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Regression of trophectodermal protrusions in the mouse blastocyst correlates with primitive endoderm formation

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This study examined trophectoderm (TE) protrusions covering the inner cell mass (ICM) during blastocyst development. Using fluorescein-labelled latex beads in E3.5 and E4.5 mouse embryos, we assessed the presence of TE protrusions (TP) and their relationship to primitive endoderm (PrE) formation. Beads were endocytosed by outer embryonic cells, enabling visualization of protrusions by confocal microscopy. We observed that TE protrusion coverage of the ICM decreased as blastocyst cell numbers increased, regressing entirely by E4.5. At this stage, no protrusions remained and the ICM exhibited a distinct PrE layer following cell sorting.

KEY WORDS: mouse embryo / blastocyst / inner cell mass / primitive endoderm / trophectoderm protrusions

The presence of cell processes on the surface of the inner cell mass (ICM), oriented towards the mouse blastocyst cavity, was first reported by Ducibella and colleagues in a study on cell junctional morphology during blastocyst development [Ducibella *et al*. 1975]. The study proposed that such processes assist in anchoring the ICM eccentrically within the trophectoderm (TE). Later, Fleming *et al*. [1984] confirmed the presence of cytoplasmic projections partially or entirely covering the surface of

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the ICM exposed to the blastocyst cavity. These cytoplasmic projections arise from TE cells, as indicated by secondary lysosomes unique to TE cytoplasm and by densely packed mitochondria, which are smaller compared to those in ICM cells. Additionally, scanning electron microscopy (SEM) images indicated the emergence of projections from outer cells [Fleming *et al*. 1984].

Based on this evidence, the primary objective of this study was to investigate whether trophectodermal protrusions (TP) contacting the ICM surface can be detected using fluorescein-labelled latex beads internalised by outer embryonic cells. Just before implantation, ICM cells segregate as the primitive endoderm (PrE) forms, separating the epiblast (EPI) from the blastocyst cavity. At E3.5, ICM cells show mutually exclusive expression of EPI- and PrE-specific transcription factors, and this pattern precedes their segregation into PrE and EPI precursors [Kurimoto *et al*. 2006, Plusa *et al*. 2008].

The second objective of this study was to examine at which developmental stage of the blastocyst TP appears and when they subsequently disappear. We also investigated whether the disappearance of TP covering the ICM correlates with the formation of the PrE in the blastocyst.

Material and methods

Animals

We used F1(C57Bl/6/Tar x CBA/Tar) mice for all the experiments. Animals were kept under a 14 h light/10 h dark cycle regime.

Collection of E3.5 mouse embryos

Embryos were obtained from natural mating. Presence of a vaginal plug on the morning after mating was taken as confirmation of successful copulation. On day 3.5 of pregnancy, females were euthanized by cervical dislocation, and uterus and oviducts were transferred to M2 medium supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma-Aldrich; Fulton and Whittingham, 1978). Embryos were collected by flushing the uterus with M2 medium using a syringe and by cutting the oviducts. The embryos were then incubated (Tissue Culture Dish 35 × 10 mm Falcon) in M2 under liquid paraffin (Sigma-Aldrich) at 37.5°C in 5% CO_2. Quality and developmental stage were assessed under an inverted microscope. Only early blastocysts were selected; delayed embryos at the morula stage and those lacking a zona pellucida were discarded.

Obtaining of E4.5 mouse embryos

To obtain E4.5 embryos, uterus and ovid ucts were isolated on day 3.5 of pregnancy as described above. Embryos were placed in drops of KSOM medium on plastic dishes (KSOM Powdered Media Kit – Millipore; Erbach *et al*. [1994] and cultured for 24 h at 37.5°C in 5% CO_2.

