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Localization of embryonic stem cells lacking E-cadherin in a mouse blastocyst

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Here we present our results regarding the role of E-cadherin (CDH1, cadherin 1) in the specification of embryonic stem cells (ESCs) in the embryo environment. It has been previously shown that, when forming embryoid bodies with wild-type ESCs, ESCs lacking a functional copy of the gene encoding the adhesive protein E-cadherin (Ecad$^{-/-}$) preferably sort out to the outside compartment, thus forming the primitive endoderm (PrE) lineage. However, little or no information is available regarding the dynamics of Ecad$^{-/-}$ cells in the actual blastocyst, and so the aim of this work was to determine in which of the three blastocyst cell lines - trophoblast (TE), epiblast (Epi), or PrE - Ecad$^{-/-}$ cells would be located in the embryo environment. For this purpose we injected ESCs into embryos at various stages of pre-implantation development. We used a H2B-GFP cell line expressing histone H2B conjugated with green fluorescent protein (H2B-EGFP), as well as a Ecad$^{-/-}$ cell line, in which cells exhibit weaker adhesive properties than wild-type Esc because of their allelic deficiency in the locus encoding cadherin CDH1. We have demonstrated that these cells exhibit a trend to locate in the TE and much less frequently in the Epi, but never in the PrE. We propose that this may be due to differences in the expression of genes characteristic of these cell lines within the Ecad$^{-/-}$ cell colony.

KEY WORDS: embryonic stem cells / pluripotency / microinjection / adhesive properties /

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epiblast / primitive endoderm / trophectoderm

Adhesion properties are crucial for organ and tissue formation, cell migration or adherence to ECM as well as cell differentiation. Within mammalian cells E-cadherin is considered as a main protein responsible for adhesion, as among the cadherin family members it forms the strongest cell-cell connection. Lack of E-cadherin was already tested in the mouse embryo [Stephenson *et al*. 2010], resulting in failure of cell differentiation, compaction and preimplantation development. However, the described study was provided with embryos, in which all blastomeres lacked E-cadherin. We asked the question on the outcome of differentiation of E-cadherin deficient cells if they are surrounded by E-cadherin wildtype neighbors. Here we present data on the cell behavior/localization/incorporation, performed with E-cadherin deficient cells injected into the wild-type embryo.

Experiments described in this paper, aiming at the elucidation of the role of E-cadherin in the specification of ES cells, were conducted using embryonic stem cells (ESCs) that were injected into embryos at the 8-cell stage and into an early blastocyst. Firstly, regardless of their pluripotency stage ESCs injected into preimplantation embryos are always located in the epiblast (Epi), and displace the embryo's own cells from that lineage [Beddington and Robertson 1989, Poueymirou *et al*. 2007]. Additionally, primitive endoderm (PrE) cells display weaker adhesion properties than Epi cells [Filimonow *et al*. 2019], similarly to what has been shown concerning ESCs that lack a functional form of the E-cadherin gene Cdh1 [Hawkins *et al*. 2012]. Moreover, E-cadherin-deficient ESCs have been demonstrated to sort out to the surface compartment and differentiate into the PrE lineage when mixed with wild-type ESCs [Moore *et al*. 2009].

Accordingly, the aim of this study was to verify the hypothesis that reducing the adhesive properties of cells promotes their differentiation towards PrE cells in the embryo. Therefore, we decided to investigate whether ESCs lacking a functional form of the E-cadherin gene would be segregated to the ICM surface and create a PrE lineage due to their reduced adhesion properties. This would help explain the importance of E-cadherin-dependent adhesive properties in the segregation of ICM cells.

Material and methods

The study presented in this paper and the employed procedures obtained the approval of the Local Ethics Committee (permit number 545/2014).

Culture of embryonic stem cells

Two lines of ESCs were used for the experiments: H2B-GFP [Hadjantonakis and Papaioannou 2004] and E-cadherin deficient ($Ecad^{-/-}$) [Hawkins *et al*. 2012]. The ESC culture was carried on the mitomycin-inactivated mouse embryonic fibroblasts

(MEFs) in a standard ESC culture medium at 37.5°C and 5% CO2 at 95% humidity.

