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Delay of polarization event increases the number of Cdx2-positive blastomeres in mouse embryo

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ABSTRACT

During preimplantation mouse embryo development expression of Cdx2 is induced in outer cells, which are the trophectoderm (TE) precursors. The mechanism of Cdx2 upregulation in these cells remains unclear. However, it has been suggested that the cell position and polarization may play a crucial role in this process. In order to elucidate the role of these two parameters in the formation of TE we analyzed the expression pattern of Cdx2 in the embryos in which either the position of cells and the time of polarization or only the position of cells was experimentally disrupted. Such embryos developed from the blastomeres that were isolated from 8-cell embryos either before or after the compaction, i.e. before or after the cell polarization took place. We found that in the embryos developed from polar blastomeres originated from the 8-cell compacted embryo, the experimentally imposed outer position was not sufficient to induce the Cdx2 in these blastomeres which in the intact embryo would form the inner cells. However, when the polarization at the 8-cell stage was disrupted, the embryos developed from such an unpolarized blastomeres showed the increased number of cells expressing Cdx2. We found that in such experimentally obtained embryos the polarization was delayed until the 16-cell stage. These results suggest that the main factor responsible for upregulation of Cdx2 expression in outer blastomeres, i.e. TE precursors, is their polarity.

Introduction

Developing mouse blastocyst consists of two distinct cell lineages: the inner cell mass (ICM) and the trophectoderm (TE). The mechanisms underlying their establishment are not fully understood. It seems that the precursors of ICM and TE diverge very early in development and that the position of the cells within the morula is important for lineage specification. The formation of two distinct populations of cells: the inner and outer cells, occurs after the 4th division, i.e. the transition from 8- to 16-cell stage. According to the "Inside–Outside" Model, the cell fate in the blastocyst is determined by its position; the inner blastomeres form ICM and the outer blastomeres are the precursors of TE (Tarkowski and Wroblewska, 1967). After the 5th division, just prior to the blastocyst formation when the embryo is composed of 32 cells, populations of outer and inner cells are singled out and follow different developmental pathways (Marikawa and Alarcon, 2009; Ralston and Rossant, 2008). Another model of lineages determination, the Cell Polarity Model, proposes that cell fate is established at the eight-cell stage, during the compaction, when the morphology of blastomeres changes and they become polarized along the apical–basal axis (Johnson and Ziomek, 1981a, 1981b).

The Cell Polarity Model assumes that subsequent cell divisions lead to the different distribution of polarity clues in descending blastomeres, depending on the inheritance of polar domain (Yamanaka et al., 2006). Accordingly, the asymmetrical (perpendicular to the apical–basal axis) division of the blastomere gives rise to two different types of cells—the outer cell, which inherits the polarization domain and stays outside, and the inner cell, which is apolar. A symmetric (parallel to the apical–basal axis) division of polarized cell generates two identical polarized cells, which remain on the embryo surface. Besides the difference in their polarity status, the outer and inner cells of mouse morula also differ in the activity of specific genes. The one of the earliest events observed during lineage determination is the expression of Caudal-like transcription factor (Cdx2) in the precursors of TE (Beck et al., 1995). In addition, TE differentiation correlates with downregulation of Oct4, the member of POU transcription factors family, which is the marker of pluripotent cells (Palmieri et al., 1994; Strumpf et al., 2005; Szczepanska et al., 2011). Eventually, at the blastocyst stage the expression of Oct4 is restricted to the ICM while Cdx2 expression occurs exclusively in TE (Niwa et al., 2005; Strumpf et al., 2005). It has been shown that Cdx2 is required for differentiation of TE and despite that Cdx2-deficient

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embryos form blastocysts with normal morphology they die before implantation (Strumpf et al., 2005). Defects in segregation of the ICM and TE lineages are also manifested in Oct4 mutants, which form functional TE but die because of decreased developmental potential of their ICM (Nichols et al., 1998). These data suggest antagonistic function of Cdx2 and Oct4 and indicate that proper interaction between these two transcription factors is necessary for the segregation of TE and ICM (Niwa et al., 2005).

It is very plausible that both mechanisms, i.e. cell polarization and function of transcription factors specific for ICM and TE, are important for early lineage specification in mouse morula. The key event in this process is the upregulation of Cdx2 expression in the TE precursors. Although it has been shown that Cdx2 expression is regulated by TEAD4 and Yap (Nishioka et al., 2008, 2009; Yagi et al., 2007) the exact mechanism of Cdx2 induction remains obscure. It was demonstrated previously that in the early mouse blastocyst the polar cells contain the apical domain which is marked by the presence of PAR3/PAR6/aPKC protein complex (Ahringer, 2003; Nelson, 2003). There are data suggesting that the apical protein complex is crucial for TE fate through its involvement in the induction of Cdx2 expression in the outside cells of mouse embryo (Jedrusik et al., 2008; Ralston and Rossant, 2008; Yamanaka et al., 2006).

In the present study we investigated the relationship between the cell position, cell polarization and the Cdx2 expression. Our experimental approach was based on the prevention of the formation of inner blastomeres, which normally develop during the 8- to 16-cell stage transition. We separated individual blastomeres from the 8-cell stage embryos before or after they polarized. Next, each set of 8 single blastomeres was cultured until they formed "embryo fragments" composed of 4 cells, i.e. until they corresponded to 32-cell developmental stage of the intact embryos. Analysis of the expression of Cdx2 within embryo fragments allowed us to conclude that at the early stages of mouse embryo development the pattern of Cdx2 expression is not determined by cell position but is the result of cell polarization. We also showed that it is possible to increase the number of Cdx2-positive cells if the polarization event is delayed from 8- to 16-cell stage.

Materials and methods

The study was approved by the Local Ethic Committee No. 1 (Warsaw, Poland).

Reagents and media

All chemicals were purchased from Sigma-Aldrich (Poznan, Poland), unless stated otherwise.

Embryos collection

Embryos were obtained from F1(C57Bl/6xCBA) 8–12-week-old female mice which were induced to ovulate by injection of 10 IU of PMSG (Intervet, Poland) and 48 h later of 10 IU of hCG (Intervet, Poland), and mated with F1(C57Bl/6xCBA) male mice. Inseminated females were selected by the presence of vaginal plug. Embryos at the 4-cell stage and compacted 8-cell stage embryos were obtained 60 and 68 h after hCG injection, respectively. Mice were sacrificed by cervical dislocation, and embryos were released from oviducts and uteri. Embryos were cultured in vitro at 37.5 °C in 5% CO_2 in air, in drops of M2 [medium 16 buffered with HEPES, Fulton and Whittingham, 1978] supplemented with 4 mg/ml BSA or KSOM [(Erbach et al., 1994), Specialty Media, USA] medium under mineral oil in 35 mm plastic dishes (BD Falcon, USA).