Labeling of TP

Zona pellucida was removed using acid Tyrode's solution (pH 2.5) – Nicolson *et al*. [1975]. Subsequently, the embryos were transferred into M2 medium. To prevent embryo adhesion, dishes were pre-coated with 1% Bacto™ agar in 0.9% NaCl (Becton, Dickinson and Company). To label TP, embryos were incubated for 10 min in the dark on a heating plate in M2 medium without BSA containing fluorescent latex beads (Fluoresbrite plain YG 0.2 µm microspheres, Polysciences, USA) at 1:20 dilution [Fleming and George 1987]. Embryos were then further incubated for 40 min in the dark on a heating plate in M2 medium with BSA to allow endocytosis of beads.

Immunofluorescent labelling

Embryos were fixed in 4% PFA in PBS without Ca^{2+} and Mg^{2+} (ThermoScientific) for 30 min, permeabilised with 0.5% Triton X-100 in PBS without Ca^{2+} and Mg^{2+} (Sigma) for 20 min and blocked overnight at 4 °C in 3% BSA to prevent non-specific binding. Subsequently, embryos were incubated with primary antibodies overnight at 4°C. For this purpose, they were transferred to a solution containing a mouse monoclonal IgG antibody against Cdx2 (1:50 in 3% BSA, BioGenex) to label TE cells. Additionally, some embryos were incubated in a solution containing a goat polyclonal IgG antibody against Sox17 (1:100 in 3% BSA, R&D Systems) to label PrE cells. The following day, the embryos were washed three times in PBS without Ca^{2+} and Mg^{2+} ions for 10 minutes each to remove unbound antibodies. They were incubated for 1 h in the dark with donkey anti-mouse IgG (Alexa Fluor 594, 1:200) in 3% BSA. PrE-labelled embryos were incubated for 1 h with donkey anti-goat IgG (Alexa Fluor 488, 1:200) in 3% BSA. Three additional washes were performed, each in PBS without Ca^{2+} and Mg^{2+} ions, for 10 min in the dark. Then, embryos were individually transferred to a glass-bottomed dish (Glass Bottom Microwell Dishes, 35mm Petri dish, 20 mm Microwell No. 1.5 cover glass 0.16 – 0.19mm, MatTek Corporation) for DRAQ (Biostatus Limited) staining to label chromatin (20 min in an incubator) and stored at 4°C in the dark. Embryos were examined using a Zeiss LSM 510 confocal microscope equipped with helium-neon and argon lasers.

Results analysis

The total number of cell nuclei and the number of TE cells were counted using Imaris and ImageJ software. Additionally, in embryos stained with the Sox17 marker, the number of PrE cells was determined. Statistical analyses were performed using Python (v3.10) with assistance from the ChatGPT language model (OpenAI, GPT-4, March 2025) for code generation and figure formatting. All results were independently verified by the authors.

Results and discussion

The experiments were conducted on two groups of embryos (Fig. 1). The first group included E3.5 embryos (n=71) and the second E4.5 embryos (n=57). To assess ICM cell sorting, embryos were labelled for Cdx2 and, in some cases, also for Sox17, a PrE marker. We found that in E3.5 embryos (n=22), all PrE and EPI cells were arranged in a mosaic-like "salt and pepper" pattern (Fig. 2). Among E4.5 embryos (n=30), 66.67% exhibited complete sorting of PrE and EPI cells within the ICM (Fig. 3).

