

DISTRIBUTION OF PRIMITIVE ENDODERM AND EPIBLAST LINEAGE SPECIFIC FACTORS IN LATE STAGE BLASTOCYSTS

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Summary: Mouse preimplantation development leads to the formation of three distinct cell types by the blastocyst, namely, the trophoblast (TE) which is an apolarized epithelial layer, the inner cell mass which forms primitive endoderm, and pluripotent lineage epiblast. Segregation of the lineages is related to the expression of some key factors such as Cdx2, Oct4, Nanog, Gata4 and Sox17. We aimed to understand the role and distribution of these factors in mouse late blastocyst stage embryos. Two cell stage embryos were flushed from mated female B6D2F1 mice 44 hours after human chorionic gonadotropin injection. They were cultured in KSOM-AA medium at 37 °C with 5 % CO₂ in humidified air up to the late blastocyst stage. We performed immunofluorescent staining with antibodies specific to these factors. After that blastocysts were imaged and assessed using a fluorescence and confocal laser scanning microscope. We show that lineage specific factors, Gata4 and Sox17, were restricted to primitive endoderm while Nanog was restricted to epiderm lineage at late blastocyst stage. Moreover, the Oct4 protein is present in the nuclei in all cells but is strongly expressed in inner cell mass cells, whereas Cdx2 is localized only in TE cells. We conclude that lineage specific factors, Gata4, Sox17 and Nanog are essential for segregation of distinct lineages and blastocyst development.

Key words: blastocyst; epiblast; preimplantation; primitive endoderm; transcription factors

Introduction

Embryonic development in mammals is initiated with a series of mitotic cell divisions and cleavages to generate blastomeres. After the end of the many rounds of cleavages with compaction, an embryo with single fluid-filled large cavity is formed, and this embryo is specifically called blastocyst (1). In eutherian preimplantation development, two extraembryonic lineages, the trophoblast (TE) and the primitive endoderm

(PrE/hypoblast) are essential to support fetal development and survival (2). The TE, outer layer of cells that forms an epithelium to surround the blastocyst cavity, is segregated from the small cluster of cells called inner cell mass (ICM). While TE establishes the connection to the mother's uterus to initiate blastocyst implantation and differentiates into trophoblast to construct the placenta, the ICM is the source of cells that give rise to the fetus and additional extra-embryonic tissues (3, 4). Several transcription factors have been identified to be involved in TE and ICM development. The essential transcription factors are Oct4 (Pou5f1) and Cdx2, which are

POU-domain and caudal-type homeodomain transcription factors, respectively. Oct4, a key regulator of pluripotency, is strongly expressed in ICM and Cdx2 is specifically expressed in TE cells (1). Oct4 protein is present in nuclei of the all cells declining gradually in TE by early blastocyst stage, while only ICM cells show intense Oct4 staining by the late blastocyst stage (3, 5). Cdx2 is essential for maintenance of the TE lineage in mouse blastocysts, and suppresses the ICM lineage formation (6, 7).

As the blastocyst cavity expands between E3.5 and E4.5, ICM needs to segregate into two distinct layers of PrE and epiblast (Epi). Cells of the PrE, as a morphologically-distinct layer, congregate on the surface of the ICM. PrE forms an epithelial layer positioned at the interface between the ICM and blastocyst cavity, and the pluripotent epiblast appears in the ICM (2, 8, 9). After the implantation of the embryo to the uterus the PrE is eventually forms the visceral and parietal endoderm layers which contributes the yolk sac. Visceral endoderm initially covers the outside of the epiblast prior to gastrulation, later it is displaced by the definitive endoderm. And ends up as the outer layer of the extraembryonic visceral yolk sac. The Epi gives rise to most of the cells of the embryo proper as well as amnion, allantois and the extraembryonic mesoderm cells that line the visceral yolk sac. (2, 10, 11). It is also known that PrE extraembryonic lineages are not only essential to perform nutritive functions but they also are sources of signals to the Epi to initiate axial patterning (2, 8, 10). During the post-implantation period, an embryo convert with two germ layers (ectoderm and endoderm) into three germ layers. This process that ectoderm, mesoderm and endoderm germ layers are established named gastrulation (12).