Embryo disaggregation and individual blastomeres culture

The 4- and 8-cell embryos were freed of zonae pellucidae using acid Tyrod's solution [pH 2.5, Nicolson et al., 1975]. In order to separate blastomeres, the embryos were transferred to M2 medium without Ca^{2+} and Mg^{2+}, supplemented with EGTA (0.2 mg/ml), and incubated for 10 min at 37.5 °C in 5% CO_2 in air, in drops of medium (each embryo in a separate drop) under mineral oil in plastic dishes. Subsequently, the embryos were disaggregated by pipetting with a narrow glass pipette (Tarkowski and Wroblewska, 1967). To facilitate separation of blastomeres some of 8-cell embryos were additionally incubated for 2–3 min in 0.5% pronase (CalBiochem, San Diego, USA) in PBS at 37.5 °C, followed by re-pipetting. After the dissociation all four blastomeres obtained from 4-cell stage embryo (called "1/4") or all eight blastomeres obtained from compacted 8-cell stage embryo (called "polar 1/8") were transferred to M2 and cultured under standard conditions (see above) for 30 h or 25 h, respectively. Immediately after the division of 1/4 blastomeres the blastomeres forming the pairs of 2/8 were separated by thin glass needle and each of obtained eight 1/8 blastomeres (called "apolar 1/8") were further cultured in separate M2 medium drops. The culture of eight polar 1/8 blastomeres or eight apolar 1/8 blastomeres resulted in the formation of the embryo fragments which were called "the full sets of embryo fragments". Each of the full set of embryo fragment was presumably composed of 4 cells. Control consisted of 32-cell embryos that developed from intact 8-cell stage embryos after 22–25 h culture ("control 1") or from intact 4-cell stage embryos after 35 h culture ("control 2"). The control embryos and experimental embryo fragments were fixed for immunodetection of Cdx2 or FITC-ConA staining.

FITC-ConA staining

Cells were fixed in 4% paraformaldehyde (PFA) in PBS (without Ca^{2+} and Mg^{2+}) for 1 h at room temperature and blocked in PBS containing 3% BSA and 0.05% Tween20. Subsequently, the blastomeres were incubated in FITC-ConA (0.7 mg/ml in PBS with 3% BSA) for 15 min. After washing with PBS without Ca^{2+} and Mg^{2+}, chromatin was visualized with DRAQ5 (10 μM in PBS; Biostatus, Leicestershire, UK) for 10 min at 37 °C. Next, control embryos and embryo fragments were analyzed with Zeiss 510 confocal laser microscope equipped with Zeiss software system (LSM Image Browser).

Immunodetection of Cdx2 protein

Control embryos and embryo fragments were fixed in 4% PFA in PBS (without Ca^{2+} and Mg^{2+}) for 1 h at room temperature, and then permeabilized with 0.3% Triton X-100 in PBS for 15 min. The nonspecific antibody binding was blocked by incubation in PBS/3% BSA/0.05% Tween, overnight, at 4 °C. Embryos were then incubated overnight at 4 °C with mouse monoclonal anti-Cdx2 antibody (BioGenex, USA) at 1:50 dilution in PBS with 3% BSA. After washing (3×15 min in PBS without Ca^{2+} and Mg^{2+}) embryos were incubated with TRITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, stock solution 0.75 mg/ml) at 1:200 dilution in PBS/3% BSA, for 1 h, at room temperature, and then washed twice in PBS (without Ca^{2+} and Mg^{2+}). Negative control was prepared by omitting the incubation with anti-Cdx2 antibody. In addition, actin localized at cell-cell contacts was visualized by incubation in FITC-conjugated phalloidin (1:1000 in

3% BSA in PBS) for 20 min at room temperature. Chromatin was visualized by incubation in DRAQ5 (10 μM in PBS; Biostatus, Leicestershire, UK) for 10 min at 37 °C. Control embryos and embryo fragments were analyzed with Zeiss 510 confocal laser microscope equipped with Zeiss software system (LSM Image Browser).