Obtaining 8-cell embryos and blastocysts

Both 8-cell stage embryos and blastocysts were isolated from hormonally stimulated female F1 (C57B1/Tar x CBAxTar) mice paired with F1 or GFP (UBI-GFP/BL6) males, respectively, for stages 64-72 and 90-95 hours after hCG administration. Embryos were cultured in M2 medium at 37.5°C with an atmosphere containing 5% CO2 until microinjection was initiated.

Microinjection of ESCs into blastocysts and in vitro embryo culture

Microinjection was performed in embryos isolated and placed in a droplet of the ESC suspension on the lid of a plastic dish covered with a layer of liquid paraffin. The batch of the ESC suspension was replaced with a fresh one every hour. A microinjection pipette (size 42, BM100T-15, BioMedical Instruments) was used to aspirate 10 ESCs and to puncture the zona pellucida - in cases when penetration appeared difficult, the procedure was assisted with the use of a piezo-injector (MW Piezo Stepper PM 10-1, Leica). ESCs were injected into the blastocyst cavity or were placed between the blastomeres of the 8-cell embryo. Both experimental (injected) and control (not injected) embryos were cultured in KSOM and fixed 48 hours after microinjection.

Detection of proteins in ESCs and ESC-derived cells in blastocysts using indirect immunofluorescence

Immunofluorescence assays were performed on ESCs cultured on cover slips (primary and secondary antibodies employed are detailed in Table 1). For the detection of Nanog, SOX2, GATA4, CDX2, E-cadherin, and N-cadherin ESCs were fixed in 4% PFA (Thermo Scientific) for 30 minutes. To label PECAM-1 protein, the ESCs were

Table 1. Primary and secondary antibodies used in this study

Primary antibodies			Secondary antibodies	
Protein	Description	Dilution	Description	Dilution
E-cadherin	rat (Takara)	1:500	goat α-rat 633 (Life Technologies)	1:200
N-cadherin	rabbit (BD Transduction Laboratories)	1:100	donkey α-rabbit TRITC (Jackson Immuno Research)	1:200
Cdx2	mouse (Biogenex)	1:50	donkey α-mouse TRITC (Jackson Immuno Research)	1:200
Gata4	rabbit (Santa Cruz)	1:100	goat α-rabbit 633 (Life Technologies)	1:200
Nanog	rabbit (CosmoBio)	1:50	donkey α-rabbit FITC (Jackson Immuno Research)	1:200
Pecam-1	rat (Becton Dickinson)	1:100	goat α-rat 633 (Life Technologies)	1:200
SSEA-1	mouse (Hybridoma Bank)	1:50	goat α-mouse (Jackson Laboratories)	1:200
Sox2	rabbit (R&D Systems)	1:100	donkey α-rabbit 594 (Life Technologies)	1:200

fixed with a 2.5% PFA for 15 minutes, while to label the SSEA-1 protein ESCs were fixed in a solution of methanol and acetic acid (7:3) for 8 minutes at -20°C. Following subsequent washes in PBS (Biomed, 3x 10 minutes), the cells were permeabilized in a 0.3% Triton X-100 (Sigma-Aldrich) solution in PBS without Ca^{2+} and Mg^{2+} ions (Biomed) for 20 minutes. Then, non-specific antibody binding sites were blocked in a 3% BSA solution with 0.01% Tween (BioRad Laboratories) in ion-free PBS overnight at 4°C. Cells were incubated with the primary antibody overnight in a humid chamber at 4°C and after washing in ion-free PBS they were incubated with the secondary antibody for 1.5 hours in the dark at room temperature. The nuclei were stained with 12.5 mM DRAQ in DAKO Fluorescent Mounting Medium. Blastocysts were fixed for 30 minutes in 4% PFA and subsequently subjected to the immunostaining procedure as described above. Chromatin staining in blastocysts was carried out using chromomycin.

Confocal microscopy

The ES cells and blastocysts were examined under a Zeiss LSM 510 confocal microscope, managed by the LSM Image Browser software. Whole embryos were imaged at 2 μm-thick optical Z sections using a 20x objective.

