. This analysis revealed that expression of Krt8 was tightly linked to Cdx2 in both wild-type and mutant embryos. Only infrequently was Krt8 expressed on its own (Fig. 1K). Notably, the proportion of Krt8 and Cdx2 double-positive cells was significantly greater in mz and zygotic mutant
embryos than in wild-type embryos (Fig. 1K). This suggests that more cells in E-cadherin mutants were initiating TE fate than in wild-type embryos. We also confirmed that when Cdx2-negative cells were present, they were Nanog- or Gata6-positive (see Fig. S5 in the supplementary material, arrows and arrowheads, respectively), confirming that Cdx2-negative cells in mz and zygotic mutants were adopting ICM fates.

Interestingly, many Cdx2-positive cells were found in the interior of mutant embryos. Whereas only 4% of wild-type inner cells were strongly Cdx2 positive, 51% and 44% of zygotic and mz mutant inside cells were strongly Cdx2 positive, respectively. Conversely, whereas Cdx2-negative cells never appeared on the surface of wild-type and heterozygous embryos, they were found in zygotic mutant embryos (6/29 Cdx2-negative cells were outside in 8 embryos) and in mz mutant embryos (3/9 Cdx2-negative cells were outside in 7 embryos). Thus, although compaction and epithelial organization are not absolutely required for the adoption of TE or ICM cell fate, the correct ratio of the two cell types and their correct positioning in the embryo are dependent upon E-cadherin-mediated cell adhesion and the development of a normal outer epithelium.

**Polarization is altered in E-cadherin mutants at E3.5**

Earlier work demonstrated that when E-cadherin-mediated cell adhesion is blocked by antibody treatment, cells in preimplantation embryos can still polarize as judged by concanavalin-A binding to the apical pole (Shirayoshi et al., 1983). However, this analysis did not assess whether basolateral polarization was affected, nor whether E-cadherin function was completely lost. We therefore examined cell polarity in E-cadherin mutant embryos using current molecular markers of apical and basolateral polarity. Specifically, we examined the distribution of the apical protein PKCζ and the basolateral proteins Na/K ATPase β1, Jam1 and Lgl1 in E3.5 (blasto cyst stage) embryos. In wild-type and heterozygous embryos, PKCζ was strongly enriched in the apical membrane domain of TE cells, whereas ICM cells showed a cytoplasmic distribution with weak
membrane enrichment (Fig. 2A), similar to previous reports (Eckert et al., 2004; Plusa et al., 2005; Ralston and Rossant, 2008). The Na/K ATPase β1 subunit and Jam1 were localized to cell-cell contacts of the ICM and were absent from the apical surface of TE (Fig. 2A; see Fig. S6A in the supplementary material), as previously reported (Madan et al., 2007; Thomas et al., 2004). Similarly, we found that Lgl1, another protein localized to the basolateral membrane in other epithelia, was associated specifically with the basolateral domain in the blastocyst TE (see Fig. S6B in the supplementary material) (Hutterer et al., 2004; Yamanaka et al., 2003; Yamanaka et al., 2006).

In both zygotic and mz E-cadherin mutants, PKCζ was strongly enriched in the membrane and lost from the cytoplasm of the majority of cells, similar to what is observed in wild-type TE cells (Fig. 2B,C). In contrast to wild-type TE cells, however, PKCζ was enriched throughout the majority of the outer cell membrane in zygotic and mz mutant cells, rather than in a specific apical domain. The Na/K ATPase β1 subunit, Jam1 and Lgl1 were still found at the surface in mutant cells; however, their localization overlapped with membrane-enriched PKCζ (Fig. 2B’,B”,C’,C”). Strikingly, these basolateral markers also accumulated in either small puncta or large vacuole-like structures within the cell (Fig. 2D). When these markers were trafficked to the cell surface they tended to colocalize with PKCζ, suggesting that sorting of apical and basolateral proteins could be disrupted in E-cadherin mutants.