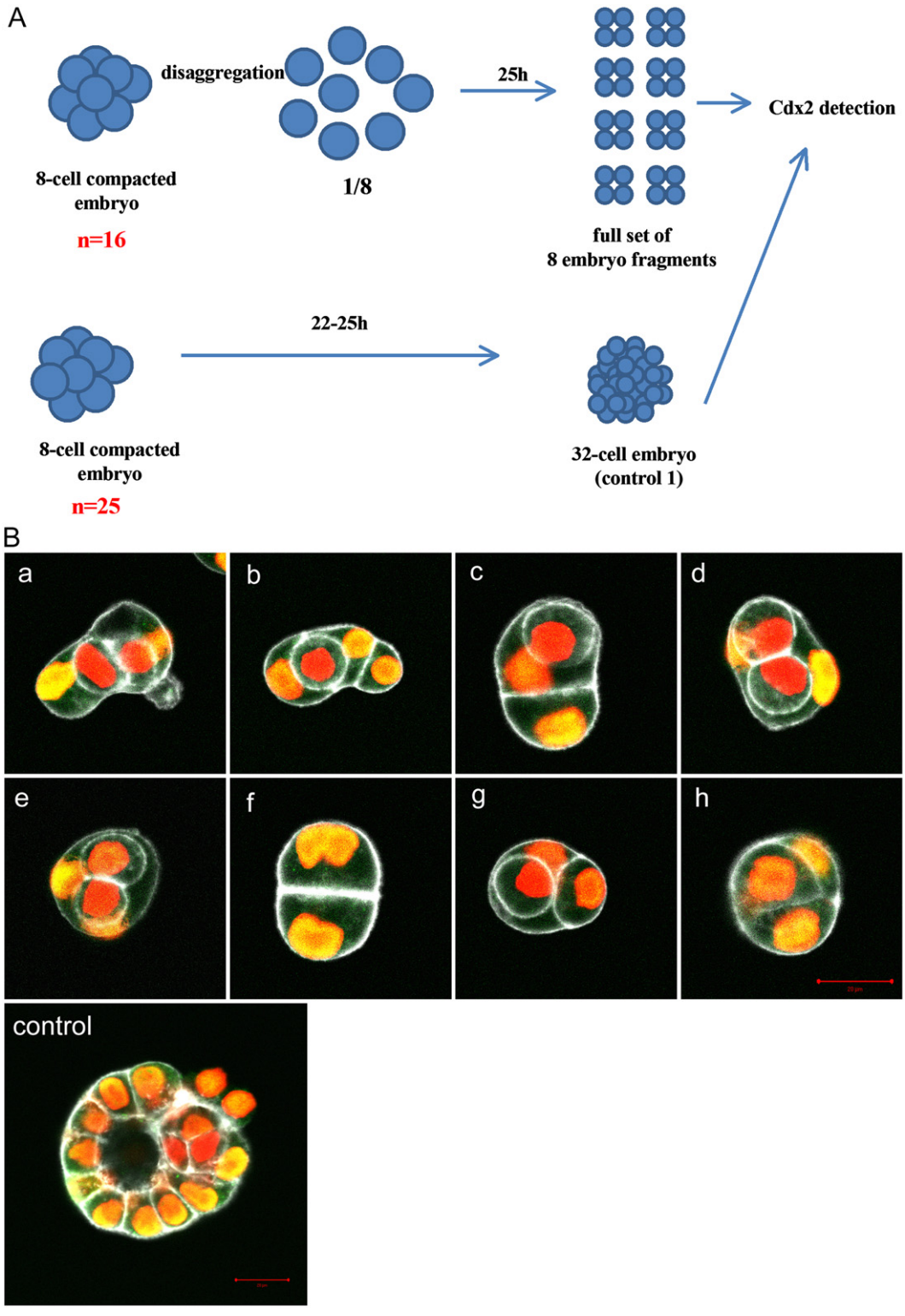

Fig. 1. Experiment 1 — embryo fragments developed from polar 1/8 blastomeres. (A) Diagram of Experiment 1. Compacted embryos at 8-cell stage were disaggregated and the individual blastomeres were cultured as a full set in vitro for 25 h to obtain 8 embryo fragments presumed to be composed of 4 cells. The intact 8-cell embryos were cultured for 22–25h to develop 32-cell embryos (control 1). (B) Cdx2 expression in control 32-cell embryo and in the embryo fragments (embryo number 9 Table 1). All images are merged single optical images of chromatin (red) and Cdx2 (green) staining. The borders of cells are visualized by actin staining with phalloidin (white). Pattern of Cdx2 expression (number of cells/number of Cdx+ cells) in the fragments is: (a) 4/2, (b) 4/3, (c) 4/3, (d) 4/2, (e) 4/3, (f) 2/2, (g) 4/3, and (h) 4/4. Scale bar 20 μm.

Results

Changing the position does not induce the Cdx2 expression in blastomeres of inner fate

In order to test whether the position of the blastomeres in the embryo is the main factor influencing the Cdx2 expression, we prevented the formation of the first progeny of inner blastomeres, the event that occurs during the 4th division i.e. the transition from 8- to 16-cell stage. For this purpose we cultured individually all polar 1/8 blastomeres obtained after the dissociation of the 8-cell compacted embryos (Fig. 1A). These blastomeres underwent mitotic division and formed 2/16 doublets in which all of the cells were outer cells, which was confirmed by confocal analysis (Fig. S1). Next, we cultured individually all 2/16 doublets (originating from a single 8-cell embryo) until they underwent additional cleavage division and formed 4 cells-embryo fragments, which development corresponded to the 32-cell control intact embryos (Fig. 1A and B). Such experimental design made possible to test whether the outer localization of blastomeres, which in intact embryo would form the population of inner cells, could influence their Cdx2 expression. The intact (control) 8-cell stage embryos were cultured until they most probably reached 32-cell stage (Fig. 1A and B). In these embryos all outer blastomeres exhibited intense nuclear Cdx2 staining and none of the inner blastomeres expressed Cdx2. Analysis of full sets of embryo fragments ($n=16$) originating from blastomeres of 8-cell embryos revealed that each of the fragment contained between 1 and 6 cells, which summarily corresponded to the embryos composed of 27–33 cells (Table 1). Similar number of blastomeres was present in the control embryos, which developed from 8-cell embryos after 22–25 h of culture (Table S1). We found that most of isolated blastomeres (82%) divided twice and formed embryo fragments composed of 4-cells (Table 1). Calculation of the proportion of the number of Cdx2 expressing cells versus total number of blastomeres within each of the 16 sets of embryo fragments showed that 64.7% of blastomeres in experimental embryo fragments (Table 1) and 59.3% of blastomeres in control 32-cell embryos expressed Cdx2 (Table S1). Observed difference was statistically insignificant ($p>0.05$). Moreover, the mean number of Cdx2-positive cells per embryo (19.8) in experimental embryo fragments was similar to that in intact embryos (18).

These data show that among the descendants of the individual polar blastomeres of 8-cell compacted embryo the number of Cdx2-positive cells is already established and does not change, even if the position of blastomeres had been changed inside out.

Preventing the polarization at the 8-cell stage results in the increase in the number of Cdx2-positive blastomeres

In order to test whether the polarity is the main factor influencing the Cdx2 expression in the embryo we prevented blastomere polarization event at the 8-cell stage. To do so we disaggregated the 4-cell embryos and cultured individual blastomeres until they divided and reached 8-cell stage (Fig. 2A). Immediately after 2/8 doublets were formed, the blastomeres were separated using thin glass needle. We proved, using the FITC-ConA staining (see the next section) that 1/8 blastomeres obtained in this way were apolar. The eight individual apolar 1/8 blastomeres originated from each 4-cell stage embryo were cultured for 30 h to obtain eight embryo fragments, each presumably composed of 4 cells, i.e. being an equivalent of control intact 32-cell embryos (Fig. 2A and B).

Analysis of full sets of embryo fragments ($n=19$) originating from the single blastomeres of 4-cell embryo revealed that each of the fragment contained between 1 and 5 cells what summarily corresponded to the embryos composed of 27–33 cells (Table 2). Similar number of blastomeres formed each of the control embryos, i.e. those ones which developed from 4-cell embryos after 35 h of culture (Table S2). We found that 80% of the isolated blastomeres have divided twice and formed 4-cell fragments (Table 2).

