Downregulation of Par3 and aPKC function directs cells towards the ICM in the preimplantation mouse embryo

Berenika Plusa[1,3,*,‡], Stephen Frankenberg[1,3,*], Andrew Chalmers[1,5], Anna-Katerina Hadjantonakis[4,§], Catherine A. Moore[1,3], Nancy Papalopulu[1,5], Virginia E. Papaioannou[4], David M. Glover[2,3] and Magdalena Zernicka-Goetz[1,3,¶]

[1]Wellcome Trust and Cancer Research UK Gurdon Institute, Tennis Court Road, Cambridge CB2 1QR, UK
[2]Cancer Research UK Cell Cycle Genetics Research Group and [3]Department of Genetics, Downing Street, Cambridge CB2 3EH, UK
[4]Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, 701 West 168th Street, New York, NY 10032, USA
[5]Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK

*These authors contributed equally to this work
‡On leave from the Department of Experimental Embryology, Polish Academy of Science, Jastrzebiec, 05-552, Poland
§Present address: Developmental Biology Program, Sloan-Kettering Institute, 1275 York Avenue, New York, NY 10021, USA
¶Author for correspondence (e-mail: mzg@mole.bio.cam.ac.uk)

Summary

Generation of inside cells that develop into inner cell mass (ICM) and outside cells that develop into trophectoderm is central to the development of the early mouse embryo. Critical to this decision is the development of cell polarity and the associated asymmetric (differentiative) divisions of the 8-cell-stage blastomeres. The underlying molecular mechanisms for these events are not understood. As the Par3/aPKC complex has a role in establishing cellular polarity and division orientation in other systems, we explored its potential function in the developing mouse embryo. We show that both Par3 and aPKC adopt a polarized localization from the 8-cell stage onwards and that manipulating their function re-directs cell positioning and consequently influences cell fate. Injection of dsRNA against *Par3* or mRNA for a dominant negative form of aPKC into a random blastomere at the 4-cell stage directs progeny of the injected cell into the inside part of the embryo. This appears to result from both an increased frequency by which such cells undertake differentiative divisions and their decreased probability of retaining outside positions. Thus, the natural spatial allocation of blastomere progeny can be over-ridden by downregulation of Par3 or aPKC, leading to a deceased tendency for them to remain outside and so develop into trophectoderm. In addition, this experimental approach illustrates a powerful means of manipulating gene expression in a specific clonal population of cells in the preimplantation embryo.

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Introduction

The two most recognizable outcomes of blastocyst formation in the mammalian embryo are differentiation of the first two cell lineages – the inner cell mass (ICM) and its outer epithelial-like coating, the trophectoderm – and establishment of the embryonic-abembryonic axis. These two processes are interconnected because trophectoderm differentiation leads to formation of the asymmetrically positioned blastocyst cavity. Thus, the trophectoderm layer envelopes the cavity in the abembryonic part of the blastocyst and the ICM in the embryonic part. Blastocyst cavity formation is the first physical manifestation of the embryonic-abembryonic axis. The first fate decision of a blastomere, whether it will become ICM or trophectoderm, appears to be specified by its position. Thus, after experimentally changing the relative position of cells in the early embryo by removal, addition or re-arrangement, their developmental fate adjusts to the new position (Tarkowski and

Wroblewska, 1967; Kelly, 1977; Hillman et al., 1972). Only the inside cells developing into ICM will form the future embryo proper, whereas the trophectoderm cells will form only extraembryonic tissues.

Numerous studies have suggested that trophectoderm differentiation requires cell polarity to be established in the outer cells of the morula (reviewed by Johnson and McConnell, 2004). Such apical-basal cell polarity develops progressively, as indicated by the distribution of a variety of cellular markers including endocytotic vesicles (Fleming and Pickering, 1985; Maro et al., 1985; Reeve, 1981), beta-catenin (Goval et al., 2000), ezrin (Louvet et al., 1996) and the localization of microvilli to outer apical surfaces (Handyside, 1980; Johnson and Ziomek, 1983). The cell-cell adhesion molecule E-cadherin is also first uniformly distributed on all cell surfaces until the early 8-cell stage. Then, after the process known as compaction, E-cadherin becomes restricted to regions of cell-

cell contact (Vestweber et al., 1987). The mechanisms that drive polarization of blastomeres in the 8-cell-stage mouse embryo are unknown, but phosphorylation changes in E-cadherin, catenins and ezrin suggest an involvement of kinases and phosphatases.

When the mouse embryo develops from the 8-cell to 16-cell stage, two types of cell division occur. Conservative divisions with the cleavage plane approximately perpendicular to the surface of the embryo result in both progeny inheriting apical and basal regions of cytoplasm. Thus, both daughter cells are polar and retain an outer apical surface. By contrast, division planes aligned approximately tangential to the surface of the embryo are differentiative and result in two types of daughter cell: one inheriting all of the apical surface and some inner surfaces, and one inheriting only inner surfaces. Consequently, one daughter cell retains an apical outer surface and is polar, whereas the other remains internal and non-polar. During the fifth division (16-cell to 32-cell stage), each outer cell again divides with its division plane either perpendicular or tangential to the cell surface, respectively, giving rise to either a pair of two outer polar cells or one outer polar and one inner non-polar cell (reviewed by Johnson et al., 1986; Fleming, 1987). By the end of the fifth cell cycle, outer cells have matured to functional trophectoderm epithelial cells and initiated Na^+/K^+-channel-mediated active transport (reviewed by Watson and Barcroft, 2001). Internal fluid accumulation results in formation of the blastocyst cavity, which forms asymmetrically and defines the orientation of the embryonic-abembryonic axis of the blastocyst.

In many model organisms, the establishment of cellular polarity cell division is regulated by the two Par (partitioning defective) molecules Par3 and Par6 that are known to form a complex with atypical protein kinase C (aPKC) (reviewed by Ahringer, 2003; Macara, 2004; Suzuki et al., 2003). As cell polarity is believed to play an important role in orientating cell divisions and therefore in establishing the ICM and trophectoderm lineages, we have examined the roles of two members of this complex, Par3 and aPKC, in allocating cells to either of these lineages. Here, we show that, when Par3 expression is downregulated by double-stranded RNA interference (dsRNAi), or when mRNA for a dominant negative form of aPKC is injected into random 4-cell blastomeres, such cells contribute an increased proportion of their progeny to the ICM. This results from an increased frequency by which such cells undertake differentiative divisions and in their marked tendency to become internalized.

Materials and Methods

Embryo collection and culture

Embryos were collected from F1 (C57BL/6×CBA) females. Females were superovulated by injection of 10 IU of pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet), followed by injection of 10 IU of human chorionic gonadotropin (hCG; Chorulon, Intervet) 48-56 hours later. Females were then mated with F1 males or transgenic histone H2B GFP males (Hadjantonakis and Papaioannou, 2004). Embryos were collected in M2 medium and cultured in drops of KSOM supplemented with amino acids (Speciality Media) and 4 mg/ml BSA, under paraffin oil in an atmosphere of 5% CO_2 in air at 37.5°C.