Results and discussion

The first step was to confirm the presence of pluripotency factors in H2B-GFP and $Ecad^{-/-}$ ES cells. In H2B-GFP cells both Nanog (Fig. 1A a-a") and SOX2 (Fig. 1A b-b") were detected in the nucleus of every cell. We also observed the presence of the adhesion protein E-cadherin in the cell membranes (Fig. 1A c-c"); however, no signal of N-cadherin was detected (Fig. 1A d-d"). We examined the location of pluripotency markers of the cell surface, including the platelet-endothelial cell adhesion molecule (PECAM-1), which presence is a typical trait of Epi cells [Robson *et al*. 2001], and SSEA-1, which is characteristic of pluripotent stem cells and has been described to play an important role in cell adhesion and migration in preimplantation mouse embryos [Furusawa *et al*. 2004, Williams and Stanley 2009]. We detected the presence of PECAM-1 (Fig. 1A e-e"), as well as SSEA-1 (Fig. 1A f-f") in H2B-GFP cells; based on these results, we confirmed that the H2B-GFP cells express pluripotency markers.

Regarding the $Ecad^{-/-}$ cell line, Nanog was present in a small number of cells (Fig. 1B a-a"). All colonies were characterized by the presence of Sox2, but this protein was not distributed uniformly within the colony - some cells showed a strong fluorescent signal indicating the presence of the protein, while others showed a much weaker signal (Fig. 1B b-b"). ESCs of the $Ecad^{-/-}$ line are characterized by the lack of the adhesion protein E-cadherin, which was confirmed by our observations (Fig. 1B c-c"). This is in agreement with previous observations [Hawkins *et al*. 2012], as we noticed

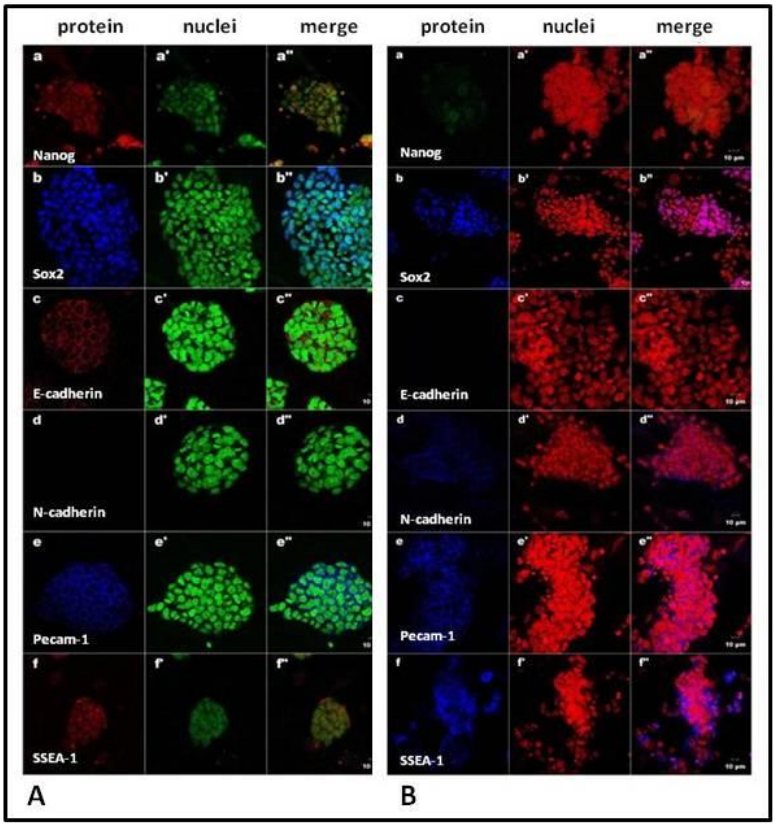

Fig. 1. (A) Localization of selected proteins in H2B - GFP cells. Photographs a-b show the presence of transcription factors Nanog and Sox2. Photographs c-f depict the location of selected surface proteins associated with intercellular adhesion. Cell nuclei (a'-f') are marked in green due to the presence of H2B histone conjugated with GFP protein. a"-f" - image overlay. (B) Localization of selected proteins in Ecad$^{-/-}$ cells. Photographs a-b show the presence of transcription factors Nanog and Sox2. Photographs c-f depict the position of selected surface proteins associated with intercellular adhesion. Cell nuclei (a'-f') were stained with DRAQ. a"-f" - image overlay.

the presence of N-cadherin in the Ecad$^{-/-}$ line (Fig. 1B d-d"). In addition, we detected the presence of PECAM-1 (Fig. 1B e-e") and the SSEA-1 protein (Fig. 1B f-f").