Calculation of the number of Cdx2 expressing cells versus total number of blastomeres within each of the 19 sets of embryo fragments showed that 78.3% blastomeres in experimental embryo fragments expressed Cdx2 (Table 2), which was higher and statistically significant ($p<0.0001$) in comparison to 59.8% of blastomeres in control 32-cell embryos and 64.7% in the fragments originating from polar 1/8 blastomeres ($p<0.05$) (Fig. 3A). Moreover, the mean number of Cdx2-positive cells per embryo in experimental embryo fragments was higher than in intact embryos (23.8 versus 19; Tables 2 and S2).

Table 1
Analysis of 16 of full sets of embryo fragments developed from polar 1/8 blastomeres. The total number of cells and number of Cdx2+ cells was estimated. In each embryo fragment the Cdx2 expression pattern is shown (number of cells/number of Cdx2+ cells).

Embryos no.	Number of cells/Cdx2+ cells in full sets of embryo fragments								Total number of cells	Number of Cdx2+ cells	%Cdx2+ cells
	1	2	3	4	5	6	7	8			
1	4/4	4/3	6/5	3/3	4/2	4/4	4/4	4/0	33	25	75.8
2	4/2	4/2	4/2	4/2	4/2	5/3	4/2	4/4	33	19	57.6
3	4/3	4/2	4/2	4/3	4/4	4/3	4/4	4/2	32	23	71.9
4	4/4	4/2	4/2	4/4	4/3	4/2	4/4	4/2	32	23	71.9
5	4/2	4/2	4/4	4/2	4/2	4/4	4/4	4/2	32	22	68.8
6	4/2	4/3	6/3	3/3	3/2	4/3	4/2	4/2	32	20	62.5
7	4/2	4/2	3/2	4/3	5/0	4/2	4/2	4/2	32	15	46.9
8	4/2	4/2	4/2	4/2	3/2	4/2	4/2	4/2	31	16	51.6
9	4/3	2/2	4/3	4/2	4/3	4/4	4/3	4/2	30	22	73.3
10	4/2	4/4	2/2	4/2	4/2	4/2	4/2	4/2	30	18	60.0
11	4/2	4/2	4/2	4/2	4/2	4/2	2/2	4/2	30	16	53.3
12	4/2	4/2	2/2	4/2	4/2	4/2	4/2	4/2	30	16	53.3
13	1/1	4/4	4/4	4/4	4/4	4/4	4/2	4/4	29	27	93.1
14	4/4	4/3	2/2	2/2	5/3	4/2	4/3	4/2	29	21	72.4
15	4/3	4/2	2/0	3/0	4/3	4/2	4/2	4/2	29	14	48.3
16	4/4	4/2	2/2	4/4	4/2	6/4	1/1	3/2	28	21	75.0
Mean									30.8	19.8	64.7