Immunolocalization of Par3 and aPKC in preimplantation embryos

Early and mid-8-cell-stage embryos were obtained by collecting 4-cell embryos 56-61 hours after hCG injection and culturing either for a further 0-1 hours or for 4-7 hours, respectively, after division to the 8-cell stage. Alternatively, embryos were collected at 60 hours or 66 hours, respectively, after hCG. Late 8-cell-stage or early 16-cell-stage embryos were obtained by collecting embryos 72-76 hours after hCG injection and fixing immediately after collection. Late morulae (>16 cells) were collected 80 hours after hCG injection. Blastocysts were collected 84-98 hours after hCG injection and fixed immediately.

Two methods of fixation were used. In the first, embryos were washed in PBS and placed on cover slips coated with concanavalin A (0.1 mg/ml), followed by fixation in methanol at –20°C for 20 minutes. In the second method, embryos were washed in PBS followed by fixation in 4% paraformaldehyde (PFA) in PBS with 0.15% glutaraldehyde and 0.07% Triton X-100. Fixation by either method was followed by three washes in PBS and 20 minutes of permeabilization with 0.25% Triton X-100 in PBS. Immunostaining was as described previously (Plusa et al., 2002). For detection of Par3, rabbit-anti-Par3 antibody (Lin et al., 2000) was used at a dilution of 1:200 and rhodamine-conjugated secondary anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:300. For detection of aPKC, rabbit- or goat-anti-aPKC antibody (specific for both aPKCλ and aPKCξ; Santa Cruz; lot no. sc-216g/r) was used at a dilution of 1:200 and, where appropriate, rhodamine-conjugated secondary anti-rabbit antibody or FITC-conjugated secondary anti-goat antibody used at a dilution of 1:300. DNA was visualized using TOTO-3 (Molecular Probes) at a concentration of 2-5 μM. Prior to examination, embryos attached to cover slips were washed with PBS and mounted on slides using Citifluor (Chem. Lab.)

Immunolocalization of aPKC, ZO1 and occludin in experimental embryos

Experimental embryos were washed in PBS followed by fixation in 4% (Par3 and aPKC) or 2.5% (ZO1 and occludin) PFA in PBS. Fixation was followed by three washes in PBS and 20 minutes of permeabilization with 0.25% Triton X-100 in PBS. Primary antibodies for Par3 and aPKC and all incubation times were as described above. For detection of ZO1, rabbit anti-ZO1 antibody (a gift from T. Fleming) was used at a dilution of 1:250; for detection of occludin, rabbit anti-occludin antibody (Zymed Laboratories) was used at a dilution of 1:300. The secondary antibody for all immunostainings was FITC-conjugated secondary anti-rabbit or anti-goat antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:300. Further processing was as described above.

Preparation of dsRNA and synthetic mRNA.

Templates used for RNA synthesis were amplified by PCR. pβGFP/RN3P plasmid (Zernicka-Goetz et al., 1997) was used to synthesize template for double-stranded *GFP* RNA (ds*GFP* RNA), using the following primers: 5′-AAATATAATACGACTCACTATA-GGGAGAGTGAAGGT-3′ and 5′-AAATATAATACGACTCACTAT-AGGGAGATCGTTGGG-3′. A pcDNA3 plasmid containing *Par3* (accession number AY026057; a gift from D. Lin) was used to synthesize template for dsRNA for *Par3* (ds*Par3* RNA), using the following primers: 5′-TAATACGACTCACTATAGGGCGAAGAA-AGTAGGCAAGAGGC-3′ and 5′-TAATACGACTCACTATAGGGG-TCATTTACCCTCAGCCTTCC-3′. All primers contained a core promoter sequence for T7 RNA polymerase. The PCR product migrated as a band of approximately 600 bp. RNA was synthesized using a Ribomay Large Scale RNA Transcription kit (Promega), purified and annealed as previously described (Wianny and Zernicka-Goetz, 2000).

Preparation of synthetic mRNA

For preparation of template for DsRed-Express mRNA, the coding region of DsRed-Express was excised using *Bam*HI and *Not*I from pDsRed-Express (Clontech) and cloned into the *Bam*HI and *Not*I sites of pRN3P vector. The template for wild-type PKCλ (*Prkci*) was a full-length His-tagged mouse PKCλ construct (Nakaya et al., 2000). The kinase-dead (K273E), dominant negative version of PKCλ was made from the full-length His-tagged mouse PKCλ construct using QuickChange Site-Directed mutagenesis kit (Stratagene) and is identical to that used previously (Akimoto et al., 1996; Nakaya et al., 2000). Capped RNA was transcribed in vitro. In brief, plasmid DNA was first linearized with *Sfi*I (DsRed-Express template) or *Sma*I (aPKC templates) and in vitro transcription performed with T3 RNA polymerase using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. Following the transcription reaction, unincorporated nucleotides were removed using MicroSpin S-200 Columns (Amersham Biosciences) and the mRNA resuspended in RNase-free water at the required concentration.

RT-PCR method

Following injection of dsRNA into zygotes and culture overnight, RT-PCR was performed on 10 pooled 2-cell embryos using SuperScript One-Step RT-PCR kit with platinum Taq (Invitrogen, catalogue number 10928-034) and 35 cycles of PCR. Reaction buffer was added to frozen embryos then divided between two tubes containing different primer mixes. β-actin primers: 5′-GAAGTGTGACGTTGA-CATCCG-3′ and 5′-ACTTGCGGTGCACGATGGAGG-3′, accession number X03672, fragment amplified=929-1200. PAR-3 primers: 5′-CGGTAAGGCTGAGCAAGAAAACC-3′ and 5′-TGAGGCGTGAG-CACTATGAGGTAG-3′, accession number AY026057, fragment amplified=1036-1393.

Injection of individual blastomeres

Late 4-cell-stage embryos were obtained by collection 56-61 hours after hCG injection, and placed in M2 medium in a manipulation chamber. Early 8-cell-stage embryos collected at this time were also used.

To examine the potential effect on cell fate in developing embryos, one randomly chosen blastomere at the 4-cell or 8-cell stage was injected. ds*Par3* RNA or ds*GFP* RNA, and dominant negative *aPKCλ* or wild-type *aPKCλ* mRNA were diluted in RNase-free water and mixed with rhodamine-dextran or DsRed prior to injection. The following final concentrations of different mixtures were used for injection of experimental groups: 0.8 mg/ml of ds*Par3* RNA and 0.4 mg/ml rhodamine-dextran; 0.8 mg/ml of ds*Par3* RNA and 0.1 mg/ml DsRed mRNA; 0.45 mg/ml of wild-type *aPKC* mRNA and 0.1 mg/ml of DsRed mRNA; 0.45 mg/ml of dominant negative *aPKC* and 0.1 mg/ml of DsRed. The following final concentrations of different mixtures were used for injection of control groups: 0.8 mg/ml of ds*GFP* RNA and 0.4 mg/ml rhodamine-dextran; 0.8 mg/ml of ds*GFP* RNA and 0.1 mg/ml DsRed mRNA. Microinjections were performed as described previously (Zernicka-Goetz et al., 1997) on a Leica inverted microscope using DIC optics. [In some experiments, dsRNA was synthesized from EGFP and in others against identical sequences in the MmGFP plasmid (Zernicka-Goetz et al., 1997). They are equally effective in RNAi and are referred to generically as GFP in the main text.]