The homeodomain transcription factors Oct4 and Nanog are expressed in the ICM and are required for proper formation of the epiblast (13-16). Nanog is expressed in ICM at E3.5 and becomes epiblast specific at E4.5 blastocyst stage (6). These transcription factors are also essential to maintain the embryonic stem cell pluripotency and self-renewal (17). The specification and differentiation of the PrE rely on the expression of key transcriptional regulators, primarily those belonging to GATA, SOX, and HNF protein families. Nanog and Gata are SOX family transcription factors that are used to mark the Epi and PrE, respectively. Epi cells are labelled by Nanog, a

homeodomain protein that is essential for the maintenance of pluripotency in mouse epiblast and embryonic stem cells (16). Sox17 is a member of SOX (SRY-related high mobility group box) transcription factor family and is first detected within the ICM, and is subsequently restricted to the PrE epithelium. Artus et al. (2011) indicate that distribution of Sox17 look like the localization of zinc finger transcription factors Gata binding protein 4 and 6 (Gata6, Gata4) in E4.5 blastocysts (11). Gata6 and Gata4 are expressed in the extra-embryonic endoderm lineages, primitive endoderm and the PrE derivatives; visceral endoderm and parietal endoderm (18, 19). Gata4 and Gata6 proteins have essential roles on differentiation of embryonic stem cells into extra-embryonic endoderm (19). It has also been suggested that Sox17 is a transcriptional regulator, and functions in the differentiation of pluripotent cells toward the extra-embryonic endoderm (17).

With this study, we demonstrate and review the current knowledge about essential transcription factors for mouse embryo development and their roles on maintenance of pluripotency in mouse late stage blastocysts (E4.5).

Material and methods

Animals

B6D2F1 (C57BL/6 x DBA/2) mice were purchased from National Cancer Institute. The protocol for animal handling and use was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Hawaii. The animals were maintained and treated according to the regulations and guidelines of the Animal and Veterinary Service at the University of Hawaii and the Committee for the Update of the Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research of the National Research Council of the National Academies (8th ed., 2011).

Embryo Collection

B6D2F1 female mice 6-8 weeks old, were induced to superovulate by intraperitoneally injections of 5 IU of equine chorionic gonadotropin (PMSG) and human chorionic gonadotropin (hCG)

at 48 hours (h) apart. Female mice were mated overnight with fertile males of the same strain. Following morning, inseminated females were selected by the presence of vaginal plug indicated the 1st day (day 1) of pregnancy. At day 2, 44 h after hCG injection female mice were sacrificed by cervical dislocation and two-cell stage embryos were flushed from the dissected oviducts with FHM HEPES-buffered medium (MR-024-D;EMD Milipore) under the stereo-microscope. After that, embryos were cultured in 20µl drops of KSOM-AA medium (MR-121-D;EMD Milipore) under mineral oil at 37 °C in a 5 % CO₂ humidified air incubator for the experiments.

Immunofluorescent Staining

Embryos were fixed in 4 % paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS) for 30 minutes (min) at room temperature. Embryos were subsequently permeabilized in PBS containing 0.5 % Triton X-100 for 15 min at room temperature. After blocking with 5 % bovine serum albumin in PBS containing 0.1 % Tween-20 (PBSw), samples were incubated in the primary antibody overnight at 4 °C and embryos were incubated in secondary antibody for 2-3 h at 25 °C. Primary antibodies used were, mouse anti-Cdx2 (1:200; Cdx2-88; BioGenex), goat anti-Gata4 (1:400; C-20, #sc-1237; Santa Cruz Biotechnology), goat anti Sox17 (1:100; S-20, # SC-17355), rabbit anti-Nanog (1:800; #RCAB0002P-F; Cosmo Bio) and goat anti-POU5F1 (1:200; N-19, #sc-8628; Santa Cruz Biotechnology), and Secondary antibodies (1:1000; Life Technologies) used were conjugated with Alexa Fluor 546 namely rabbit anti-mouse, donkey anti-goat and conjugated with Alexa Fluor 488, namely donkey anti-rabbit, rabbit anti-goat. Stained samples were mounted in ProLong Gold antifade reagent containing 4',6'-diamidino-2-phenylindole (DAPI; Life Technologies) (2).