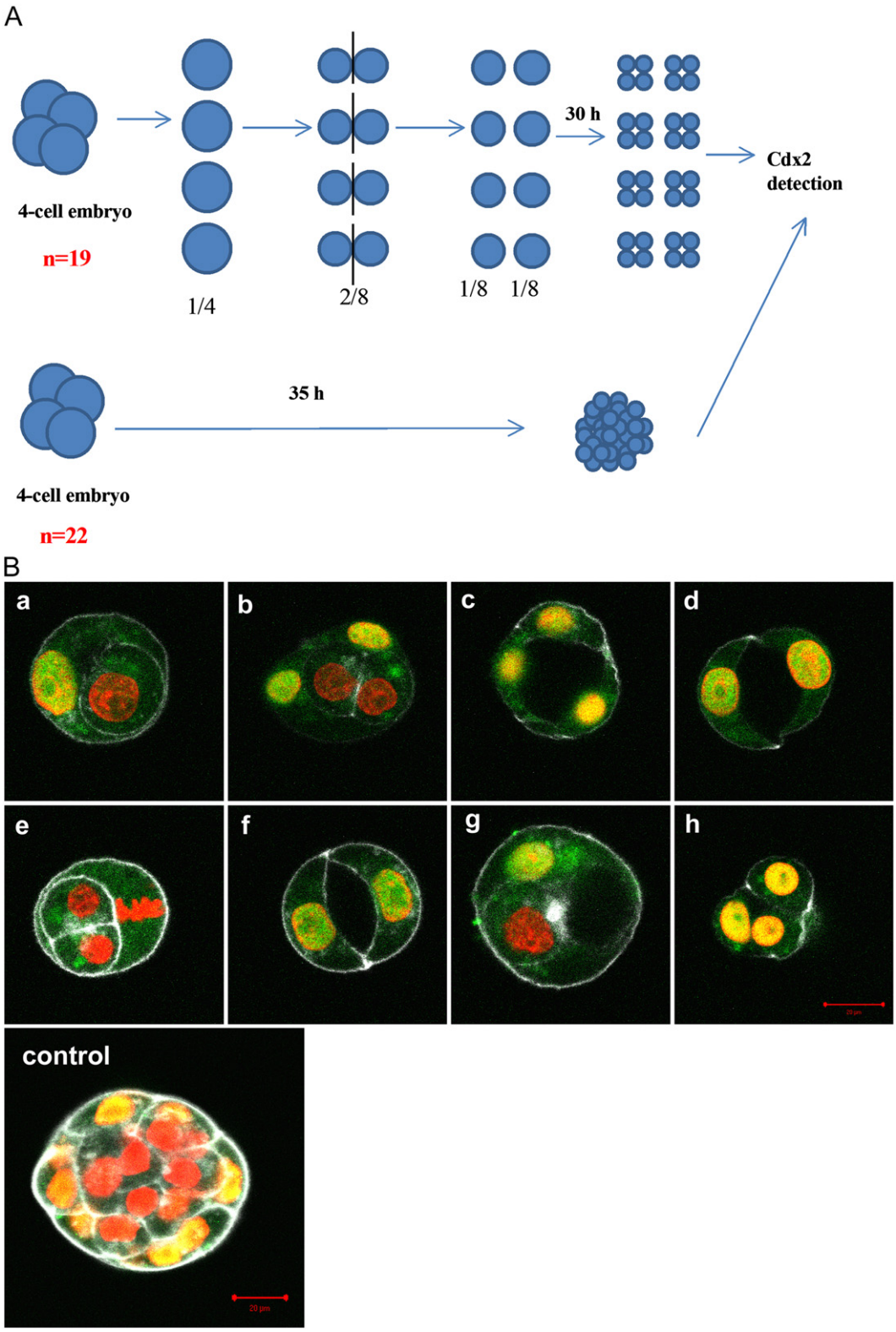

Fig. 2. Experiment 2 — embryo fragments developed from apolar 1/8 blastomeres. (A) Diagram of experiment 2. Embryos at the 4-cell stage were disaggregated and the individual blastomeres were in vitro cultured as a full set. Immediately after division the pairs of 1/8 blastomeres (2/8) were separated and the individual blastomeres 1/8 were cultured as a full set in vitro for 30 h to obtain 8 fragments (presumably composed of 4 cells). The intact 4-cell embryos were cultured for 35h to develop 32-cell embryos (control 2). (B) Cdx2 expression in the embryo fragments and control 32-cell embryo. All images are merged single optical images of chromatin (red) and Cdx2 (yellow) staining Pattern of Cdx2 expression (number of cells/number of Cdx+ cells) in the fragments is: (a) 2/1, (b) 4/2, (c) 3/3, (d) 2/2, (e) 3/1, (f) 2/2, (g) 3/1, and (h) 4/4. Scale bar 20 μm.

Table 2
Analysis of 19 of full sets of embryo fragments developed from 1/8 apolar blastomeres. The total number of cells and number of Cdx2+ cells was estimated. In each embryo fragment the Cdx2 expresion pattern is shown (number of cells/number of Cdx2+ cells).

Embryos no.	Number of cells/Cdx2+ cells in full sets of embryo fragments								Total number of cells	Number of Cdx2+ cells	%Cdx2+ cells
	1	2	3	4	5	6	7	8			
1	5/4	4/3	4/4	4/4	4/2	4/4	4/2	4/4	33	27	81.8
2	4/2	4/4	5/5	4/2	4/4	4/4	4/2	4/2	33	25	76
3	4/4	4/4	4/2	4/4	4/4	4/4	4/4	4/4	32	29	94
4	4/2	4/4	4/4	4/4	4/4	4/4	4/3	4/4	32	29	90.6
5	4/2	4/4	4/2	4/3	4/2	4/2	4/2	4/4	32	21	66
6	4/4	4/2	4/4	4/4	4/4	3/3	4/3	4/4	31	28	90
7	4/2	4/4	4/4	4/4	4/4	4/4	4/2	3/2	31	26	87
8	3/3	4/3	4/4	4/4	4/2	4/2	4/4	4/4	31	26	84
9	4/4	4/2	4/2	4/4	4/4	4/3	4/2	3/3	31	24	77
10	4/4	4/2	4/4	4/4	2/1	4/4	4/2	5/3	31	24	77
11	4/4	4/2	3/2	4/2	4/2	4/4	4/2	4/4	31	22	70.9
12	4/2	4/2	3/2	4/2	4/2	4/2	4/4	4/2	31	18	58
13	4/4	3/2	3/3	4/4	5/5	2/1	4/2	5/3	30	24	80
14	4/4	4/3	2/1	4/3	4/2	4/2	4/3	4/4	30	22	73
15	2/1	4/2	4/4	4/4	4/2	4/3	4/4	3/3	29	23	79
16	4/2	4/4	4/4	4/2	4/4	3/2	4/2	2/2	29	22	76
17	4/3	4/2	4/4	4/2	2/1	2/1	4/2	4/2	28	17	61
18	2/1	3/3	4/4	3/3	4/2	4/4	4/4	3/3	27	24	89
19	2/0	4/2	2/2	5/5	4/2	2/2	4/4	4/4	27	21	78
Mean									30.5	23.8	78.3