It is worth noting that we observed no colonies with a complete lack of the Sox2 protein. Only a portion of cells in the colony was devoid of this protein, or contained it in lesser amounts. This means that the cell, from which a given colony originated, must have been initially characterized by the presence of SOX2 at the time of seeding onto the culture dish and during culture the pluripotency of some SOX2-expressing cells decreased. In contrast, this was not the case with colonies of H2B-GFP cells grown under the same conditions, which indicates that cells lacking E-cadherin have a more labile phenotype than wild-type ESCs containing the GFP gene.

Our results confirmed previous observations by other authors, who reported a lack of E-cadherin leading to the presence of N-cadherin in Ecad$^{-/-}$ cells. It was shown that this compensation mechanism is responsible for maintaining the pluripotency of these cells via the STAT3 pathway [Hawkins *et al*. 2012]. However, it should be noted that N-cadherin has weaker adhesive properties than E-cadherin, so Ecad$^{-/-}$ cells are characterized by weaker adhesion than wild-type ESCs. In order to investigate whether these different adhesive properties can affect the location of Ecad$^{-/-}$ cells, we introduced ESCs into embryos at the 8-cell stage and the early blastocyst stage.

Embryos at the 8-cell stage, into which ESCs were injected, developed into early blastocysts within a 48h in vitro culture. We found that the ratio of the number of early blastocysts containing H2B-GFP cells to the total number of embryos subjected to microinjection was 92.8%. In 21.4% of the embryos, the number of ESCs and their progeny exceeded the number used for microinjection (10 cells), which indicates an integration efficiency of Ecad$^{-/-}$ cells amounting to 100%. To determine the lineage identity of injected H2B-GFP cells into the Epi, PrE, and TE lineages of the embryo, we immunostained for Cdx2, a TE marker, and Gata4, a protein specific to PrE. Cells that remained unlabeled were considered to be part of the Epi lineage. We then counted the number of ESC-derived cells in each cell lineage of the embryos (Tab. 2) and found that H2B-GFP cells were located exclusively in the Epi of all analyzed blastocysts, sometimes even completely displacing embryonic cells from that lineage (Fig. 2A). Additionally, no contribution of Ecad$^{-/-}$ cells to the PrE lineage was observed. The frequency of Ecad$^{-/-}$ cells in the Epi was minor; however, presence of Ecad$^{-/-}$ cells was detected in the TE (Tab. 2). Ecad$^{-/-}$ cells never seemed to contribute exclusively to the Epi lineage; rather, they were present in this lineage only when they also contributed to the TE lineage (Fig. 2B a-e). Nevertheless, even when integrating into the TE,

Table 2. Contribution of H2B-GFP ESCs and Ecad-/- ESCs, as well as embryo derived cells, to blastocysts developed from 8-cell embryos injected with ESCs

Item	Mean number (range) of ESC derived cells contributing to:			Mean number (range) of embryo derived cells contributing to:	
	EPI	PE	TE	EPI	PE
ESC H2B-GFP (n=14)	7.35±6.58 (0-23)	0	0	4.64±3.17 (0-10)	10.21±3.21 (6-15)
ESC Ecad$^{-/-}$ (n=11)	0.36±0.92 (0-3)	0	2.45±1.36 (1-5)	10.36±6.39 (1-21)	4.90±5.12 (1-14)

Ecad$^{-/-}$ cells never dominated this lineage; the highest number of these cells observed within the TE after microinjection was 5, but it rarely exceeded 2.