We found that DsRed was generally a better marker then rhodamine-dextran. DsRed gave stronger signals 24 hours after injecting its synthetic mRNA, making it easier to identify the borders of cells. However, residual RNAase activity in preparations of dsRNA led to degradation of synthetic mRNA. Thus, in experiments analysing the effects of injection of ds*Par3* RNA, rhodamine-dextran was added as a lineage marker.

Both experimental and control embryos were cultured until appearance of a blastocyst cavity and then observed under an inverted confocal microscope. Sections were recorded every 0.4 μm. Data were processed using the Huygens deconvolution system.

Data analysis on cell fate experiments

Data were analysed using IMARIS software with 3D options to allow precise localization of cells in the blastocyst. All cells in each blastocyst were identified and scored as ICM or trophectoderm depending on whether any part of the cell surface formed part of the conceptus surface. Trophectoderm cells were scored as mural if they formed any part of the lining of the blastocyst cavity. Progeny of injected cells were distinguished by the presence of rhodamine-dextran or DsRed fluorescence. The presence of wild-type and dominant negative aPKC in injected cells was confirmed by fixing blastocysts after confocal analysis in 4% PFA and processing as described above for immunolocalization.

Time-lapse microscopy

Injection of 4-cell embryos was as described above. After 21 hours of culture, embryos were briefly checked for fluorescence by confocal microscopy. Positive embryos were then cultured on a Zeiss Axiovert 200M inverted microscope in KSOM at 37.5°C and 5% CO_2 in air. Bright field and fluorescent images were captured using an ORCA Hamamatsu Orca ER CCD camera and AQM Advance 6 image software package (Kinetic Imaging) at five focal planes, each separated by approximately 10 μm, every 10 minutes for 20 hours. Time-lapse series were then analysed using Volocity software (Improvision).

Results

Par3 and aPKC become progressively localized to the apical plasma membrane and tight junctions during preimplantation development

As the Par complexes are known to become localized to specific regions of several polarized cell types, we wished to determine whether this also happens in the preimplantation mouse embryo since polarized cells develop during the process of compaction. To this end, we chose to examine the subcellular localization of Par3 and aPKC, two members of one of the Par complexes (Izumi et al., 1998; Lin et al., 2000). We found that, from the 8-cell stage, Par3 localized to parts of the apical surfaces of blastomeres (Fig. 1A). At the 16-cell stage, it extended towards lateral surfaces (not shown); by the blastocyst stage, it was more concentrated at apico-lateral surfaces and intercellular contacts, giving punctate staining suggestive of localization to tight junctions (note differences in staining of individual cells in optical sections of embryo in Fig. 1B-D). Immunolocalization of aPKC revealed that it was also diffusely distributed throughout the cell in addition to being concentrated at the cell membrane both in regions of cell-cell contact and on outward-facing surfaces of the 8-cell blastomeres (Fig. 1E). As previously described, the enzyme was also present at higher levels in the nucleus (Pauken and Capco, 2000). In the mid-8-cell to 16-cell stages, aPKC localization extended to the apico-lateral surfaces (Fig. 1F). In blastocysts, it was localized to the apical surface of trophectoderm cells, and more densely to the apico-lateral borders at presumed sites of tight-junction formation (Fig. 1G, arrowhead). It was sparser at regions of inner cell-cell contact and absent from basal surfaces of trophectoderm, although present at surfaces of ICM cells facing the blastocyst cavity.

Fig. 1. Localization of Par3 (A-D) and aPKC (E-H) in preimplantation mouse embryos. (A) Par3 (red) localizes to apical cell surfaces (arrow) at the 8-cell stage. (B-D) Three optical sections of the same blastocyst at 5 µm, 24 µm and 42 µm showing concentration of Par3 at apico-lateral surfaces of cells (arrowheads). DNA is stained blue. (E,F) aPKC is concentrated at apical cell surfaces (arrow on this glancing optical section) and nuclei (arrowhead) at the 8-cell stage. (G,H) Confocal sections of the same blastocyst at 14 µm and 26 µm showing concentration at outer apico-lateral cell borders (arrowheads). The inset to panel H shows a mitotic spindle in a dividing blastomere revealed by staining of aPKC (red), Par3 (green) and DNA (blue). Bar, 10 µm.
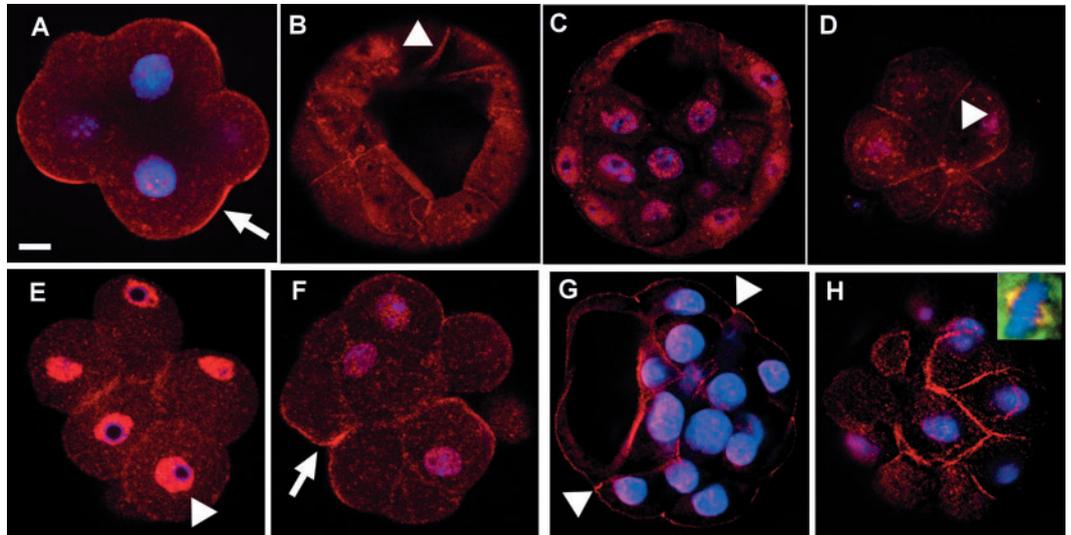
We also found some aPKC and Par3 associated with the mitotic spindle in these cells (inset to Fig. 1H). Thus, by the morula-blastocyst stages, Par3 and aPKC have very similar patterns of subcellular localization consistent with both the previously observed polar nature of 16-cell-stage blastomeres and the epithelial-like nature of trophectoderm cells at the blastocyst stage.

Par3 RNAi and dominant negative aPKC influence cell positioning

To test the role of Par3 and aPKC in the first cell fate decisions of the mouse embryo, our strategy involved downregulating Par3 by RNAi or expression of a dominant negative form of aPKC in a randomly targeted 4-cell-stage blastomere. We combined this with lineage tracing to monitor the allocation of the progeny of injected cells within the blastocyst. For all experiments, we used a transgenic mouse line expressing GFP fused with histone H2B to facilitate accurate scoring of all cells and their positions within the newly formed blastocyst (Hadjantonakis and Papaioannou, 2004).