Microscopy and Image Analysis

Embryos were imaged using an Axiovert 200 fluorescence microscope (Carl Zeiss) and FV1000 confocal laser scanning microscope (Olympus). For confocal microscopy, serial optical sections were imaged at 2 µm intervals under a 40x objective lens with oil.

Results

Expression of Cdx2 and Oct4 proteins in blastocyst stages of mouse embryos

Cdx2 expression is known as a marker of TE and TE precursors is absent from ICM (6, 20). Moreover, Oct4 is regarded as a marker of pluripotent cells, and is also expressed in TE cells of mouse blastocysts (3). We firstly examined Cdx2 and Oct4 protein expression of blastocysts in different embryos by immunofluorescence staining. Strong Cdx2 protein localization persisted in the nuclei of the TE cells (Figure 1A) while Oct4 protein localization was seen in both TE and ICM cells (Figure 1B). We next analyzed the Oct4 and Cdx2 expression in the same embryo. TE cells showed clear nuclear Cdx2 staining whereas Oct4 expression was revealed in nuclei of all the cells in blastocyst. Moreover, comparison of mean fluorescence intensities of Oct4 within the nuclei of ICM and TE cells revealed that ICM cells showed intense Oct4 staining (Figure 1C). We did not observe co-existence of Cdx2 within the ICM cells, suggesting that Cdx2 is localized exclusively in the nuclei of TE and is a TE specific transcription factor (Figure 1C). We also noted the same results in the literature that Cdx-2 staining cells in blastocysts were trophectoderm while the ICM was positive only for Oct4 (21).

Expression of lineage-specific transcription factors for PrE and Epi Tissue Segregation

To elucidate the PrE and Epi tissue segregation, we assessed the localization of the EPI-specific homeobox transcription factor Nanog and the PrE-specific factors Gata4 and Sox17 during this period (9, 22). To determine the expression domain of these transcription factors, E4.5 stage blastocysts were immunostained with specific antibodies and imaged using confocal and immunofluorescence microscopy. We confirmed that E4.5 blastocysts had an ICM whose cells were clumped and consisted of two distinct PrE and Epi cell lineages. PrE cells that are in contact with the blastocyst cavity, form the superficial layer of the ICM and while Epi cells form the deeper layer of ICM cells (Figure 2 and 3).