We found a significant increase in the proportion of cells with membrane enrichment of the apical protein PKCζ and a paucity of PKCζ in the cytoplasm in E-cadherin mutants. In wild-type embryos, 64% of cells had membrane-enriched PKCζ whereas 87% of zygotic mutant cells and 88% of mz mutant cells had membrane-enriched PKCζ (Student’s t-test, P<0.01; n=7, 8 and 7 embryos, respectively; see Fig. S7A in the supplementary material). We then scored cells as Cdx2 positive or negative and overlaid these data with those for PKCζ expression. Cells with membrane-enriched PKCζ of wild-type, zygotic mutant and mz mutant embryos were almost all Cdx2 positive (99%, 98% and 99%, respectively). Some cells
lacking PKCζ enrichment also expressed Cdx2 (16% of wild-type, 67% of zygotic mutant and 89% of mz mutant apolar cells). However, examination of the intensity of Cdx2 expression demonstrated that Cdx2 expression levels were significantly higher in cells with membrane-enriched PKCζ in comparison to those without in wild-type, zygotic mutant and mz mutant embryos (Fig. 2E). Given that Cdx2-expressing cells are still observed in E-cadherin mutants, the formation of a restricted apical domain per se is not necessary for Cdx2 expression. However, apical membrane specification itself could still be important for TE specification, as expression of apically localized markers and Cdx2 expression remain tightly linked in E-cadherin mutants.

Examination of Yap localization in cells with and without membrane-enriched PKCζ in E-cadherin mutants revealed that Yap was localized to the nuclei of cells with membrane-enriched PKCζ and to the cytoplasm of cells without membrane-enriched PKCζ, independent of their spatial distribution in the embryo (Fig. 3B’,C’,D,E). Strikingly, Yap was enriched in the cytoplasm of cells lacking membrane-enriched PKCζ even when they were found on the surface of E-cadherin mutant embryos (Fig. 3B’,C’, arrows). This indicates that localization of apical markers, and not just cell position, is important for Yap nuclear localization. There was also a greater number of cells with nuclear localized Yap in E-cadherin mutants (62% of cells in wild-type embryos had nuclear localized Yap, in comparison to 87% and 82% for zygotic and mz mutants, respectively; see Fig. S7B in the supplementary material). Thus, increased Yap activity could account for the increased proportion of Cdx2-positive cells in these embryos.

**Polarization anticipates Cdx2 expression and upregulation in isolated ICMs**

Clearly, intracellular polarization can occur in the absence of E-cadherin, but the normal segregation of the apical and basolateral domains is disrupted. Apical membrane marker localization is associated with strong Cdx2 expression, suggesting that apical polarity might drive upregulation of Cdx2. To test this further we examined whether polarization precedes Cdx2 upregulation in other contexts. Recent work from our laboratory has demonstrated that polarization of early blastomeres precedes upregulation of Cdx2 in undisturbed embryos (Ralston and Rossant, 2008). We tested whether the same was true in a situation in which de novo regeneration of TE occurs. When ICMs are immunosurgically isolated from early blastocysts, they normally regenerate a TE layer on their surface after culture (Hogan and Tilly, 1978; Spindle, 1978). It was also recently demonstrated that cells on the surface of aggregates formed from inner cells isolated from 16- and 32-cell embryos can initiate Cdx2 expression and generate an apparently morphologically normal TE (Suwinska et al., 2008).

To examine the relationship between polarization and Cdx2 expression in this context, we isolated ICMs at the ~32-cell stage, when embryos had either no blastocoel or the cavity constituted less than half the total embryo volume (late morula stage or early blastocyst stage). Isolation and screening for efficiently isolated ICMs took ~1 hour. ICMs were fixed immediately after isolation and testing whether the same was true in a situation in which de novo regeneration of TE occurs. When ICMs are immunosurgically isolated from early blastocysts, they normally regenerate a TE layer on their surface after culture (Hogan and Tilly, 1978; Spindle, 1978). It was also recently demonstrated that cells on the surface of aggregates formed from inner cells isolated from 16- and 32-cell embryos can initiate Cdx2 expression and generate an apparently morphologically normal TE (Suwinska et al., 2008).