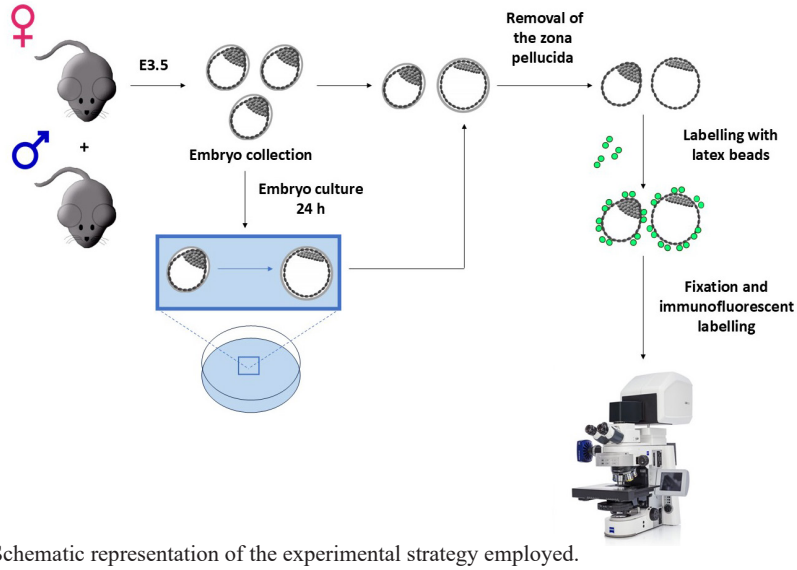

Fig. 1. Schematic representation of the experimental strategy employed.

The embryos were analysed for the presence of latex beads in cells located on the surface of the ICM, which was interpreted as the evidence of the presence of TP. After analysing 49 E3.5 blastocysts labelled with the antibodies against Cdx2 protein, TP were observed in 53.06% of the embryos (Fig. 4). Due to technical limitations (an insufficient number of latex beads bound to the outer cells of the embryo or an inadequate number of latex beads that underwent endocytosis), it was not possible to definitively determine the presence or absence of TP in 14.29% of the embryos. In the remaining 32.65%, TP were not detected.

In a group of 22 E3.5 blastocysts labelled with antibodies against Cdx2 and Sox17 proteins, TP were detected in 54.55% of the embryos (Fig. 2). In 13.64% of blastocysts, the presence of latex beads on the surface of the ICM could not be determined due to technical limitations. The remaining 31.81% showed no presence of TP.

No fluorescent labeling was detected on the ICM surface in any of the 57 E4.5 blastocysts, indicating the absence of TP at this stage (Fig. 3, 5).

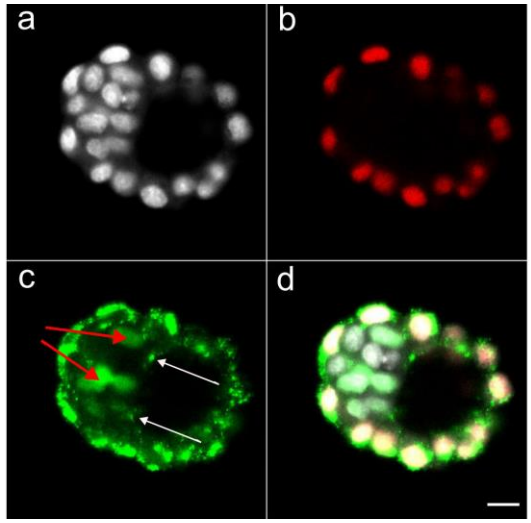

Fig. 2. Section through E3.5 embryo. (a) Nuclear chromatin is labelled in white. (b) Cdx2 protein is visualised in red within the nuclei of TE cells. (c) Sox17 protein is labelled in green within the nuclei of PrE cells, and latex beads, also labelled in green, are present in the cytoplasm of TE cells. White arrows indicate TE cytoplasmic protrusions. Red arrows indicate PrE cells. (d) Merged images. Scale bar: 20 µm.