As we wished to use RNAi to downregulate *Par3*, we first carried out control experiments in order to show that dsRNA against *Par3* was indeed effective in reducing *Par3* transcripts (Fig. S1A, in supplementary material). As a control for the spatial elimination of a gene product irrelevant for development, we injected single blastomeres of a group of the transgenic GFP-histone H2B embryos with dsRNA for *GFP* and mRNA for DsRed at the 4-cell stage. Later examination of such embryos by fluorescence microscopy showed that this treatment was effective in reducing levels of GFP-histone fluorescence in the progeny of the injected cells (Fig. S1B-E, in supplementary material).

To investigate the effects of downregulating *Par3* in individual 4-cell-stage blastomeres, we co-injected dsRNA for *Par3* and rhodamine-dextran as a lineage tracer into such cells, cultured the embryos to the blastocyst stage and scored the labelled progeny as occupying either the ICM or trophectoderm. In this group of embryos and a group injected with ds*GFP* RNA, the mean proportion of labelled cells in the blastocyst was similar (t=1.2, P>0.1), indicating that ds*Par3* RNA had no effect on cell division compared with this control. However, the contribution to ICM of labelled cells was significantly increased in embryos injected with ds*Par3* RNA. This was apparent from optical sectioning of such embryos that showed the clone derived from the injected cell marked in red occupied a much greater proportion of the ICM than in control embryos (Figs 2, 3). Whereas in control embryos the mean proportions of labelled ICM cells to total labelled cells was 0.34 for DsRed-injected embryos (*n*=38), in embryos injected with ds*Par3* RNA this proportion increased to 0.53 (*n*=16; t=5.2, P<0.01; Fig. 2; Table 1; Tables S1-S6, in supplementary material). Thus, inhibiting Par3 function had biased cells towards becoming ICM.

In the second series of experiments, we injected mRNA for a dominant negative form of aPKC together with mRNA for DsRed to one blastomere of the 4-cell-stage embryo. One series of control embryos was injected with mRNA for DsRed alone and another with a mixture of mRNA for wild-type aPKC and DsRed (Fig. S2, in supplementary material). When the mean relative contributions of labelled cells to the blastocyst were calculated, they were similar in all groups to the expected proportion of 25%, indicating that none of the injections had any detectable effect on division rate (Fig. 3). Optical sectioning of embryos again indicated a greater contribution of labelled cells to the ICM in the dominant negative aPKC-injected group, compared with control embryos (Fig. 2). The mean proportions of labelled ICM cells to total labelled cells increased from 0.33 for DsRed-injected embryos (see above) to 0.71 (*n*=27; t=8.0, P<0.01) for dominant negative aPKC-injected embryos (Fig. 2; Table 1). Thus, interfering with both Par3 and aPKC biased the progeny of treated cells towards becoming ICM. The effect of dominant negative aPKC injection was stronger than that observed for ds*Par3* interference and reduced the proportion of the clone derived from the treated cell that contributed to trophectoderm (Fig. 3).
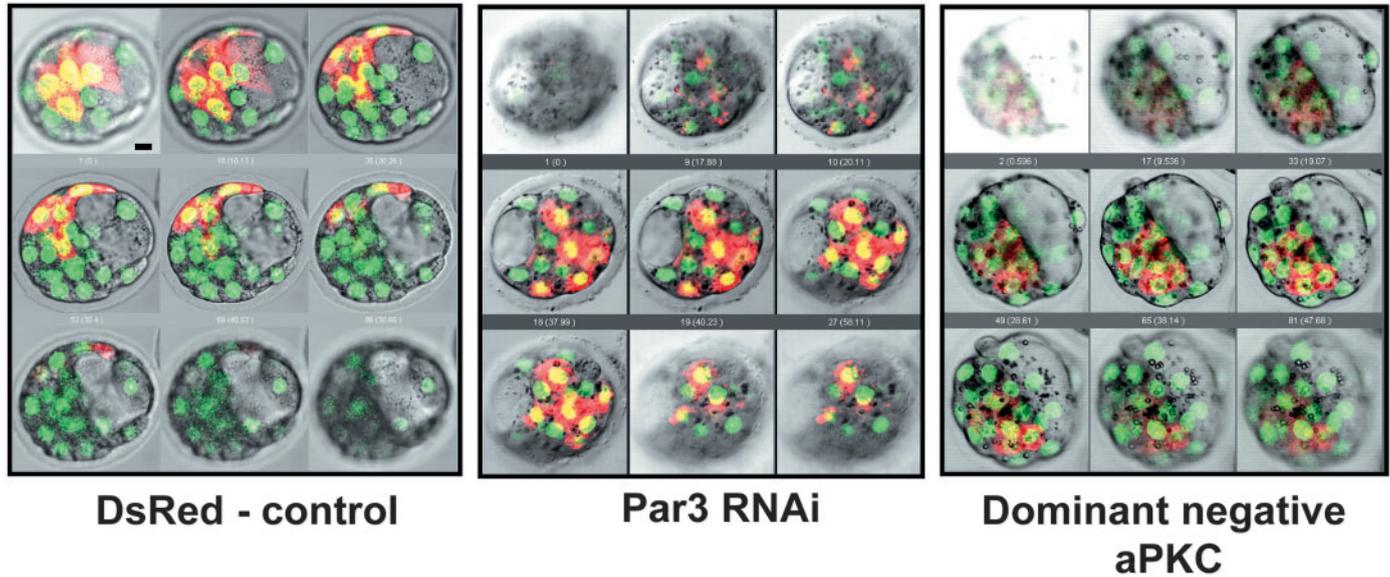
Fig. 2. Representative series of confocal sections of blastocysts injected at the 4-cell stage with DsRed mRNA, ds*Par3* RNA and dominant negative aPKC mRNA. Nuclei fluoresce green, reflecting expression of GFP-tagged histone H2B. The clone formed by the progeny of the injected cells is marked in red. The mean proportions of labelled ICM cells to total labelled cells was 0.34 for DsRed-injected embryos (n=38); 0.53 for ds*Par3* RNA-injected embryos (n=16); and 0.71 for dominant negative aPKC-injected embryos (n=25). Bar, 10 μm.

Influence of differential cell polarity on the establishment of the embryonic-abembryonic axis

As an alternative way of analysing these data, we examined the distribution of the clonal progeny of individual control 4-cell blastomeres and those in which Par3 or aPKC function was downregulated, with respect to the site of formation of the blastocyst cavity that is flanked by mural trophectoderm. To this end, we related the proportional contribution of the clone to mural trophectoderm to the proportional contribution of the clone to the trophectoderm in total (n.b. M, mural; TE, trophectoderm). Specifically, we represented the former by the ratio M as follows:

$$M=[(\text{no. labelled mural TE})/(\text{total no. labelled TE})]/[(\text{no. mural TE})/(\text{total no. TE})]$$

This controls for variation between blastocysts in the degree of cavity expansion. Thus, in any given embryo, a value of $M>1$ indicates a relatively high contribution of labelled cells to mural versus polar trophectoderm and a value of $M<1$ indicates a relatively low contribution of labelled cells to mural trophectoderm. This value is independent of the contribution of labelled progeny to ICM. To measure the latter, the proportion of the clone comprising trophectoderm, we calculated another ratio, F (fate), to represent the relative contribution of labelled cells to ICM and trophectoderm: $F=(\text{no. labelled TE})/(\text{total no. labelled cells})$.