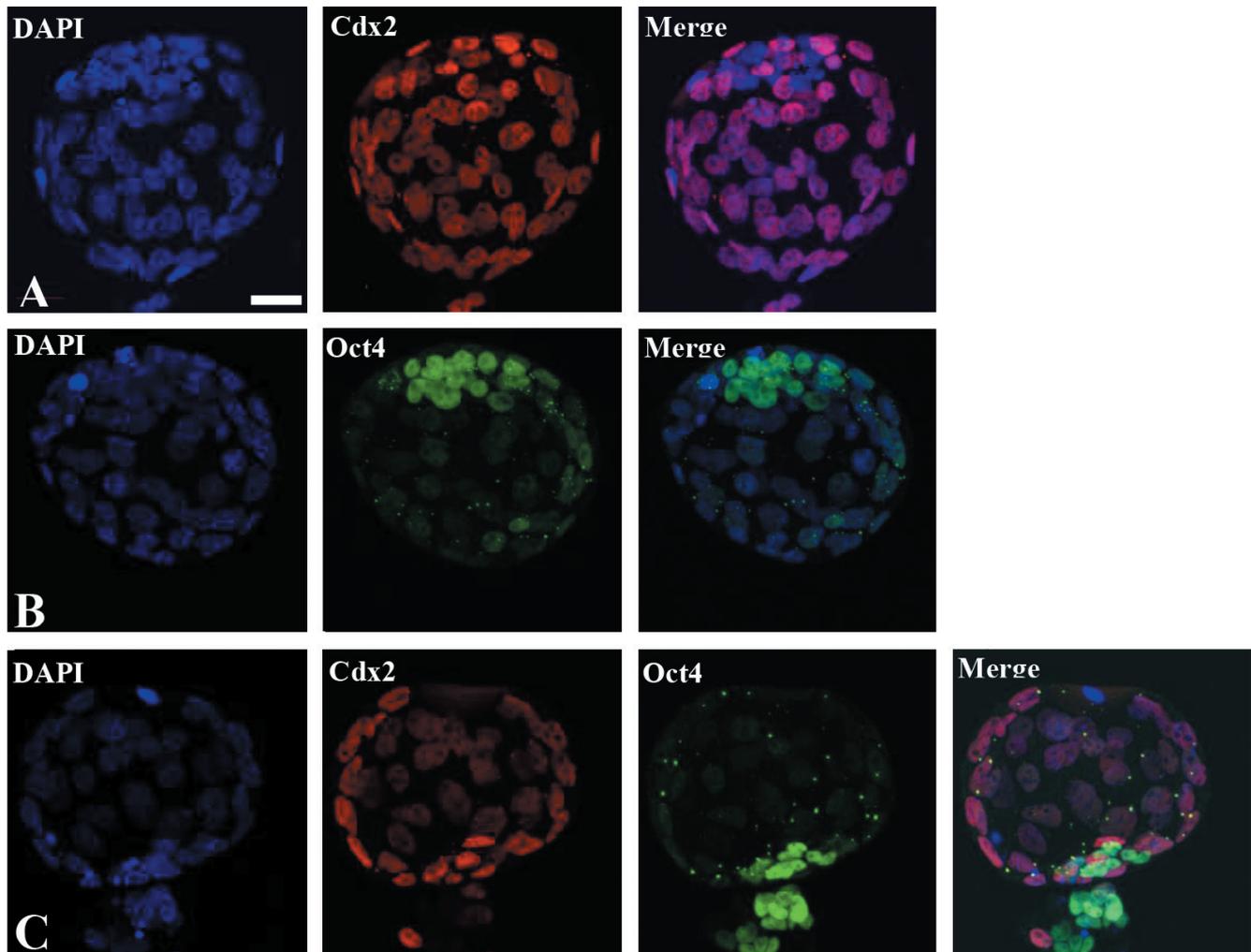


Figure 1: Localisation of transcription factors, Cdx2 and Oct4 in blastocyst

Embryos were imaged by confocal microscopy. (A)-TE cells were immunostained with Cdx2 (red) protein. (B)- Blastocyst was immunostained for pluripotency marker Oct4 (green) protein. (C)- Blastocyst was double labeled for Cdx2 (red) and Oct4 (green). Oct4 was predominantly located in the ICM, but some weak Oct4 staining was detected in the TE and Cdx2 is exclusively expressed in TE with a clear lineage segregation (A-C) Nuclei were stained with DAPI (blue). Confocal images are z-series projections. Scale bar represents 20 μ m.

By the E4.5 blastocyst stage, nuclei of the Epi cells were positively stained with Nanog protein. We demonstrate that Gata4, a zinc-finger-containing transcriptional regulator, and Sox17 were localised in the nuclei of the PrE, the ICM cells immediately adjacent to the blastocyst cavity (Figure 2 and 3). These data revealed that Sox17 and Gata4 expression is specific to PrE cells while Nanog is specific to Epi cells. We also did not observe any coexpression of Nanog with Sox17 nor Gata4, suggesting that these factors are specific for PrE and Epi cell lineages and demonstrate salt and pepper distribution during late blastocyst stage.

Discussion

We detailed the formation of three distinct cell lineages by the late blastocyst: the pluripotent epiblast, trophoblast and primitive endoderm. We demonstrated that cells from late blastocyst stage express several key transcription factors to achieve segregation of the lineages. We observed that the distribution of the Epi-specific transcription factor Nanog and the PrE-specific factors Gata4 and SOX17 during the late blastocyst stage. Expression of Nanog, Gata4 and Sox17 have previously been reported in late blastocysts (2, 5, 11, 17) and our