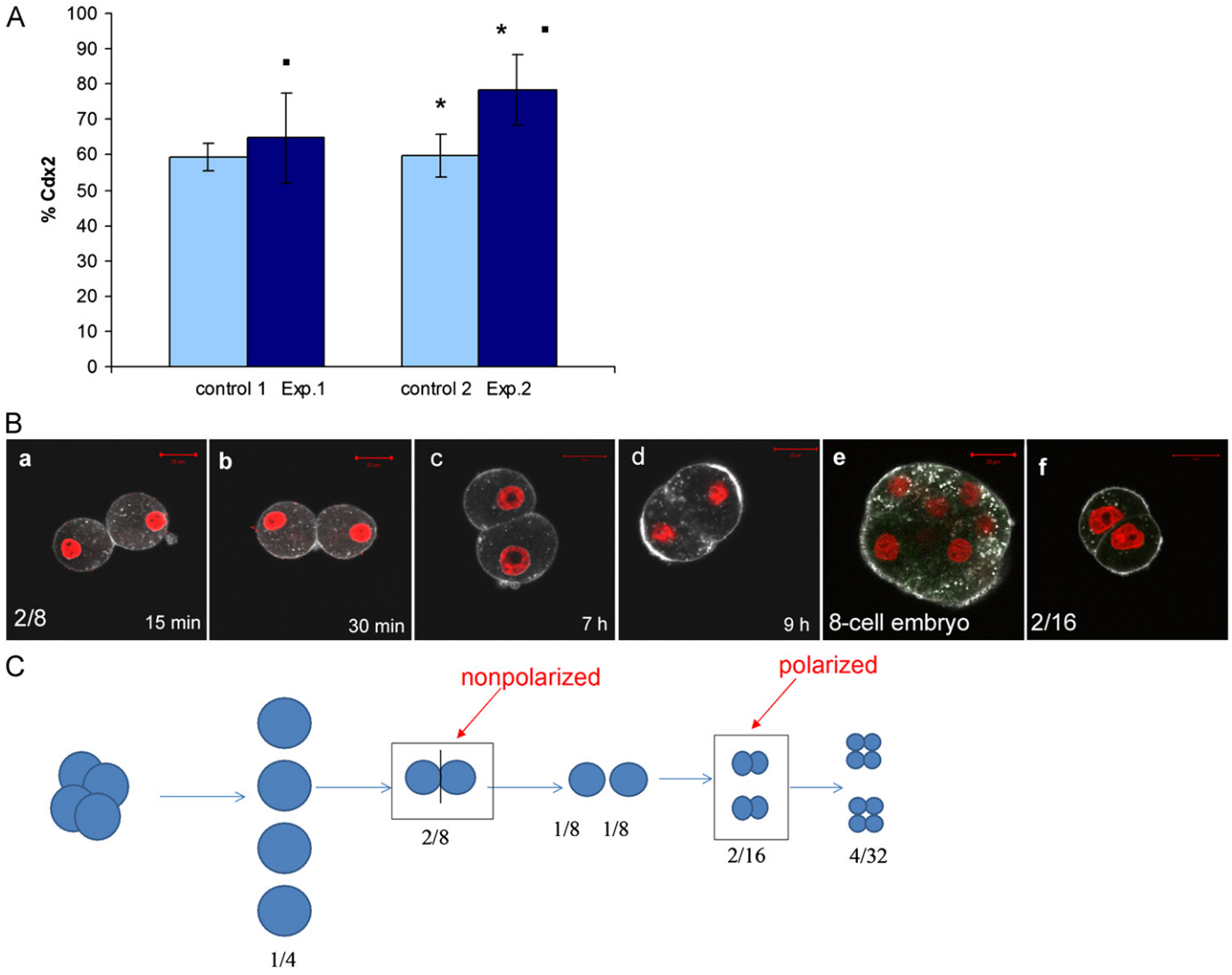

Fig. 3. (A) Analysis of Cdx2-positive cells in embryo fragments. In each of full sets of fragments (experimental embryo) which developed from blastomeres of 8-cell stage embryo (polar in Exp. 1, or apolar in Exp. 2), the total number of cells and the frequency of Cdx2+ cells were estimated (Tables 1 and 2) and compared to the Cdx2 frequency in intact control embryos (control 1 and control 2, Tables S1 and S2). Statistical significance: $^{*}p < 0.0001$, $^{\bullet}p < 0.05$. (B) Detection of polarization of blastomeres—in 2/8 couplets and in 2/16 couplets using FITC-ConA labeling. The 2/8 couplets, obtained from a individual 1/4 blastomeres after dissagregation of 4-cell embryo, were cultured for (a) 15 min, (b) 30 min, (c) 7 h, (d) 9 h, (e) control, intact 8-cell stage embryo, and (f) the couplets of 2/16 blastomeres became polarized during in vitro culture. The caps of microvilli are indicated by white arrows. Scale bar 20 μm. (C) Diagram depicting the polarization of cells in Exp. 2. The couplets of 2/8 blastomeres were apolar during their separation. The cells forming the 2/16 couplets polarized during in vitro culture.

Preventing the polarization at the 8-cell stage results in delaying the polarization to the 16-cell stage

To obtain apolar 1/8 blastomeres described in the previous section we separated them from the pairs of 2/8 resulting from single 1/4 blastomere division. As the procedure of separation takes about 15–30 min, we had to be sure that during that time the pair of 2/8 blastomeres did not polarize. We used the FITC-ConA staining, which detects formation of the cap of microvilli on the apical part of polar cell which is the early sign of polarization. We found that 2/8 couplets resulting from single 1/4 blastomeres cultured for 15 min and 30 min, remained apolar (Fig. 3B). The first signs of polarization were detected after 7 h of culture, and distinct cap of microvili was noticeable after 9 h. Similar sign of polarization was observed in control intact 8-cell embryos that developed from 4-cell stage embryos (Fig. 3B).

This indicates that the 1/8 blastomeres separated from the 2/8 pairs within 15–30 min after their formation, were apolar (Figs. 2A and 3C). However we found that during the next 10–12 h, when the 1/8 divided and the embryo fragments were composed of two 1/16 blastomeres (2/16 pair), both of the cells became polarized, as was confirmed by ConA labeling (Fig. 3B and C).

Taken together, we found that when embryo fragments were obtained from apolar 1/8 blastomeres, the polarization of blastomeres was delayed to the 16-cell stage. Because in the embryo fragments (in contrast to the intact embryos) all of the 1/16 blastomeres were polarized, this results in increase of number of Cdx2-positive cells

Delayed polarization results in changes in Cdx2 expression pattern

In full sets of fragments obtained from both polar 1/8 blastomeres (Fig. 1A, Table 1) and apolar 1/8 blastomeres (Fig. 2A, Table 2), the 4-cell fragments (4/32 quartets) were the most frequent (82% and 80%, respectively). We divided all of the 4-cell embryo fragments into three groups depending on Cdx2 expression pattern — those in which 2 out of 4 (4/2), 3 out of 4 (4/3) or all 4 (4/4) cells were Cdx2 positive. We found that among all of the 4-cells fragments obtained from polar 1/8 blastomeres, 62.8% of fragments showed the 4/2 pattern of Cdx2 expression, while the patterns 4/3 and 4/4 were observed in 15.2% and 22% of fragments, respectively (Fig. 4A and B). In contrast, in the group of the 4-cells embryo fragments, which developed from apolar 1/8 blastomeres, the most frequent was the 4/4 pattern of Cdx2 expression (52%) (Fig. 4A and B), although the fragments in which 2 out of 4 cells were Cdx2-positive (4/2) were also represented with high frequency (39%). Fragments, in which 3 out of 4 cells (4/3) were Cdx2 positive were observed with only 9% frequency. To explain the origin of Cdx2 expression pattern in the 4-cells fragments developed from polar 1/8 blastomeres we propose the model of inheritance of polar domain during cell division (Fig. 5, Exp. 1). This model includes all possibilities of symmetric (A–C) and asymmetric (D and E) divisions of polar 1/8 blastomeres, and subsequent divisions of 1/16 blastomeres. Symmetric division generates two polar cells while the asymmetric division results in one polar and one apolar cell. In our experiments the most of the 4-cell fragments were composed of two bigger, Cdx2-positive cells and two smaller, Cdx2-negative cells (pattern 4/2, Fig. 4A and B). This is in good agreement with our model (Fig. 5, Exp. 1) predicting that such pattern of Cdx2 expression is the result of either symmetrical (C) or asymmetrical (D) division of the 1/8 blastomere. Because in intact embryos at least 5 out of 8 blastomeres undergo asymmetrical division (Suwinska et al., 2008) we can speculate that embryo fragments with the 4/2 pattern of Cdx2 expression developed mostly from the asymmetrical division of 1/8 blastomeres (Fig. 5 Exp. 1, D). The fragments with four