Thus, a high F value (close to 1) represents a relatively high contribution to trophectoderm rather than ICM. The values M and F should vary independently if there is no relationship between the relative contribution of any randomly labelled 4-cell blastomere to the ICM and the position of its trophectoderm progeny with respect to the embryonic-abembryonic axis. However, values of F and M showed a positive correlation in the control groups of embryos (Fig. 4A,

orange points; 4B, purple points; 4C, all values) that was highly significant (see legend to Fig. 4). This indicates that, when a randomly labelled 4-cell blastomere gives rise to a relatively high proportion of ICM, the trophectoderm progeny of that blastomere have a strong tendency to be polar rather than mural.

Examination of these ratios following downregulation of the function of either Par3 or aPKC also indicates that these treatments result in an increased contribution of cells to the ICM (note the distribution of the pale blue and dark blue points along the F axis in Fig. 4A,B). Consistent with the earlier analysis, in 20% (n=25) of embryos in the dominant negative aPKC-injected group, there was no contribution at all of labelled cells to the trophectoderm and, in a further 20% of embryos, only a single cell made such a contribution. Thus, in 40% of embryos injected with dominant negative aPKC, most progeny of the injected cell become internalized. In those embryos where trophectoderm cells were labelled, they were predominantly polar. Although the effect was not as strong, the proportion of cells contributed by the labelled clone to the ICM was similarly increased in embryos injected with ds*Par3* RNA. In such cases, the clones often occupied ICM together with adjoining mural and adjoining polar trophectoderm.

Dominant negative aPKC influences cleavage orientations

The allocation of cells to the inside of the embryo occurs as a result of differentiative divisions of surface cells from the 8-cell to 16-cell and 16-cell to 32-cell stages. These divisions result in dissimilar progeny, one cell remaining outside and the other being directed inside. We wished to examine the extent to which the increased numbers of inside cells we observed following downregulation of Par3 or aPKC function resulted from
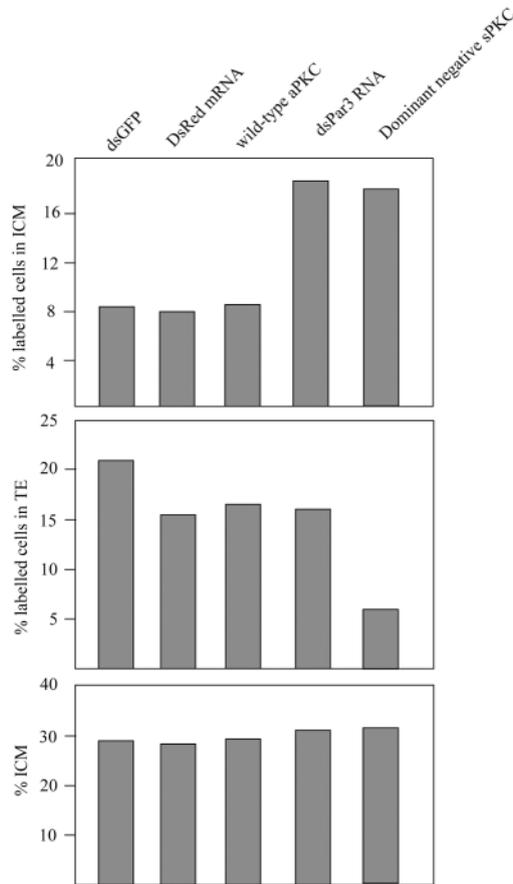
Fig. 3. Proportions of labelled and unlabelled ICM and trophectoderm cells in experimental and control groups of embryos. Note that the relative proportions of ICM versus trophectoderm cells remain almost constant, whereas the relative numbers of labelled cells in ICM or trophectoderm differ between groups.

changes in the relative frequency of conservative versus differentiative divisions. We addressed this question following the injection of mRNA for the dominant negative form of aPKC. As in the previous experiments, we co-injected one 4-cell-stage blastomere with synthetic mRNA for DsRed in order to be able to identify progeny of the injected cell by their fluorescence and followed the divisions of such cells by time-lapse microscopy (17 dominant negative aPKC injected and 18 control embryos). We followed embryos on multiple focal planes and noted both the orientation of the cleavages of labelled cells and the placement of daughter cells in either the outer cell layer or the ICM. Examples of a conservative and differentiative divisions visualized in this way are shown in Figs 5A' and B' respectively. We assessed the number of divisions of these two types in the 8-cell to 16-cell and 16-cell to 32-cell stages and also scored the number of divisions of inner cells generated in the clone in the latter cycle (Table 2). It can be seen that the frequency of conservative divisions is lower in cells expressing dominant negative aPKC. The effect is seen one division cycle following injection of the synthetic mRNA for the mutant aPKC at the 8-cell to 16-cell stage, but is stronger at the 16-cell to 32-cell divisions. This might indicate that interfering with the function of this protein kinase leads to a change in spindle orientation

through either a direct or indirect effect. Whereas outer cell spindles orientated parallel to the cortex would result in conservative divisions, the randomization of division orientation would result in more individual daughter cells leaving the surface layer. These experiments also allowed us to follow the divisions of labelled cells that became internalized. These appeared to divide at a frequency similar to the inside cells of control embryos that had been injected with mRNA for the fluorescent marker protein alone. Thus, the increase in numbers of labelled inside cells expressing dominant negative aPKC did not appear to be a consequence of any increased rate of cell division of inside cells.

Our time-lapse observations also enable us to notice that outer cells expressing dominant negative aPKC had a tendency to become internalized within the embryo. An example of this is shown in the series of time-lapse images presented in Fig. 5C'. In this time-lapse series, it can be seen that flanking outer cells become extended (arrows in panels C and D) to replace the labelled cell formerly in the outer position. In 17 embryos analysed, we observed 18 cells to become displaced from the outer layer in this way. Thus, expression of the dominant negative aPKC appears not only to decrease the proportion of conservative divisions, but also to change the properties of cells such that they cannot any longer remain in the outer cell layer.

Effects of dominant negative aPKC upon tight junction formation

As Par3 and aPKC are involved in the later stages of tight junction formation (Suzuki et al., 2002; Eckert et al., 2004), we wondered whether the consequences of downregulation of Par3 or expression of dominant negative aPKC upon allocation of cells to the inside of the embryo might also be influenced by effects of these molecules on tight junction formation. Tight junctions begin to form at the 8-cell stage with the recruitment of one of the isoforms of ZO1alpha and rab13 (Fleming et al., 2000). This is followed at the 16-cell stage by recruitment of cinguilin. Finally, assembly is completed at about the 32-cell stage by recruitment of a second ZO1alpha isoform and occludin. aPKC has been shown to be one of the later tight junction components to be recruited (Suzuki et al., 2002). As dominant negative aPKC gave a stronger response than downregulation of Par3 in the allocation of cells to the ICM, we investigated tight junction formation following such treatment (Fig. 6). To this end, we again injected a random 4-cell-stage blastomere with synthetic mRNAs for both aPKC and DsRed, and allowed such embryos to develop to the late 8-cell or blastocyst stages before undertaking immunostaining to reveal ZO1 or occludin, respectively. We found that recruitment of these tight junction components does occur at the appropriate stages both in outer cells expressing dominant negative aPKC (that fluoresce red) and surrounding outer cells that are the progeny of uninjected blastomeres. Thus, it would seem that, whereas expression of dominant negative aPKC results in an increased allocation of cells to the 'inside' of the embryo, it does not appreciably affect the aspects of formation of tight junctions that we monitored.