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hours in culture, we found that mutant ICMs lacking Cdx2 expression regenerated a cell layer on their surface that resembled TE (77% of all mutants compared with 79% of wild-type ICMs and 93% of heterozygous ICMs; n=13, 14 and 14 ICMs for each genotype, respectively). This cell layer was polarized as judged by PKCζ localization and was negative for the primitive endoderm marker Gata6 (Fig. 5). In some cases, embryos even generated blastocoels (54% of all mutants formed at least a small cavity, compared with 57% and 79% of all wild-type and heterozygous ICMs, respectively; Fig. 5). This result demonstrates that Cdx2 expression is not required for polarization of ICM cells, nor for regeneration of a morphologically normal TE-like epithelial layer.

**DISCUSSION**

We have investigated in detail the role that E-cadherin-mediated cell adhesion plays in the restriction of cell fate in preimplantation mouse embryos. We demonstrate that loss of both maternal and zygotic E-cadherin leads to both the absence of normal segregation of apical and basolateral domains and a failure to generate a normal, organized epithelium at the blastocyst stage. However, cells in mutant embryos can still initiate TE and ICM fate, although the normal ratio of these cell types is disturbed. In E-cadherin mutants more cells express Cdx2 than in wild-type embryos, suggesting that E-cadherin-mediated cell adhesion is important for the restriction of Cdx2 expression and TE fate. Moreover, the normal spatial distribution of TE- and ICM-like cells to the outside and the inside of the blastocyst, respectively, did not occur. This result extends an earlier study demonstrating that delaying blastocyst formation by temporarily blocking E-cadherin activity with antibodies biases cells in blastocysts towards TE fate (Shirayoshi et al., 1983).

Lats kinase is required to repress the expression of Cdx2 via phosphorylation and nuclear exclusion of Yap (Nishioka et al., 2009) in the preimplantation embryo. Lats kinase is activated by upstream Hippo signaling in mammalian cells and Hippo signaling itself is dependent upon cell-cell contact (Zhao et al., 2007). Thus, lack of E-cadherin-mediated cell contact could lead to reduced Hippo signaling and account for the increased proportion of Cdx2-positive cells compared with wild-type embryos. Consistent with this, we observed nuclear localization of Yap in Cdx2-positive cells throughout E-cadherin mutant embryos, suggestive of reduced Hippo signaling. However, in the few remaining apolar cells observed, Yap was enriched in the cytoplasm, even when these cells were found on the surface of the embryo. This indicates that apical membrane polarization, and not just reduced cell contact, is important for repression of Hippo signaling.

The polarization hypothesis states that outer cells that polarize de novo or inherit an apical domain through cell division will become TE, whereas inside apolar cells occupy the ICM (Johnson and McConnell, 2004; Johnson and Ziemek, 1981; Yamanaka et al., 2006). Previous work has shown that early blastomers can polarize in the absence of E-cadherin-mediated cell adhesion (Shirayoshi et al., 1983; Ziemek and Johnson, 1980). We confirmed in this study that, even in the complete absence of E-cadherin, cells still express apical markers on their surface, although they were more broadly distributed across the cell surface than in wild-type TE. We also found that although basolateral markers could accumulate on the cell surface, their distribution partially overlapped with that of apical markers. This suggests a defect in the basolateral sorting machinery. Indeed, interference with the transport of basolateral proteins by disrupting clathrin-dependent traffic can lead to an accumulation of basolateral proteins.
By the blastocyst stage of development, we can detect no evidence demonstrating that polarity reinitiation is also independent of Cdx2. -null ICMs also regenerate a polarized epithelium, embryos (Ralston and Rossant, 2008). Importantly, we found that observations that polarization anticipates Cdx2 expression in intact Cdx2 expression in many cells. This is consistent with previous Spindle, 1978). We found that during TE regeneration, polarization (Handyside, 1978; Hogan and Tilly, 1978; Rossant and Lis, 1979; isolated early ICMs are cultured, they regenerate an outer TE layer process of regenerating a TE layer. Studies have shown that when and Cdx2 expression, we examined isolated ICMs during the area of the apical domain on the cell surface and for the restricted results confirm that E-cadherin is not required for apical membrane target proteins to the cell membrane (Shaw et al., 2007). These cadherins are important for the delivery of basolaterally targeted vesicles that failed to dock on the plasma membrane (Fig. 2D) (Grosshans et al., 2006; Zhang et al., 2005). Further support for this idea comes from a recent study showing that caderins are important for the delivery of basolaterally targeted proteins to the cell membrane (Shaw et al., 2007). These results confirm that E-cadherin is not required for apical membrane domain formation during polarization but is essential to restrict the area of the apical domain on the cell surface and for the restricted accumulation of basolateral proteins within the plasma membrane.