Embryos injected with ESCs at the early blastocyst stage were allowed to develop up to the late blastocyst stage. Analysis of these embryos revealed the presence of H2B-GFP cells only in 29 blastocysts out of the total number of 95 (30.5%). In microinjected embryos Ecad$^{-/-}$ cells were observed in 37 out of 46 (80.7%). In none of the embryos did we notice that the number of incorporated cells exceeded the number

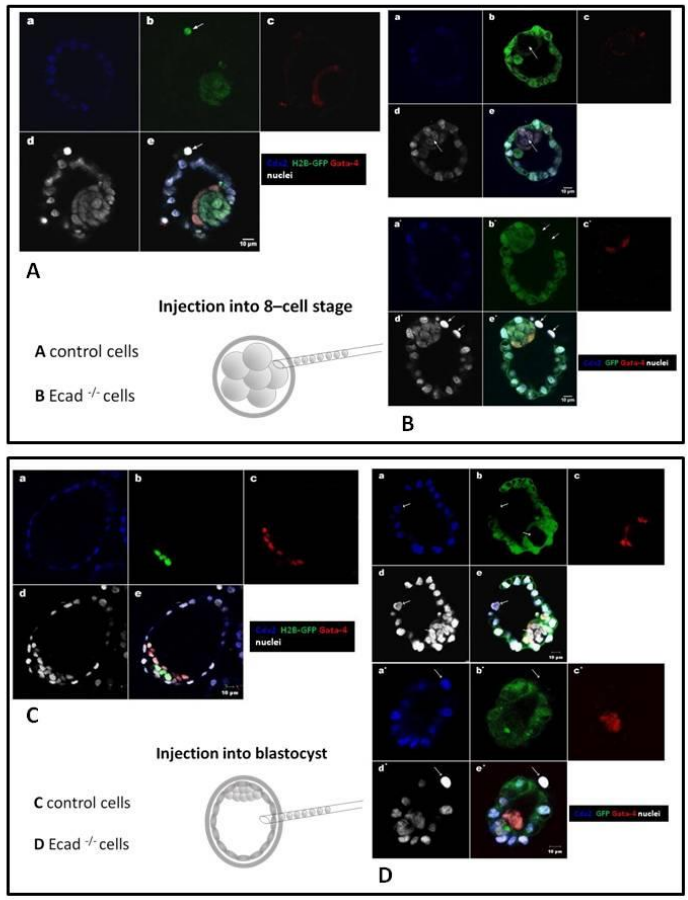

Fig. 2. (A) Localization of H2B-GFP cells in blastocysts derived from 8-cell F1 embryos. a) Cdx2 protein marked in blue in trophoblast cells; b) H2B-GFP ESCs expressing green fluorescent protein; c) Gata4 protein marked in red, a marker of primitive endoderm; d) white cell nuclei stained with chromomycin; e) overlay of all images. A white arrow points to an ESC „stuck" outside the embryo. (B) Localization of Ecad$^{-/-}$ cells in blastocysts derived from 8-cell GFP expressing embryos. a-e - blastocyst with embedded ESCs in both epiblast and trophoblast; a'-e' - blastocyst with ESCs present only in the trophoblast; a; a') Cdx2 protein marked in blue in trophoblast cells; b; b') embryo cells expressing green fluorescent protein; c; c') Gata4 protein marked in red, a marker of primitive endoderm; d; d') white cell nuclei stained with chromomycin; e; e') overlay of all images. White arrows indicate the location of embedded Ecad$^{-/-}$ ESCs in the embryo. (C) Localization of H2B-GFP cells after injection into early blastocysts. a) Cdx2 protein marked in blue in trophoblast cells; b) H2B-GFP ESCs expressing green fluorescent protein; c) Gata4 protein marked in red, a marker of primitive endoderm; d) white cell nuclei stained with chromomycin; e) overlay of all images. (D) Localization of Ecad$^{-/-}$ cells after injection into GFP expressing early blastocysts. a - e blastocyst with embedded ESCs both in the epiblast and trophoblast; a'- e' blastocyst, in which ESCs are present only in the trophoblast; a; a' in blue, the Cdx2 protein is marked in trophoblast cells; b; b' embryo cells expressing green fluorescent protein; c; c' in red, Gata4 protein, a marker of primitive endoderm is marked; d; d' white cell nuclei stained with chromomycin A; e; e' overlay of all images. The white arrows indicate the position of Ecad$^{-/-}$ cells.