Discussion

Progenitors of the ICM of the mammalian blastocyst arise after

Table 1. Contributions of labelled cells to specific regions of the blastocyst following injection of ds*Par3* RNA, mRNA for aPKC, or control molecules

| | ICM | | | Trophectoderm (TE) | | | | | | | | | | Total labelled/ | Labelled ICM/total |
| | | | | Mural | | | Polar | | | Total | Total | | Total | | |
Treatment (*n*)	Labelled	Unlabelled	Total	Labelled	Unlabelled	Total	Labelled	Unlabelled	Total	labelled	unlabelled	Total	cells	total cells (%)	labelled (%)
ds*GFP* (*n*=14)	2.2	5.6	7.8	1.7	3.4	5.1	4.4	9.1	13.5	6.1	12.6	18.6	26.4	31%	27%
ds*Par3* (*n*=16)	5.1	3.8	8.9	1.8	3.9	5.8	2.6	9.6	12.3	4.4	13.6	18.0	26.9	35%	53%
DsRed (4-cell) (*n*=38)	2.4	6.3	8.7	2.5	8.3	10.8	2.3	9	11.3	4.9	17.3	22.1	30.9	24%	33%
DsRed (8-cell) (*n*=9)	1.1	7.8	8.7	2.4	7.8	10.2	0.7	10	11	3.1	18.4	21.6	30.4	14%	26%
aPKC dom. neg. (*n*=27)	5.8	4.8	10.6	0.4	6.9	7.3	1.8	12.9	14.6	2.2	19.7	21.9	32.5	24%	73%
aPKC wild-type (*n*=28)	2.6	6.5	9.1	2.1	6.0	8.1	3.1	9.3	12.4	5.3	15.3	20.6	29.7	26%	33%
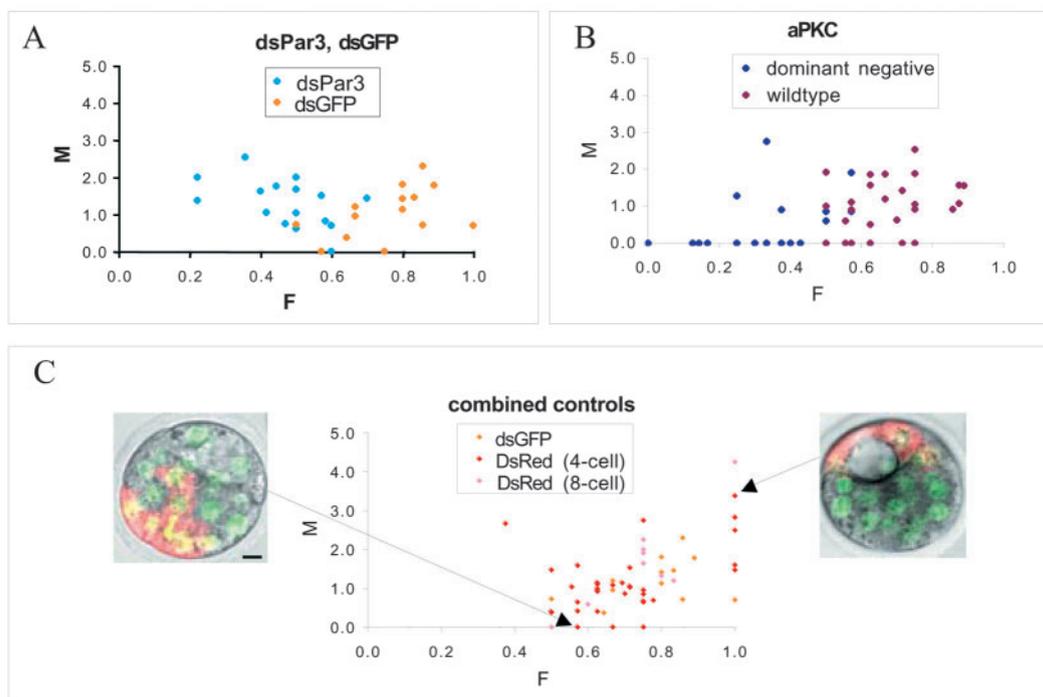
Fig. 4. Proportional contribution of labelled cells to mural trophectoderm (*M*) against relative contribution of labelled cells to trophectoderm versus ICM (*F*). The derivation of the values *M* and *F* is described in the text. (A) Values of *M* and *F* for a group of 16 experimental embryos injected at the 4-cell stage with ds*Par3* RNA (pale blue). A group of 14 control embryos were injected with ds*GFP* RNA (orange). (B) Values of *M* and *F* for a group of 25 experimental embryos injected at the 4-cell stage with mRNA for dominant negative aPKC (blue). A group of 26 embryos were injected with mRNA for wild-type aPKC (purple). (C) Combined groups of control embryos injected with DsRed mRNA at either the 4- or 8-cell stage (red and pink, respectively) or ds*GFP* RNA at the 4-cell stage (orange). (C, left panel) A control embryo (corresponding to the indicated data point) representing a clone of cells making a major contribution to the ICM and also the polar trophectoderm. (C, right panel) A control embryo (corresponding to the indicated data point) representing a clone of cells contributing predominantly to mural trophectoderm. Bar, 10 µm. Positive rank (Spearman) correlation coefficients were obtained for the values of *F* and *M* in both the ds*GFP* and DsRed control groups (r=0.44, 0.10<*P*< 0.20; and r=0.37, *P*<0.05, respectively). Control blastomeres injected with DsRed at the 8-cell stage, or with wild-type aPKC at the 4-cell stage, also gave respective positive correlation coefficients of r=0.52 (0.10<*P*< 0.20) and r=0.35 (0.05<*P*< 0.10). When all control groups were combined (panel C), the correlation was highly significant (r=0.51, *P*<0.001).

cells first become polarized at the 8-cell stage and then undergo differentiative divisions in the next two division cycles to produce inside cells. We have now found that functional downregulation of both Par3 and aPKC in randomly selected 4-cell-stage blastomeres increases the probablity that the progeny of these cells will become internalized and as a secondary consequence adopt an 'inside' cell fate. Several

mechanisms could account for this loss of the ability to retain outside identity and it seems likely that these are not exclusive. One possibility is that Par3 and aPKC are needed to develop cellular polarity in early mouse blastomeres, as is known in other cell types. Thus, their downregulation could lead to a failure to develop the apical cellular structures required in trophectoderm cells. The increased tendency of cells to