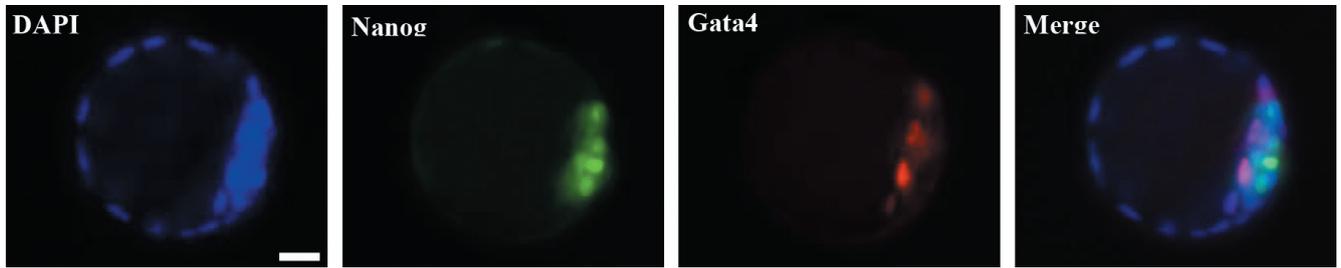


Figure 2: Salt and pepper distribution of Nanog and Gata4 in E4.5 blastocysts. Gata4 is expressed in PrE cells (green) and Nanog is expressed in Epi cells (red). Nuclei were stained with DAPI (blue). Embryos were imaged by fluorescence microscopy. Scale bar represents 20 μ m.

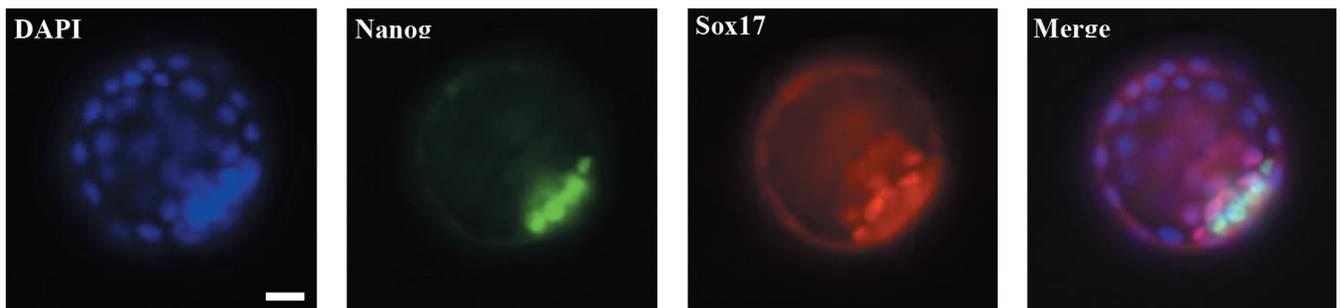


Figure 3: Sox17 and Nanog distribution pattern in E4.5 blastocysts. SOX17 is restricted to PrE epithelium and Nanog is restricted to Epi lineage. PrE marker (Sox17) is exclusive from the Epi marker (Nanog). Sox17 positive cells (red), Nanog-positive cells (green) and nuclei were labeled with DAPI (blue). Embryos were imaged by fluorescence microscopy. Scale bar represents 20 μ m.

data correlate well with these (22). Our results revealed that Gata4 and Sox17 were restricted to PrE epithelium reported results while Epi marker Nanog was restricted to Epi lineage. We observed that cells expressing PrE and Epi markers, Gata4 and Sox17, Nanog, respectively are distributed in a salt and pepper pattern, showed that these lineages have a mutually exclusive distribution and these data are consistent with the cell sorting hypothesis (23, 24).

Several transcription factors are known to play important roles in TE and ICM fate. Pou5f1 deficient embryos, which encode Oct4, generate an ICM that expresses TE markers (13, 25, 26). Loss of Cdx2 embryo forms an expanded blastocyst including TE, but inability to sustain TE function and development. In addition, Oct4 is strongly expressed in the external cells of these embryos suggesting that Cdx2 is essential to repress the expression of Oct4 in TE (1, 6, 20).

By the E3.5, Cdx2 expression becomes restricted exclusively to the cells located on the

outside TE cells, whereas Oct4 protein is present in all cells until late blastocyst stage, declining gradually in the TE thereafter (3, 5). The early restriction of Cdx2 expression indicates that Cdx2 is an essential factor for divergence of TE and ICM lineages (26). By the E3.5 stage, Cdx2 expression becomes restricted exclusively to trophectoderm (3, 20, 25). As expected, we demonstrated that Oct4 was present in nuclei of all cells, but stronger staining was associated with ICM cells and Cdx2 was localized exclusively to the nuclei of TE cells at E4.5 blastocyst stage.