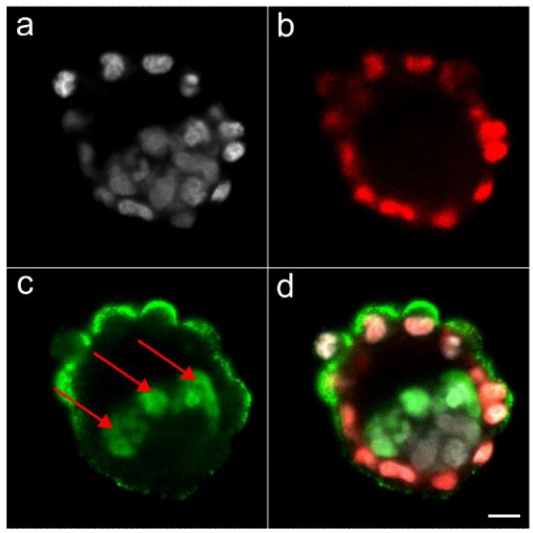

Fig. 3. Section through E4.5 blastocyst. (a) Nuclear chromatin is labelled in white. (b) Cdx2 protein is visualised in red within the nuclei of TE cells. (c) Sox17 protein is labelled in green within the nuclei of PrE cells. Red arrows indicate PrE cells. (d) Merged images. Scale bar: 20 µm.

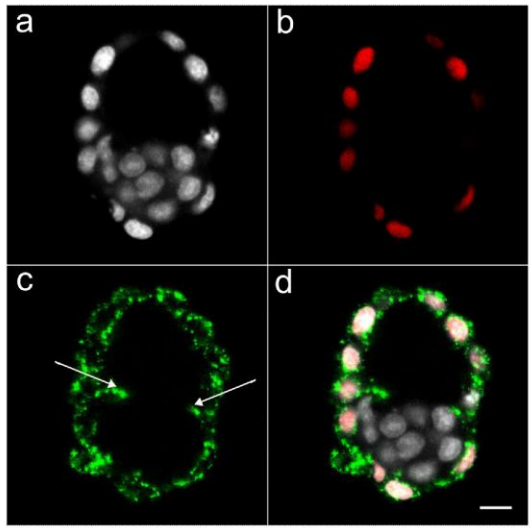

Fig. 4. Section through E3.5 blastocyst. (a) Nuclear chromatin is labelled in white. (b) Cdx2 protein is visualised in red within the nuclei of TE cells. (c) Latex beads are labelled in green within the cytoplasm of TE cells. Arrows indicate TE cytoplasmic protrusions. (d) Merged images. Scale bar: 20 µm.

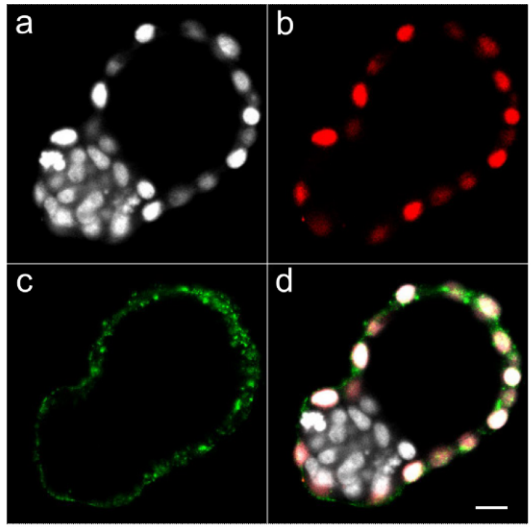

Fig. 5. Section through E4.5 blastocyst. (a) Nuclear chromatin is labelled in white. (b) Cdx2 protein is visualised in red within the nuclei of TE cells. (c) Latex beads are labelled in green within the cytoplasm of TE cells. (d) Merged images. Scale bar: 20 µm.