Fig. 5. Dominant negative aPKC changes cleavage orientations and leads to cells 'falling' from the outside cell layer into the inside part of the embryo (A',B'). Examples of the conservative (A') and differentiative (B') divisions in embryos followed by time-lapse microscopy through five focal planes after injection of a single 4-cell-stage blastomere with mRNA for DsRed and dominant negative aPKC. Images A-D represent stages of development of a single embryo at a central focal plane. The parental cells being followed are outlined with red and the daughter cells with blue and yellow dashed lines. (C') Example of the internalization of a cell followed by time-lapse microscopy through five focal planes in a pre-cavitation embryo injected with mRNA for DsRed and dominant negative aPKC into a single 4-cell-stage blastomere. Images A-D represent stages of development of a single embryo at a central focal plane. Bar, 10 μm.
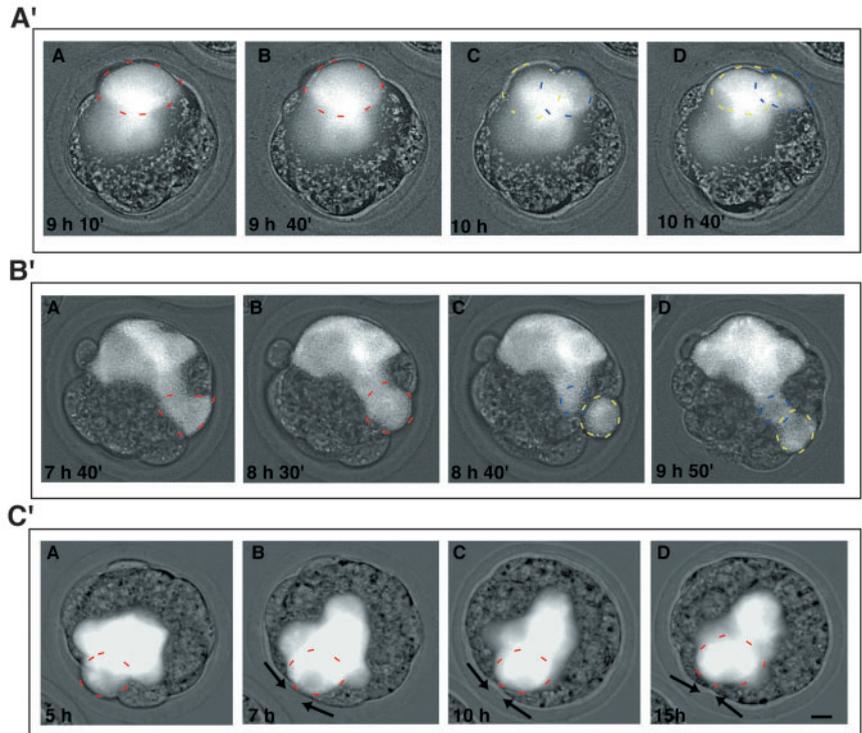
adopt an inside position following this downregulation might also be a consequence of a failure to develop effective tight junctions between cells that would otherwise have remained in the trophectoderm even though the early stages of tight junction formation did not appear to be affected. Finally, downregulation of Par3 or aPKC could reorientate the spindle and thereby the plane of cleavage such that, below a threshold level, a differentiative division would occur. Such a reorientation of cleavage could also be an indirect effect resulting from a change in cell shape or other cellular properties.

To what extent might the effects we see following the functional downregulation of Par3 or aPKC be attributed to one or more of the overlapping processes associated with cell polarization and compaction of the embryo at the late 8-cell stage? We first consider whether the effects we observed could overlap with functions ascribed to E-cadherin, a molecule postulated to be a player in both compaction and polarization. This molecule becomes restricted to baso-lateral regions at the 8-cell stage (Vestweber et al., 1987). As it is possible to use RNAi to knock-down E-cadherin in the mouse zygote (Wianny and Zernicka-Goetz, 2000), we considered whether the effects of knocking-down E-cadherin expression in individual

blastomeres might have a similar effect to functional downregulation of Par3 or aPKC. By contrast, however, we found that the progeny of cells injected with E-cadherin dsRNA formed clones of distinctive cells on the outside of the embryo that had failed to compact (Fig. S3, in supplementary material). As progeny of cells injected with ds*Par3* RNA or mRNA for dominant negative aPKC do compact, this suggests that E-cadherin might be involved in a distinctive stage of the polarization process.

A second overlapping process associated with cell polarization is the orientation of cell division. In common with other epithelial cell layers, outer blastomeres can undergo conservative divisions in which both daughter cells remain in the epithelium. If this division orientation is lost, one of the daughter cells will have a greater chance of being directed inside. We found that expression of dominant negative aPKC led to a greatly increased tendency for the orientation of the cleavage plane in the outer cells to be lost, resulting in

Table 2. Proportions of cells undertaking differentiative or conservative divisions following expression of dominant negative aPKC

	Dominant negative		Wild-type	
Division type	8- to 16-cell	16- to 32-cell	8- to 16-cell	16- to 32-cell
Differentiative	14 (47%)	22 (73%)[†]	13 (37%)[‡]	9 (22%)[§]
Conservative	16 (53%)[*]	8 (27%)	22 (63%)	32 (78%)[¶]
Inner	–	16	–	11
No division	3	18	1	17
Undetermined	1	4	0	3
Total	34	68	36	72

[*]In 2 embryos, the division plane appeared oblique but the final positions of daughter cells reflected a conservative division.
[†]In 5 embryos, the division plane appeared oblique but the final positions of daughter cells reflected a differentiative division.
[‡]In 2 embryos, the division plane appeared oblique but the final positions of daughter cells reflected a differentiative division.
[§]In 1 embryo, the division plane appeared oblique but the final positions of daughter cells reflected a differentiative division.
[¶]In 1 embryo, the division plane appeared oblique but the final positions of daughter cells reflected a conservative division.

differentiative divisions. At present, we cannot say whether this is an effect directly on the spindle orientation mechanism or whether it is mediated as a secondary response to cell polarization or cell shape.

Interestingly, in almost 40% (10/25) of embryos injected with dominant negative aPKC-labelled cells, they either made no contribution or made only a single cell contribution to trophectoderm. In normal development, all 8-cell-stage blastomeres give rise to at least one outer cell at the fourth division, and all outer 16-cell-stage cells give rise to at least one outer cell at the fifth division (reviewed by Johnson et al., 1986; Fleming, 1987). Thus, at least in some embryos, the dominant negative aPKC-injected outer progeny must have become completely internalized during cleavage. This implies that the observed 're-direction' towards ICM fate cannot be explained solely by loss of spindle orientation leading to more-differentiative divisions. Indeed, our time-lapse imaging directly demonstrated that the outer progeny of either conservative or differentiative divisions could be displaced from the outer layer into the inside of the embryo. Thus, it appeared as though neighbouring outside cells 'out-competed' injected cells for outside positions. This could be a result of the change in polarity of progeny of the injected cells that therefore can become internalized, causing neighbouring outside cells to flatten more to make up the depleted trophectoderm. Such 'cell sorting' at this stage, whereby non-polar cells become engulfed by more polar cells, is in agreement with previous observations (Johnson and Ziomek, 1982; Kimber et al., 1982). Moreover, it may be accentuated at later stages when the known functions of Par3 and aPKC in tight junction formation (Suzuki et al., 2002; Hirose et al., 2002) become more significant.