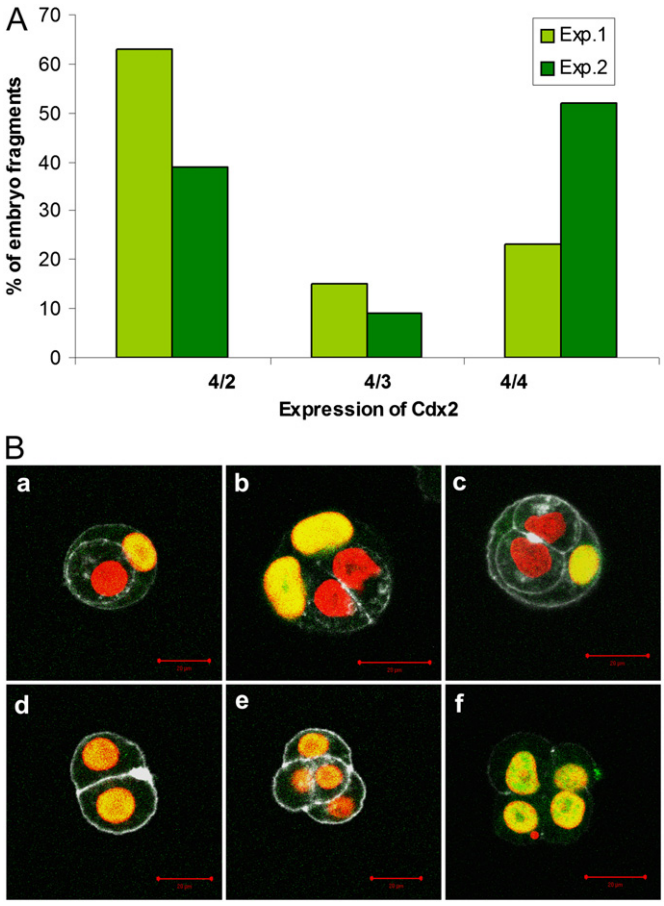

Fig. 4. (A) Pattern of Cdx2 expression in 4-cells fragments of embryos developed in vitro from 1/8 blastomeres in Exp. 1 and Exp. 2. The 4-cell embryo fragments were divided into three groups depending on Cdx2 expression pattern: 4/2, 4/3 and 4/4 (number of cells/number of Cdx+ cells). In Exp. 1 the most frequent was the 4/2 pattern (62.8%) and in Exp. 2 — the 4/4 pattern (52%) of Cdx2 expression. (B) Pattern of cleavage and Cdx2 expression among the embryo fragments. All images are merged single optical images of chromatin (red) and Cdx2 (yellow) staining (a)–(c) After asymmetric divisions the bigger cells are Cdx2-positive and the smaller cells are Cdx2-negative. The bigger cells surrounded the smaller cells. (a) 2-cell fragment (2/1 pattern); (b) 4-cell fragment with the 4/2 Cdx2 expression pattern (see Fig. 5 Exp. 1C and D); (c) 3-cell fragment (3/1 pattern); (d)–(f) after symmetric divisions the cells are of equal size and all are Cdx2-positive. D/2-cell fragment (2/2), (e) 4-cell fragment (4/4), and (f) 4-cell fragment (4/4) (see Fig. 5 Exp. 1, A, Exp. 2, A). Scale bar 20 μm.

(4/4) or three (4/3) Cdx2-positive cells were less frequent, and we believe that they were the result of symmetrical divisions of 1/8 blastomeres (Fig. 5 Exp. 1, A and B). In the intact 8-cell embryo symmetrical division occurs in no more than 3 out of 8 blastomeres (Suwinska et al., 2008).

In our second experiment, when the embryo fragments developed from apolar 1/8 blastomeres, polarization and compaction took place when the embryo fragments were composed of two 1/16 blastomeres (Fig. 3C). Because both of the blastomeres in the 2/16 pair were polarized, such delayed polarization resulted in increase number of polar cells in comparison to intact 16-cell embryo where at least 5 apolar blastomeres were present, resulting from the asymmetric division (Fig. 5, Exp. 1, D, Exp. 2). We conclude that the higher number of polar cells in Exp. 2 leads to increase in number of Cdx2-positive blastomeres in 4/32 quartets. We observed that most of the fragments were composed of 4 Cdx2-positive cells (pattern 4/4), which is consistent with the scheme of divisions of two polar 1/16 cells (Fig. 5, Exp. 2). If one of the cells in the 2/16 doublet was apolar then it would give rise to two Cdx2 expression patterns: 4/1 and 4/2 with all cells of equal size. Because