Table 3. Contribution of H2B-GFP ESCs and Ecad-/- ESCs, as well as embryo derived cells, to late blastocysts developed from early blastocysts injected with ESCs

Item	Mean number (range) of ESC derived cells contributing to:			Mean number (range) of embryo derived cells contributing to:	
	EPI	PE	TE	EPI	PE
ESC H2B-GFP (n=40)	3.17±3.13 (0-11)	0	0	10.55±5.00 (2-24)	17.25±6.41 (8-35)
ESC Ecad$^{-/-}$ (n=41)	0.63±1.44 (0-6)	0	2.41±2.38 (1-8)	8.58±4.88 (0-22)	15.75±6.14 (3-27)

of H2B-GFP or Ecad$^{-/-}$ cells introduced during microinjection. The lineage of injected ESCs in blastocysts was determined as described above (Tab. 3). We found that ESCs of the H2B-GFP line injected into early blastocysts and integrated into the embryo are only found in the Epi cells of late blastocysts (Fig. 2C). Following injection of Ecad$^{-/-}$ cells, only a few of them were detected in the Epi (Tab. 3), averaging 0.6 of such cells per Epi in one embryo. Similarly to what was described earlier, no contribution of Ecad$^{-/-}$ cells to the PrE line of late blastocysts was observed. However, we did register the frequent presence of Ecad$^{-/-}$ ESCs in the TE (85.4% of blastocysts), where they were present in numbers ranging from 1 to 8, with an average of 2.4 (Tab. 3). Ecad$^{-/-}$ cells injected into early blastocysts were rarely found to incorporate exclusively into the Epi lineage. More commonly, we observed blastocysts, in which Ecad$^{-/-}$ ESCs were present in both the Epi and TE lineages (Tab. 3, Fig. 2D. a-e). In most cases the number of Ecad$^{-/-}$ cells that contributed to the TE lineage did not exceed 2 cells and therefore did not dominate this lineage.

As expected, control ESCs of the H2B-EGFP line injected into embryos were located exclusively in the Epi [Poueymirou *et al.* 2007]. The main premise for conducting experiments to determine in which blastocyst lineage ESCs lacking E-cadherin would be located was connected with the results of previous experiments conducted on embryoid bodies. In embryoid bodies formed from wild-type ESCs and those lacking the E-cadherin gene it was observed that cells lacking this protein were found on the surface of the aggregate, forming an outer differentiating layer in the PrE [Moore *et al.* 2009]. Another justification for conducting our experiments was also that it was shown that in the peri-implantation mouse blastocyst the amount of E-cadherin in the connections between PrE cells is lower than between Epi cells [Filimonow *et al.* 2019]. However, the results of the experiments presented in the current study showed that despite the lack of E-cadherin and the consequent reduction of adhesive properties resulting from the presence of only N-cadherin, Ecad$^{-/-}$ ESCs were not found in the PrE lineage. Within our research we never observed Ecad$^{-/-}$ ESCs incorporated into the PrE; therefore, it seems that the lack of E-cadherin and its replacement with N-cadherin, and the consequent weaker adhesion properties of the cells do not affect the location and differentiation of ESCs. We observed that Ecad$^{-/-}$ cells are predominantly located in the TE of manipulated embryos and express the transcription factor Cdx2, characteristic