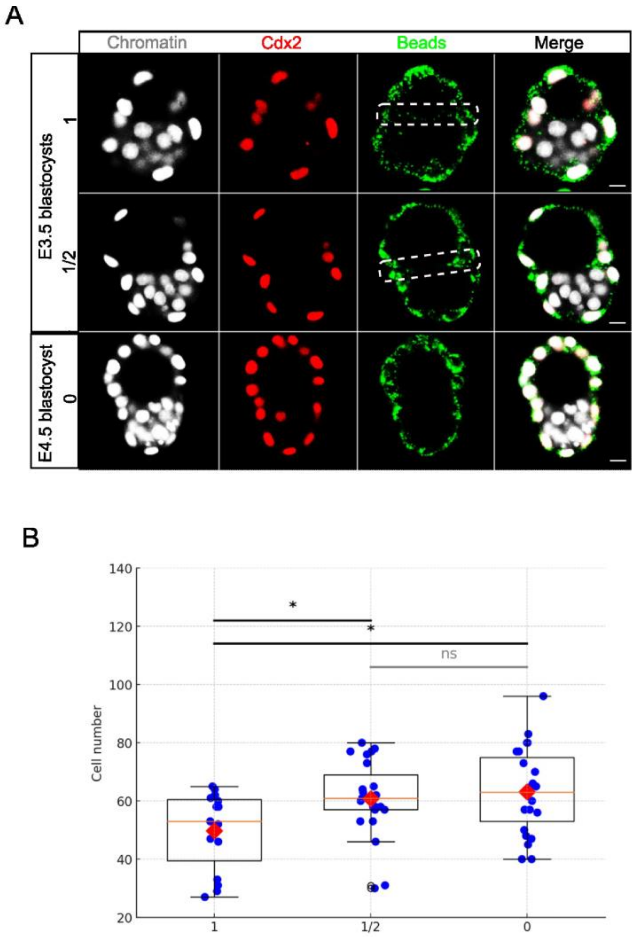

Fig. 6. Sections through blastocysts (A) and box plot of cell numbers (B) across three experimental groups: Group 1 ("1" - embryos with TP completely covering the ICM), Group 2 ("1/2" - embryos with TP covering half the ICM surface), and Group 3 ("0" - embryos without TP). (A) Detection of Cdx2 (red) and latex beads (green) in representative blastocysts. Chromatin (white) was labelled with DRAQ. Localisation of TP marked by dashed-line rectangles. (B) Individual data points are shown as blue dots, with red diamonds indicating the mean for each group. The boxes represent the interquartile range (IQR), with the median marked by the horizontal line inside the box. The whiskers extend to 1.5 times the IQR. Statistical significance, based on the Mann-Whitney test, is indicated by stars ("*") for significant pairwise differences ($p < 0.05$). Non-significant differences are labelled as "ns". The Y-axis shows the cell number, ranging from 20 to 140. Scale bar: 20 µm.

In E3.5 embryos with detectable TP, the extent of ICM surface coverage varied. Embryos with TP completely covering the ICM were designated as "1". Embryos with TP covering half the ICM surface were labelled as "1/2". Embryos without TP were marked as "0" (Fig. 6A).

The overall average cell counts of E3.5 embryos without TP was 63.41, ranging from 40 to 96 cells. Blastocysts with TP covering half of the ICM had an average cell count of 61.92, varying from 30 to 80 cells. Embryos with TP completely covering the ICM had an average cell count of 49.73, ranging from 27 to 65 cells. To examine the relationship between the degree of ICM coverage by TP and the total number of embryo cells, a Kruskal-Wallis test was conducted. Since $p<0.05$, this indicated that there was a statistically significant difference between at least one pair of groups. To identify which groups differed, a Mann-Whitney test was performed, showed that embryos with TP completely covering the ICM had significantly higher cell numbers than blastocysts without TP or with TP covering half of the ICM. (Fig. 6B).

The study first aimed to test whether latex bead labelling of TE cells can reveal TE cytoplasmic protrusions (TP) on the ICM surface. This method labels all TE cells without bead endocytosis into the blastocyst cavity or adjacent ICM cells as shown in earlier studies [Fleming and George 1987, Szczepanska *et al*. 2011], which reported that beads endocytosed by outer cells were found on the ICM surface, suggesting the presence of TP. Since beads label only TE cells, their detection on the ICM surface indicates TP. Our results confirm that bead endocytosis by outer TE cells enables identification of TP, formed by TE but projecting into the embryo.

During our experiments on two groups of E3.5 embryos, we detected TP in over a half of the embryos. In some embryos, when the image was taken from above, it was not possible to determine the presence of TP, thus such blastocysts were excluded from further analysis. Importantly, no TP were observed among E4.5 embryos, identified as late blastocysts.