Although tight junction assembly begins at the 8-cell stage with the recruitment of an isoform of ZO1alpha and rab13, final assembly is not achieved until around the 32-cell stage after aPKC has been recruited (Fleming et al., 2000). Consistent with this, we see the normal timing of recruitment of both early and late assembled tight junction components (ZO1 and occludin, respectively) in cells expressing dominant negative aPKC that is comparable to neighbouring control cells. Thus, the primary effect of dominant negative aPKC upon inner cell allocation occurs at a stage early in the formation of tight junctions. Although we do not know the effects of dominant negative aPKC upon the tight junction assembly at this stage, we cannot rule out its participation in this process and indeed the generation of cell polarity might be related to tight junction formation.

Our experiments indicate that dominant negative aPKC has a stronger effect than Par3 RNAi. This graded series of phenotypes allow us to hypothesize that in normal development a subset of outer cells that either are less polarized or have less effective tight junctions may be more likely to divide differentiatively. This would be in agreement with a previous report that cells with a smaller domain of external microvilli, and therefore less polarized, tend to undertake differentiative divisions (Pickering et al., 1988). Thus, one can envisage gradational effects on cell behaviour: maximum cellular polarity and most effective tight junctions would permit conservative divisions that generate outer cell progeny; moderate polarity would permit the differentiative/oblique divisions that generate polar outer cells and sister non-polar
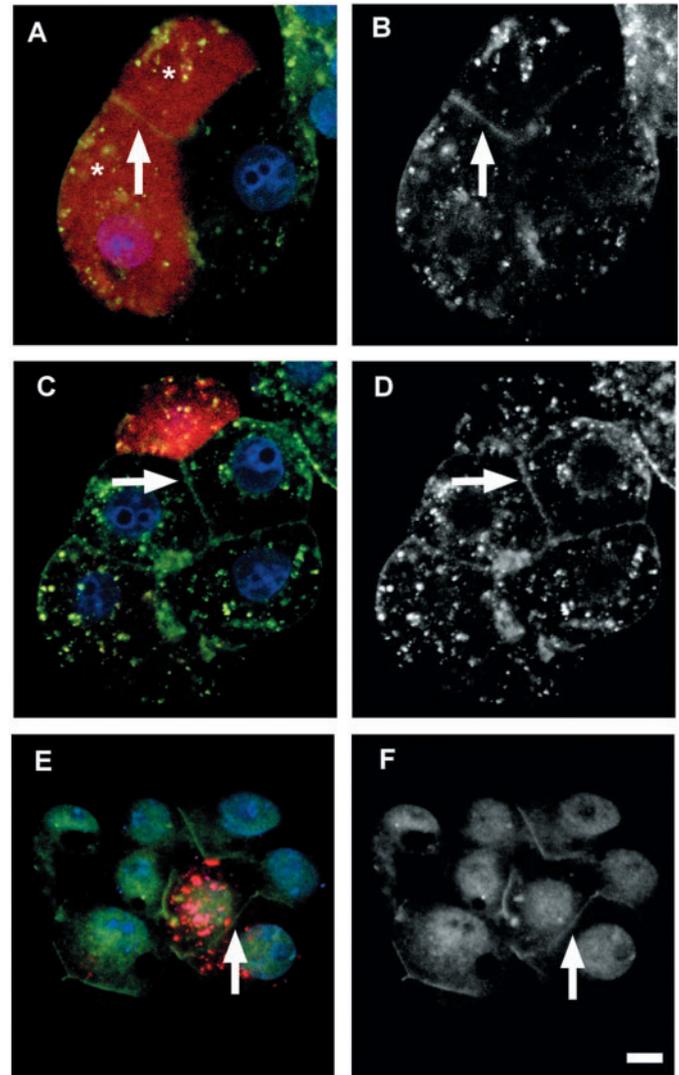

Fig. 6. Recruitment of tight junction proteins following expression of dominant negative aPKC. (A-D) Localization of the tight junction protein ZO1 at the late 8-cell stage in embryos injected with mRNA for dominant negative aPKC and DsRed into one 4-cell-stage blastomere. ZO1 (green and monochrome) localizes to apposing cell surfaces (arrows) between both injected (A,B; injected cells are red and marked with an asterisk) and non-injected (C,D) progeny; 11 embryos were analysed. (E,F) Similar embryo at the blastocyst stage. Occludin (green and monochrome) localizes to the tight junction (arrow) in both non-injected and injected progeny; 9 stained embryos were analysed. The latter are red and marked with an asterisk. Bar, 10 μm.

inner cells, but would nevertheless be sufficient to maintain the outer cells in position; minimal polarity and loss of tight junctions would be insufficient for maintaining an outside position, thus leading to internalization. Since *Par3* RNAi is likely only to downregulate this target protein partially, the increased numbers of ICM cells might be generated more through decreased numbers of conservative divisions rather than through more-complete loss of polarity and tight junctions as might occur with expression of dominant negative aPKC.

In addition to these perspectives on the roles of Par3 and aPKC in early mouse development, the lineage studies we

present for control embryos demonstrate that, when a randomly labelled 4-cell-stage blastomere contributes to a relatively high proportion of ICM, it has a strong tendency to contribute to polar rather than mural trophectoderm. This suggests a reciprocal relationship between the generation of ICM cells and the site of cavity formation and thus offers support to earlier work (Kelly et al., 1978; Graham and Deussen, 1978; Spindle, 1982; Surani and Barton, 1984; Fleming et al., 1987; Piotrowska et al., 2001). It has been suggested that the site of cavitation, and hence the appearance of mural trophectoderm, may be determined by the region of the morula in which outer cells are at the most advanced state of trophectoderm maturation (Garbutt et al., 1987). Another possibility is that two distinct populations of inner cells arise from the fourth and fifth divisions. The population of inner cells generated at the fourth division would be uniformly distributed irrespective of the location of their sister outer cells as they are the only inside cells at this stage. However, after the fifth division, inner cells comprise two populations; those generated from divisions of inside cells and those generated de novo from outside cells. Could inner cells formed de novo at the fifth division differ from their more interior cousins because they were more recently generated? Such a difference could reflect their different properties or a stronger connection of inner cells generated at the fifth division to their outer cell sisters affecting the site of cavitation.

The major findings of this study suggest that common mechanisms might be involved in regulating asymmetrical cell division in the development of both invertebrates and vertebrates. The functions of the Par complexes in such processes are well established in *Caenorhabditis elegans*, a model organism highly suited to assign function to molecules through genetic analysis (Guo and Kemphues, 1996; Wodarz, 2002). Although the apical cellular localization of aPKC has been reported to correlate with development of cell fate in *Xenopus* embryos (Chalmers et al., 2003), a role for Par3 and aPKC in the early development of any vertebrate embryo has not previously been tested directly. Here, we have applied two methods for functionally downregulating these proteins in a surrogate genetic approach that suggests a function for them in regulating the polarized and junctional properties of the cell, the orientation of its division, and hence its position in the normal development of the mammalian embryo. Functional downregulation of Par3 or aPKC leads to cells undertaking a higher frequency of differentiative divisions that direct at least one daughter inside. Such cells also become less competitive in retaining outer positions in the developing blastocyst. In the preimplantation mouse embryo, the fate of a cell is generally thought to be specified by its position. Thus, experimentally changing the relative position of a cell will adjust its fate (Tarkowski and Wroblewska, 1967; Hillman et al., 1972). Our study shows that, by interfering with genes that regulate cell polarity it is possible to direct defined clones of cells to new positions and hence redirect their fate in the preimplantation embryo.

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