To gain further insight into the relationship between polarization and Cdx2 expression, we examined isolated ICMs during the process of regenerating a TE layer. Studies have shown that when isolated early ICMs are cultured, they regenerate an outer TE layer (Handyside, 1978; Hogan and Tilly, 1978; Rossant and Lis, 1979; Spindle, 1978). We found that during TE regeneration, polarization is strongly linked with Cdx2 expression and polarization precedes Cdx2 expression in many cells. This is consistent with previous observations that polarization anticipates Cdx2 expression in intact embryos (Ralston and Rossant, 2008). Importantly, we found that Cdx2-null ICMs also regenerate a polarized epithelium, demonstrating that polarity reinitiation is also independent of Cdx2. By the blastocyst stage of development, we can detect no evidence of maternal Cdx2 protein in Cdx2 mutant embryos (Strumpf et al., 2005), and yet ICMs from these stages are fully capable of reforming a polarized outside epithelium. Cdx2 mutant embryos do show subsequent breakdown of the integrity of the outer TE epithelium, suggesting that Cdx2 plays a role in the ongoing maintenance of polarity (Strumpf et al., 2005). This latter finding might help explain data in a recent publication that supports a more direct role for Cdx2 in promoting polarization (Jedrusik et al., 2008). Notably, although previous studies found no sign of Cdx2 protein before the 8-cell stage (Jedrusik et al., 2008; Strumpf et al., 2005), a recent study has suggested that a faint amount of maternally loaded transcript in this study implicated maternally derived Cdx2 protein in cell survival, polarization and even TE lineage specification (Jedrusik et al., 2010). Based on these results it would be informative to examine maternal Cdx2-null embryos for similar defects. Interestingly, TE regeneration is only found in ICMs isolated from early blastocysts, a time at which Cdx2 expression is still apparent at low levels within the ICM (Dietrich and Hiiiragi, 2007; Ralston and Rossant, 2008); if ICMs are isolated from later blastocysts, a primitive endoderm layer is generated on their surface, rather than a TE layer (Handyside, 1978; Hogan and Tilly, 1978; Spindle, 1978). Since most Cdx2-null ICMs still regenerate a TE-like epithelium, we conclude that although Cdx2 expression is required to maintain TE, it is not the sole factor determining whether ICM cells retain the ability to regenerate TE.

The results of this study demonstrate that cells of the preimplantation embryo can initiate TE fate in the absence of epithelium formation and E-cadherin-mediated cell adhesion. They also demonstrate that these processes are important for the restriction of apical membrane formation and for the correct segregation of TE and ICM cell fates. Finally, these results provide support for a role for apical membrane polarization in the upregulation and/or maintenance of Cdx2 expression. Although Cdx2 expression can initiate prior to polarization (Dietrich and Hiiiragi, 2007; Ralston and Rossant, 2008), maintenance and upregulation of expression appear to occur only in polarized cells. Future work will need to address how apical membrane polarization acts to facilitate Cdx2 expression; for example, whether it facilitates/represses signaling involved in Cdx2 expression, or whether it is involved in the segregation of a determinant required for expression.

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