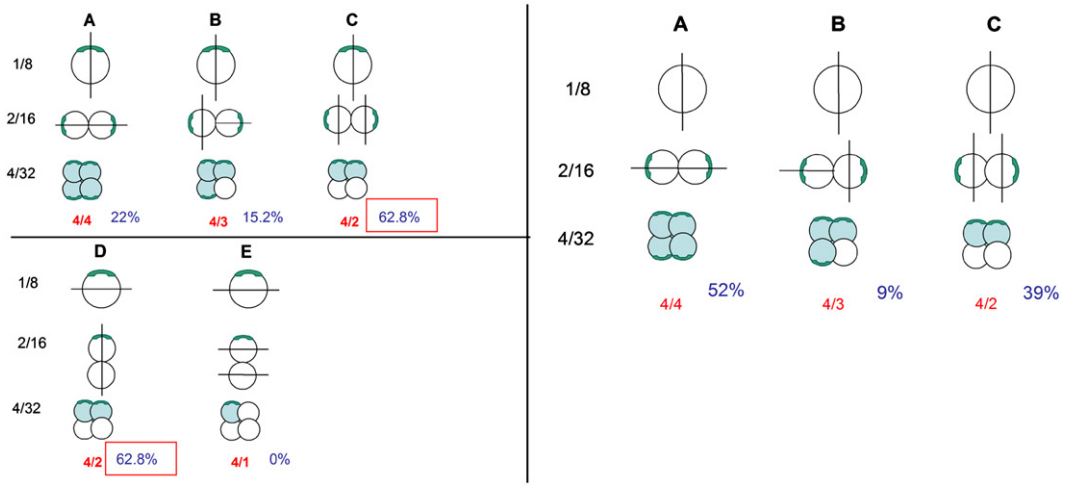

Fig. 5. Exp. 1. The model of cells division of 1/8 polar blastomeres in the Experiment 1. The pattern of Cdx2 expression depends on the pattern of the inheritance of polar domain (marked as yellow cap) during two rounds of divisions (1/8–2/16 and 2/16–4/32). Model shows all possibilities of symmetric (A–C) and asymmetric (D and E) divisions of polar 1/8 blastomeres, and during subsequent round of divisions of 1/16 blastomeres. As the result the 4/32 fragments develops with different Cdx2 expression patterns: 4/4, 4/3 and 4/2 (A–C) and 4/2 and 4/1 (D and E). The most frequent pattern of Cdx2 expression is 4/2 (62.8%). Exp. 2. The model of cells division of polar 2/16 couplets of blastomeres in the Experiment 2. It was assumed that all of 1/16 blastomeres were polarized before divisions leading to 4/32 fragments (see Fig. 2B). Model shows all possibilities of symmetric and asymmetric divisions of polar 1/16 blastomeres. The pattern of Cdx2 expression in 4/32 fragments depends on the pattern of the inheritance of polar domain (marked as a red cap). The 4/4 Cdx2 expression patter is the most frequent (52%).

we never found the 4/1 pattern, and in all of 4/2 fragments the Cdx2-positive cells were bigger than Cdx2-negative cells, we conclude that the 2/16 doublets were always composed of two polar cells (Fig. 5, Exp. 2).

Discussion

The crucial event responsible for the segregation of the first two cell lineages in preimplantation mouse embryo is the upregulation of Cdx2 expression in outer cells observed at the 16- to 32-cell stage transition. This results in the outer cells becoming the TE precursors and inside cells contributing to the ICM of developing blastocyst. The mechanisms underlying the induction of Cdx2 expression in the outer cells remains unclear, but it seems that fate of outer cells is determined not only by their position in embryo (Tarkowski and Wroblewska, 1967; Yagi et al., 2007), but also by the polarization event at the 8-cell stage (Jedrusik et al., 2008; Johnson and Ziomek, 1981b; Ralston and Rossant, 2008).

In the present study we investigated which one of these two factors – cell position or cell polarization – is the most important for the upregulation of Cdx2 in the outer cells of the mouse embryo during TE formation. The main goal of our study was to generate and analyze separately a full set of embryo fragments originating from each of the blastomeres of 8-cell embryo. We prevented the formation of inner cells during two subsequent cleavages of blastomeres, as all of the cells in embryo fragments were exposed to the embryo surface. When we used blastomeres isolated from 8-cell embryos at the stage in which all blastomeres are already polarized (Johnson and Ziomek, 1981b) the proportion of cells which express Cdx2 in embryo fragments was similar to that observed in intact embryos. Our results are consistent with the Cell Polarity Model, which implies that during the asymmetric division of polar blastomeres at the 8-cell stage, the only cells able to express Cdx2 will be those descendants of the outer cells who inherited the polarity domain (Yamanaka et al., 2006). We conclude that among the descendants of the individual polar blastomeres of 8-cell compacted embryo the number of Cdx2-positive cells is already established and does not change, even if the position of blastomeres had been changed inside out. While studying the 2/16 doublets, Dietrich and Hiiragi (2007) came to the similar conclusion—the outer position was not sufficient to change the fate of "inner" blastomeres. The advantage of our experimental design was that we obtained full sets of embryo fragments originating from all the blastomeres of one 8-cell embryo and this allowed us to precisely differentiate between the effect of blastomere position and polarization on its subsequent differentiation.

Previous studies showed that the polarity established at the 8-cell stage is conserved throughout the subsequent division (Johnson and Ziomek, 1981a, 1981b). It was also observed that separated polarized 1/8 blastomere after subsequent division produced a pair of 2/16 blastomeres, which in most cases consisted of a bigger polar and a smaller nonpolar cell. Moreover it was shown that the inequality in size was paralleled by differential Cdx2 expression—the bigger cell showed higher level of Cdx2 expression than the smaller one (Dietrich and Hiiragi, 2007). Our results indicates that the polarity inherited by 2/16 doublets is also conserved throughout the next division, when 4/32 quartets are formed, because we observed the correlation between cell size and Cdx2 expression—two bigger cells were always Cdx2-positive and two smaller cells were Cdx2-negative. These embryo fragments were probably in most cases descendents of these 2/16 doublets, which were unequal in size as the result of asymmetric division of polar 1/8 blastomere.

The mechanism controlling the pattern of the cleavage from 8- to 16-cell stage in mouse embryo is unclear, but there are data showing that the higher level of Cdx2 protein and/or RNA expression promotes asymmetric cleavages (Jedrusik et al., 2008). It is known that the ratio of inside to outside blastomeres in intact 16-cell stage embryo varies from 8:8 to 5:11 (Handyside, 1981; Johnson and Ziomek, 1981a; Suwinska et al., 2008). This means that at least 5 blastomeres of 8-cell embryo divide asymmetrically, allocating descending cells into the inner and outer populations. After the subsequent division the number of outside cells in 32-cell embryo averages 19.5 (Suwinska et al., 2008), which is in good agreement with the mean number of Cdx2-positive cells observed in intact embryos in our experiments. Moreover, in our experiments, the mean number of Cdx2-positive cells per embryo in the embryo fragments developed from blastomeres isolated from compact 8-cell embryos was

similar to that in intact embryos (19.8). This indicates that the fate of descendants of 1/8 polar blastomeres did not change, despite the fact, that all or at least most of blastomeres in the embryo fragments become the outer cells. It seems that the outer position is not sufficient to induce the Cdx2 expression in the blastomeres that correspond to the inner blastomeres of the 16- and 32-cell stage of intact mouse embryo.

Our results indicate that the pattern of division (asymmetric or symmetric) in polar blastomeres of 8-cell embryo is already established, and it is the main factor responsible for the regulation of the pattern of Cdx2 expression in descending cells. In addition, when we experimentally prevented polarization of 1/8 blastomeres, so they polarized only after the division to 16-cell stage (i.e. as 2/16 doublets), we observed that the number of Cdx2-positive cells increased. Therefore, we believe that the time of the first polarization event is crucial for the pattern of Cdx2 expression.

Taken together our experiments suggest that although polarization event depends on the outer position of the cell, when polarization is established, it is the main factor affecting Cdx2 expression in the TE precursors.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.013.

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