The establishment of ICM and Epi relies on the interactions of transcription factors like Oct4, Nanog and Sox2 (13, 14, 16, 27). It has been demonstrated that Oct4, Nanog and Sox2 are also key players and promote embryonic stem cell pluripotency and self-renewal (17). Oct4 deficient embryos fail to form pluripotent ICM cells, which will differentiate into the extraembryonic trophoblast lineage (13). On the other hand, ablation of Nanog in ICM cells of embryonic

stem cells cause the loss of self-renewal and differentiation of endoderm-like cells (16, 28).

At the late blastocyst stage, extraembryonic endoderm is differentiated from the ICM to generate visceral and parietal endoderm. Gata4 and Gata6 have essential roles on differentiation of visceral endoderm. Moreover, Gata4 and Gata6 in embryonic stem cells are sufficient for controlling differentiation program towards extra-embryonic endoderm (17, 19). It is known that Sox17 acts in the differentiation of mouse embryonic stem cells toward the extra-embryonic endoderm (17, 29) and embryonic stem cells deficient in Sox17 fail to differentiate into extraembryonic cell types (17). Niakan et al. (2010) also demonstrate that Sox17 is a transcriptional regulator of differentiation in embryonic stem and ICM cells (17).

In this study, we showed the distribution of Cdx2, Oct4, Nanog, Gata4 and Sox17 in blastocyst stage murine embryos. Our data showed that lineage specific factors Gata4, Sox17 and Nanog are expressed by PrE and Epi cells respectively, and these lineage specific factors, Gata4, Sox17 and Nanog play essential roles in regulating and maintaining the characteristics of each lineage in blastocyst stage embryos.

Acknowledgements

I would like to thank Dr. Vernadeth B. Alarcon for opening up her laboratory and providing all the equipments and antibodies to do this research.

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RAZPOREDITEV PRIMITIVNEGA ENDODERMA IN EPIBLASTNIH RODOVNO SPECIFIČNIH DEJAVNIKOV V POZNI FAZI BLASTOCIST

D. Mutluay

Povzetek: Mišji predimplantacijski razvoj vodi do tvorbe treh različnih tipov celic blastociste - trofektoderma (TE), ki je nepolarizirana epiteljska plast, notranje celične mase, ki tvori primitivni endoderm in pluripotentni epiblast. Ločevanje teh celičnih linij je povezano z izražanjem nekaterih ključnih genov oziroma prepisovalnih dejavnikov, kot so Cdx2, Oct4, Nanog, Gata4 in Sox17. Naš namen je bil raziskati vlogo in porazdelitev izraženosti teh genov v mišjih zarodkih v pozni fazi blastociste. Zarodki so bili izprani iz brejih samic miši B6D2F1 44 ur po injiciranju humanega horionskega gonadotropina. Do faze pozne blastociste so bili zarodki gojeni v mediju KSOM-AA pri 37 °C s 5 % CO₂ v navlaženi atmosferi. Izvedli smo imunofluorescenčno barvanje s protitelesi, specifičnimi za prej našete gene oziroma beljakovine. Blastociste so bile opazovane in ocenjene s pomočjo fluorescenčnega in konfokalnega mikroskopa. Pokazali smo, da je bila izraženost rodovno specifičnih genov Gata4 in Sox17 omejena na primitiven endoderm, medtem ko je bil gen Nanog omejeno izražen v epidermu v poznem stadiju blastociste. Poleg tega je bila beljakovina Oct4 prisotna v jedrih v vseh celicah, vendar najmočnejše v notranji masi, medtem ko je bil Cdx2 lokaliziran samo v celicah TE. Iz rezultatov sklepamo, da je rodovno specifična izraženost genov Gata4, Sox17 in Nanog bistvenega pomena za ločevanje različnih linij in razvoja blastociste.

Ključne besede: blastocista; epiblast; predimplantacija; primitivni endoderm; prepisovalni dejavniki