Fleming *et al.* [1984] examined TE cytoplasmic protrusions in HRP-labelled embryos using SEM and TEM. SEM showed protrusions converging toward the ICM centre, some interconnected and others separated by gaps. HRP accumulation within these structures, absent in ICM cells, identified them as TE-derived. TEM revealed only fragmentary sections, later merged to reconstruct protrusions. From these analyses, the ICM surface was concluded to be covered by either a single continuous protrusion or two radially arranged protrusions narrowing toward the ICM [Fleming *et al.* 1984].

In another work, TP were visualised using transgenic mice with GFP expressed under the *Cdx2* promoter. 2A sequence inserted between GFP and H2B enabled releasing GFP into the cytoplasm, which allowed to observe the morphology and position of GFP-positive cells both outside and inside the embryo. TP were identified in all early and mid-blastocysts, while they were considered barely noticeable in late blastocysts. Confocal microscopy images showed that protrusions originated from GFP-positive cell precursors and surrounded GFP-negative ICM cells. These protrusions became thinner and eventually disappeared from the surface of the ICM in the late blastocyst stage. Additionally, phalloidin staining of the blastocysts cell boundaries confirmed that protrusions separated ICM cells from the blastocyst cavity [Toyooka *et al*. 2016].

Comparing the detectability of TP, which Toyooka *et al.* [2016] reported as reaching 100%, with our current results, where protrusions were visualized in just over half of the analysed E3.5 blastocysts, it becomes evident that the endocytosis of latex beads facilitates the detection of TP. However, limited TP detection in E3.5 blastocysts using latex beads compared to genetically modified animals may represent a false negative result due to the limited sensitivity of latex beads. Therefore, the use of transgenic mice with GFP expressed under the *Cdx2* promoter is probably the best method for this purpose. Nevertheless, using beads is undoubtedly cheaper and simpler than purchasing and maintaining transgenic mouse lines.

Toyooka *et al*. [2016] observed TP in early and mid-blastocysts, but they were absent or barely visible in late blastocysts. TP also disappeared during TE cell mitosis in some early and mid-blastocysts, though this did not appear to affect ICM surface cells, likely due to their brief exposure to the blastocyst cavity when protrusions were absent.

The TP observed in the present study covered the surface of the ICM to varying degrees. The extent of ICM surface coverage varied depending on the size of the blastocyst, with some showing full coverage of the ICM surface by TP, and others only half. However, no TP were observed in late blastocysts. These observations are consistent with the results of Toyooka *et al*. [2016], where it was noticed that TP became thinner and receded from the surface of the ICM with blastocyst development. Therefore, it was suggested that TP in the late blastocyst stage were replaced by a layer of PrE cells.

To test this hypothesis, embryos were additionally labelled for the PrE marker to track ICM cell sorting. In all late blastocysts with sorted ICM, TP were absent. Correlation between blastocyst cell number and TP extent further indicated that TP separating the ICM from the cavity diminish during development. Previous studies showed that ICM isolated from early blastocysts regenerates a TE layer, whereas late ICM forms a PrE layer [Hogan and Tilly 1978, Fleming *et al*. 1984, Szczepanska *et al.* 2011, Toyooka *et al*., 2016]. According to Fleming *et al*. [1984] TP may act as a protective barrier for ICM cells, separating them from the blastocyst cavity environment, thereby maintaining a population of pluripotent cells. This indicates that the role of TP is to separate ICM cells from the blastocyst cavity until the sorting of cells in the ICM and the formation of the PrE layer are complete.

In summary, our results show that TP disappear from the ICM surface as PrE formation completes. Their reduction and loss correlate with increasing blastocyst cell numbers, suggesting a transient role in shielding pluripotent ICM cells before lineage segregation. These findings support the idea that TP are replaced by the PrE layer and may function as structural or signaling mediators during early lineage specification.

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Ethics approval

All experiments were conducted in accordance with the ARRIVE guidelines and national and European regulations.

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