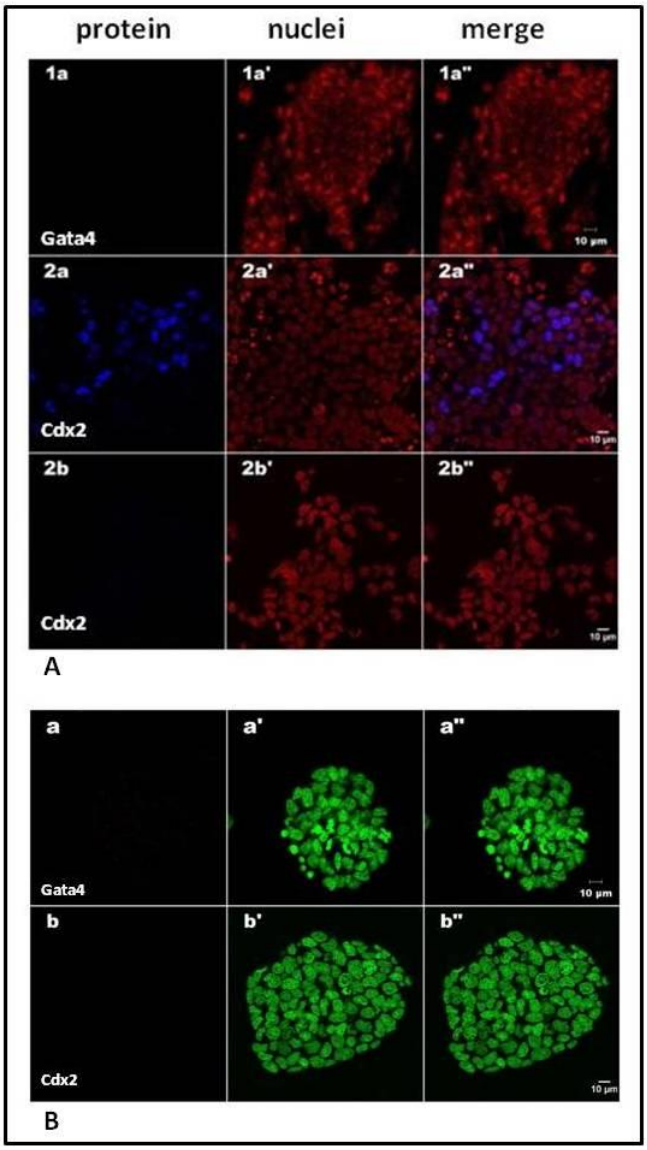

Fig. 3. (A) Localization of transcription factors Gata4 and Cdx2 in two cell colonies of Ecad$^{-/-}$ cells. Figures 1a - 1a" show the labeling of ESCs for the presence of the PE marker Gata4. Figures 2a - 2a" and 2b - 2b" show two different colonies of Ecad$^{-/-}$ ESCs labeled against the Cdx2 protein. Cell nuclei (1a'-2b') were labeled with DRAQ. Figures 1a" - 2b" show the overlay of images. (B) Lack of transcription factors Gata4 and Cdx2 in two cell colonies of H2B-GFP cells. Images a-a" show the labeling of ESCs for the presence of the primary endoderm marker Gata4. Images b-b" show a colony labeled against the Cdx2 protein. Cell nuclei (a'; b') are visible in green due to the presence of H2B histone conjugated with GFP protein. Images a"; b" show the overlay of all images.

of this lineage. ESCs have been long known to have the ability to spontaneously differentiate into lineages that normally originate from the Epi under culture conditions, but the differentiation into the extraembryonic TE was an unexpected observation. Therefore, we decided to verify whether CDX2 expression is triggered in these cells after their injection into embryos and integration into this lineage, or whether it occurs before injection, i.e. during Ecad$^{-/-}$ cell culture. We also decided to check whether the lineage marker is present in ESCs during culture. We found that neither Ecad$^{-/-}$ nor H2B-EGFP cells show the PrE marker Gata4 (Fig. 3AB). However, we detected the expression of Cdx2 - a marker characteristic of TE - in one third of the Ecad$^{-/-}$ cell colonies, whereas the remaining two thirds showed no signal of the protein (Fig. 3A). As for H2B-EGFP cells, we never observed the presence of Cdx2 after injection into embryos (Fig. 3B).

Unlike H2B-EGFP cells, Ecad$^{-/-}$ cells showed heterogeneous expression of Sox2. The question that arises then is whether Ecad$^{-/-}$ ESCs with high levels of SOX2 lack (or show low levels of) Cdx2 protein, and conversely, whether cells with reduced SOX2 expression also express CDX2. It is known that the differentiation of ESCs is accompanied by a decrease in the expression of pluripotency genes, but during the initial phase of their differentiation these cells exhibit proteins responsible for both pluripotency and cell differentiation [Ohinata *et al*. 2022]. Because the analysis of a potential correlation between SOX2 and CDX2 in E-cadherin-deficient cells falls beyond the scope of this study, in order to better understand the dynamics of these cells in the embryo it will be worthwhile to analyze the levels of SOX2 and the presence of CDX2 protein in Ecad$^{-/-}$ cells.

In conclusion, the results of our study do not provide a definitive answer to the question of how E-cadherin expression is linked to the expression of lineage-specific markers during mouse embryo pre-implantation development, but they provide a helpful starting point to direct further research to the relationship between E-cadherin and the differentiation of ESCs and mouse embryo cells towards the TE lineage and potential links with their pluripotency status.

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